Available online at www.sciencedirect.com
SCIENCE DIRECT®
Forest Ecology and Management 196 (2004) 143–158
Forest Ecology and Management
www.elsevier.com/locate/foreco

Response of soil microbial biomass and community composition to chronic nitrogen additions at Harvard forest

Jana E. Compton[a,*], Lidia S. Watrud[a], L. Arlene Porteous[a], Shira DeGrood[b]

[a]U.S. Environmental Protection Agency, National Health and Environmental Effects Research Laboratory, Western Ecology Division, 200 SW 35th St., Corvallis, OR 97333, USA
[b]Department of Land, Air, and Water Resources, University of California, Davis, CA 95616, USA

Abstract

Soil microbial communities may respond to anthropogenic increases in ecosystem nitrogen (N) availability, and the microbial response may ultimately feed back on ecosystem carbon and N dynamics. We examined the long-term effects of chronic N additions on soil microbes by measuring soil microbial biomass, composition and substrate utilization patterns in pine and hardwood forests at the Harvard Forest Chronic N Amendment Study. Functional and structural genes for important N cycling processes were studied using DNA community profiles. In the O horizon soil of both stands, N additions decreased microbial biomass C as determined by chloroform fumigation-extraction. Utilization of N-containing substrates was lower in N-treated pine soils than in the controls, suggesting that N additions reduced potential microbial activity in the pine stand. Counts of fungi and bacteria as determined by direct microscopy and culture techniques did not show a clear response to N additions. Nitrogen additions, however, strongly influenced microbial community DNA profiles. The ammonia monooxygenase gene (*amoA*) generally was found in high N-treated soils, but not in control soils. The *nifH* gene for N_2-fixation was generally found in all soils, but was more difficult to amplify in the pine N-treated soil than the controls, suggesting that the population of N_2-fixers was altered by N additions. The 16S rDNA gene for *Nitrobacter* was found in all samples, but distinct differences among DNA profiles were observed in the pine B horizon in the control, low N, and high N-treated plots. Our findings indicate that chronic N additions decreased chloroform microbial carbon and altered microbial community profiles. These changes in microbial community structure may be an important component of the response of terrestrial ecosystems to human-accelerated N supply.

Keywords: Microbial biomass; Fungi; Bacteria; Colony-forming units; Chloroform fumigation-extraction; Substrate utilization; Ammonia-oxidizing bacteria; Nitrite-oxidizing bacteria; Nitrogen-fixing bacteria; *amoA*; *NOR*; *nifH*; Community DNA profiles

1. Introduction

Humans have dramatically altered the supply of nutrients to terrestrial ecosystems (Vitousek et al., 1997; Galloway and Cowling, 2002), yet few studies have examined the widespread effects of increased nutrient supply on microbial biomass, function and community composition. Changes in microbial community structure and function may have important consequences at a variety of scales. Specific groups of microbes are responsible for key carbon and nitrogen cycling processes such as lignin degradation, nitrogen fixation and nitrification. Collectively, changes in these small-scale processes have large-scale implications, by influencing the production of greenhouse gases and the ability of terrestrial ecosystems to retain added N.

* Corresponding author. Tel.: +1-541-754-4620; fax: +1-541-754-4799.
E-mail address: compton.jana@epa.gov (J.E. Compton).

0378-1127/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.foreco.2004.03.017

Nitrogen additions may alter microbial biomass and activity in several ways. The availability of N constrains primary production in most temperate ecosystems (Vitousek and Howarth, 1991), and in turn N may limit decomposition and microbial activity (Kaye and Hart, 1997; Hobbie and Vitousek, 2000). If N limits microbial growth, then activity and populations would increase directly after N addition. However, a large number of studies have shown that N additions decreased or did not influence decomposition and soil respiration (reviewed by Fog (1988); see also Aber et al. (1998); Micks et al. (2004) and Bowden et al. (2004)). Interactions between N and lignin may contribute to the stabilization of soil organic matter by increasing the production of recalcitrant organic matter compounds (Ågren et al., 2001) or decreasing lignin-degrading enzyme activity (Carreiro et al., 2000), such that decomposition may decrease in response to N deposition. Ågren et al. (2001) concluded that the major reasons for slower decomposition after N fertilization include increased decomposer efficiency, more rapid formation of recalcitrant organic matter and decreased growth rate of decomposers. These changes in decomposer growth rate and efficiency could be the consequence of shifts in microbial community structure and function, and have important implications for ecosystem-level C and N cycling.

Due to the limitations of culture-dependent techniques to study soil microbes, molecular based polymerase chain reaction (PCR) methods are now widely used. A growing number of recent studies have assessed the functional and taxonomic diversity of environmental N cycling microbes based on sequencing and other molecular techniques. These include the study of ammonia-oxidizing microbes in native, tilled and fertilized soils (Bruns et al., 1999; Mendum et al., 1999; Hermansson and Lindgren, 2001); *Nitrosospira* in acid soils (Stephen et al., 1998); *nifH* bearing bacteria in forest soils and rice roots (Ueda et al., 1995; Widmer et al., 1999; Shaffer et al., 2000); and *Nitrobacter* in soil (Degrange and Bardin, 1995; Grundmann and Normand, 2000). In order to understand the links between microbial community composition and ecosystem function, it may be necessary to combine molecular and ecosystem approaches (Jackson et al., 2002).

Here we examine the long-term influence of N additions on microbial community structure and function at the Harvard Forest Chronic N Amendment Study. By examining soil microbes using a variety of techniques, we can assess their relative sensitivity to the effects of N loading and site conditions. We examined the response of soil microbes to chronic N deposition using conventional direct microscopy and culture techniques, chemical extraction, substrate utilization (BIOLOG) and molecular techniques. Molecular community DNA fingerprints based on specific 16S and functional DNA genes were used to evaluate microbial nitrification and N_2-fixation capabilities.

2. Methods

2.1. Site description

This work was conducted at the Harvard Forest Chronic N Amendment Study in Petersham, MA, USA. The purpose of the chronic N study is to simulate accelerated atmospheric N deposition. The N additions began in 1988 (described in detail by Magill et al. (1997) and Magill et al. (2004)), and are conducted in two stand types: red pine and mixed hardwoods. The red pine (*Pinus resinosa*) plantation was established in 1926, and this area was historically cultivated, as evidenced by the plow layer and lack of surface stones. Oaks (*Quercus rubra* and *Quercus velutina*), red maple (*Acer rubrum*) and black birch (*Betula lenta*) dominate the 90-year-old mixed hardwood stand; the area now occupied by the hardwood stand was historically used for pasture until the late 1800s. Each stand has three treatment plots (30 m × 30 m) representing a control, low and high N addition rate (0, 5 and 15 g N m^{-2} per year, added six times per year as ammonium nitrate).

2.2. Soil sampling and processing

Soil samples were collected in 1999 (17 September and 29 November), 2000 (18 September and 6 November), and 2001 (4 June) by driving a 5 cm diameter PVC pipe into the ground from the soil surface to 20 cm soil depth (2 cm diameter corer was used in 1999). Not all sample collection dates were used for all analyses (see individual methods sections). Three replicate soil cores were collected at each plot, and all

replicates were used in the analyses for a given date. The cores were sent in an ice chest by overnight mail to the EPA laboratory, and refrigerated upon arrival. Within 72 h of receipt, the cores were separated into O, A and B horizon soils. The samples were gently homogenized, and stored at 4 °C until processing. Large roots, wood and litter were removed from the samples. Results are presented per unit oven-dry soil (105 °C), except where noted.

2.3. Microbial biomass carbon by chloroform fumigation-extraction

Extractable C and chloroform microbial C were determined using the chloroform fumigation-extraction method (Vance et al., 1987). For this analysis, we used the O or A horizon samples collected in November 1999, November 2000 and June 2001. The samples were processed within approximately 72 h after collection. To determine extractable dissolved organic carbon (extractable DOC), field moist soils were shaken with 0.5 M potassium sulfate (7:1 solution:-soil) for 1 h on a reciprocating shaker, and then filtered through pre-rinsed filters (Whatman #1, Whatman Inc., Newton, MA, USA). To a separate sub-sample, we added 1 ml of ethanol-free chloroform, kept in a sealed container for 48 h at room temperature, and then extracted as described above. Organic carbon in the extracts was determined by UV-persulfate digestion. We calculated chloroform microbial C as the difference in C concentration between the fumigated and unfumigated samples. Since no correction was applied for extraction efficiency, we refer to our estimates of microbial biomass C as chloroform microbial C. The influence of N additions, stand type and sample date were examined by three-way ANOVA using SYSTAT version 10 (SPSS Inc., Chicago, IL).

2.4. Direct microscopy of fungi and bacteria

The number of bacteria and lengths of fungal hyphae were estimated by direct microscopy using the September 1999 and September 2000 samples. Soil Foodweb Incorporated (Corvallis, OR) conducted these counts. For each sample, 1 g of fresh material was placed in 9 ml of 200 mM phosphate buffer (pH 7.2). A 1:100 dilution was prepared from this slurry. Fungal hyphae were quantified in an agar film prepared from 0.5 ml of the 1:10 soil suspension and 1 ml molten 1.5% (w/v) agar (Ingham and Klein, 1984; Lodge and Ingham, 1991). Hyphal lengths were counted using differential interference contrast microscopy (200×). The number of bacteria were counted after staining 0.5 ml of 1:100 soil suspensions with fluorescein isothiocyanate and filtering onto a 0.4 μm polycarbonate filter. Filters were examined using epifluorescent microscopy at 1000× with oil immersion (Babiuk and Paul, 1970). Bacterial and fungal biomass were calculated from the volume of bacterial cells and fungal hyphae in 1 g dry soil using the visual estimates, and assuming bacterial cell density averages 330 mg cm^{-3}, and fungal tissue averages 410 mg cm^{-3} (Ingham et al., 1991). The influence of N additions, stand type and soil depth were examined for each sampling date by three-way ANOVA using SYSTAT version 10 (SPSS Inc., Chicago, IL).

2.5. Culturable fungi and bacteria

Subsamples of soil were placed in dilution bottles with 90 ml extraction buffer (0.2% sodium hexametaphosphate and 6 μM Zwittergent detergent) and shaken for 5 min at a setting of 8 on a Multi-Wrist Shaker (Lab-Line Instruments, Inc., Melrose Park, IL). Samples collected during all collection dates were used for these analyses. Tenfold dilutions were spread-plated in duplicate to determine total bacteria (1/10 tryptic soy agar, Difco Manual, 1984, with 100 μg ml^{-1} cyclohexamide), total fungi (dichloran-rose bengal agar with 50 μg ml^{-1} chlorotetracycline and 100 μg ml^{-1} streptomycin) and spore-forming bacteria (5 min heat treatment at 85 °C before plating on the media used for total bacteria above). Plates were incubated for 5–7 days at 25 °C and the colony-forming units (CFU g^{-1} soil) were counted. The influence of N additions, stand type and soil depth were examined for each sampling date by a multi-way ANOVA using SYSTAT version 10 (SPSS Inc., Chicago, IL).

2.6. Substrate utilization patterns

The substrate utilization potential of microbial communities was determined for the A horizon samples collected in November 1999 using BIOLOG microtitre sole-source nitrogen (EN) plates (BIOLOG

Inc., Hayward, CA). BIOLOG EN substrates represent 95 unique N-containing compounds, including inorganic compounds, amino acids, amines, carbohydrates, nucleosides and dipeptides. One gram of soil was diluted tenfold into an inoculum solution containing pyruvate, ferric chloride and tetrazolium dye. The pH was adjusted to 7.0, soil minerals were removed via centrifugation and filtration, and the extract was incubated for 24 h. Following adjustment of the turbidity to 85% transmittance at 590 nm (Di Giovanni et al., 1999), 150 μl was plated into each well. The plates were incubated at 25 °C and the optical density read at 590 nm every 4 h for a total 116 h using a microplate reader (Molecular Devices, Sunnyvale, CA). We determined the percent change in optical density (OD) by correcting for microbial growth with a control well without an N substrate. To simplify the data presentation, the 95 BIOLOG EN substrates were grouped into nine categories based on their chemical composition.

2.7. Molecular methods

The DNA was extracted within 72 h of receiving the samples using a small-scale extraction method (Porteous et al., 1997). The DNA extraction followed a series of steps including heated cell lysis (68 °C), sodium dodecyl sulfate (SDS), guanidine isothiocyanate, freeze-thaw cycles (−80 and 68 °C), separation with polyethylene glycol (PEG 8000), hexadecyltrimethylammonium bromide (CTAB), chloroform and isopropanol precipitation, and purification by filtration (MicroconTM-100 microconcentrators). DNA visualized on 1% agarose gels consisted of high-molecular weight DNA about 20–23 kb in size with some shearing for all samples. Mean DNA yields determined by gel electrophoresis were 2–10 μg DNA g^{-1} O horizon (fresh weight), 5–10 μg DNA g^{-1} soil A horizon, and 2–5 μg DNA g^{-1} soil B horizon.

Ammonia-oxidizing proteobacteria (including *Nitrosospira*, *Nitrosococcus* and *Nitrosomonas*) carry the *amoA* gene that encodes for ammonia monooxygenase, and are primarily responsible for the first step of autotrophic nitrification, the conversion of ammonia to nitrite (Purkhold et al., 2000). Fragments of *amoA* were amplified with the forward primer *amoA*-1F 5′-GGGGHTTYTACTGGTGGT-3′ (Stephen et al., 1998) and the reverse primer *amoA*-2R 5′-CCCCTSKGSAAAGCCTTCTTC-3′ (Rotthauwe et al., 1997) and resulted in a 490 bp product. Mixtures of primers at 0.4 μM each (Center for Gene Research and Biotechnology (CGRB), Oregon State University, Corvallis, OR), bovine serum albumin (BSA) at 4.0 mg ml^{-1}, and 0.5 μl of the DNA extract with a DyNAwax overlay (Aldrich Chemical Co., Milwaukee, WI) were heated at 85 °C for 3 min. After cooling, 100 μM of each deoxynucleoside, 1.5 mM $MgCl_2$, and 2 U of *Taq* DNA polymerase in 1X reaction buffer (Roche Diagnostics Corporation, Indianapolis, IN) were added to a final 50 μl volume. The following conditions for amplification were performed after an initial denaturation for 2 min at 95 °C: 10 cycles of 95 °C for 40 s, annealing touchdown from 64 to 61 °C, and extension for 40 s at 72 °C; then, 25 cycles of 40 s at 95 °C, 40 s at 61 °C, and 40 s at 72 °C + 1 s per cycle. A final 7 min extension step was performed at 72 °C. Restriction analysis was performed overnight on ethanol precipitated PCR products using a combination of 3 U each of *Msp*I (Amersham Pharmacia Biotech, Piscataway, NJ) and *Cfo*I in 1X buffer L (Roche Diagnostics Corporation) at 37 °C.

Nitrite-oxidizing microbes (e.g., *Nitrobacter* and *Nitrococcus*) convert nitrite to nitrate in the second stage of nitrification, and carry the *NOR* gene that encodes for ribulose 1,5 bisphosphate carboxylase (Bock et al., 1989). Fragments of *Nitrobacter* 16S rDNA genes were amplified with the forward primer 5′-CTAAAACTCAAAGGAATTGA-3′ and the reverse primer 5′-TTTTTTGAGATTTGCTAG-3′ and resulted in a 400 bp product (Degrange and Bardin, 1995). The PCR cocktails contained 100 μM of each deoxynucleoside (Promega, Madison, WI), 1 μM of each primer (CGRB), 3 mg ml^{-1} BSA, 0.5 U of FastStart™ *Taq* DNA polymerase in 1X reaction buffer containing 2.0 mM $MgCl_2$ (Roche Diagnostics Corporation), and from 1 to 3 μl sample DNA. Amplification for 40–45 cycles was performed following an initial denaturation for 6 min at 95 °C: 30 s at 95 °C, annealing for 1 min at 50 °C, and extension for 1 min at 72 °C. Following a 10 min final extension at 72 °C the samples were held initially at 10 °C for 10 h and then stored at −20 °C. Restriction analysis of PCR products was performed overnight using 5 U each *Cfo*I and *Alu*I in 1X buffer A (Roche Diagnostics Corporation) at 37 °C.

Free-living N_2-fixation is performed by a large diverse group of non-symbiotic microbes including *Azospirillum*, *Azobacter*, *Bacillus*, *Bradyrhizobium*, *Clostridium*, *Cyanobacteria*, *Enterobacter*, and *Klebsiella* that carry the *nifH* iron protein gene that encodes nitrogenase reductase (Eady, 1991). Fragments of *nifH* genes were amplified using nested PCR with the forward primer 5′-GCIWTITAYGGNAARGGNGG-3′ and the reverse primer 5′-GCRTAIABNGCCATCATYTC-3′ for the first PCR and the second (nested) PCR was performed with the same reverse primer and the forward primer 5′-GGITGTGAYCCNAAVGCNGA-3′ and resulted in a 370 bp product (Widmer et al., 1999). The PCR cocktails contained 1 μM of each primer (CGRB), 200 μM of each deoxynucleoside, 1 U of FastStart™ *Taq* DNA polymerase in 1X reaction buffer containing 2 mM $MgCl_2$ (Roche Diagnostics Corporation) and from 0.5 to 2 μl sample DNA. For the first PCR step, 3.6 mg ml^{-1} BSA were added, and for the nested PCR step, 0.3 mg ml^{-1} BSA was added. After initial denaturation for 5 min at 95 °C, the amplification proceeded as follows: 11 s at 94 °C and 15 s at 92 °C, annealing for 8 s at 48 °C and 30 s at 50 °C, and extension for 10 s at 74 °C and 10 s at 72 °C. A 10 min final extension at 72 °C was after either 40 or 30 cycles for the first or nested PCR reaction, respectively. PCR products were purified with polyethylene glycol and NaCl precipitation followed by an ethanol wash. Restriction analysis was performed overnight on DNA pellets resuspended in 40 μl using 2 U *Hae*III in 1X reaction buffer M at 37 °C.

Following the PCR cycling in model MJR PT-100 thermal cyclers (MJ Research, Inc., Watertown, MA), the quality of amplified products was analyzed on 2% agarose gels with ethidium bromide. The community fingerprints (restriction analysis patterns) of the amplified and restricted gene pool complexities were visualized on 3% Synergel (Diversified BioTech)/0.7% HMP (Gibco BRL) and 3.5% MetaPhor gels (FMC Bioproducts, Rockland, ME) with ethidium bromide. Molecular weight markers for all PCR products and community DNA fingerprints were a mixture of pUCBM21 DNA (cleaved with *Hpa*II) and pUCBM21 DNA (cleaved with *Dra*I and *Hind*III) and ϕX174 DNA (cleaved with *Hae*III) (Roche Molecular Biochemicals, Indianapolis, IN). Molecular weights of restriction fragments were determined using GeneProfiler 3.56 (Scanalytics, CSPI, Billerica, MA). Community DNA fingerprints were compared to simulated restriction fragment length polymorphisms (RFLPs) of published Genbank (Benson et al., 2002) microbial sequences that were cut to the desired PCR fragment size target region and restricted with the appropriate enzymes using Dnasis Max v.1 (Hitachi Software Engineering America, Ltd., San Bruno, CA). Dominant combinations of restriction fragments in the fingerprints that reflected different phylotypes were identified, labeled, and compared with published Genbank sequence profiles. Electrophoretic gel images were scanned and inverted using a densitometer (BioRad Model GS-670, Hercules, CA) and the Molecular Analyst™ image analysis software version 1.5 for PC (Biorad, Hercules, CA). Photographic contrast and brightness auto adjustments were performed with Adobe Photoshop 4.0 software (Adobe Systems Inc., San Jose, CA). Community DNA fingerprints were presented as fragment sizes on a logarithmic scale and shown as inverted electrophoretic scans.

3. Results

3.1. Chloroform microbial C and extractable C

Chloroform microbial C was strongly influenced by stand type, date of sampling, and chronic N additions (Table 1). In the O horizon samples, average microbial C in the pine control plot was nearly eight times greater than in the pine high N plot (Fig. 1). The hardwood control plot had 1.6 times more microbial C than the hardwood high N plot. While N additions strongly reduced microbial biomass in the O horizon,

Table 1
Probability values of factors influencing extractable DOC and chloroform microbial biomass C

Factor	Extractable DOC	Chloroform microbial C
Stand	NS	0.003
N level	0.000	0.003
Date	0.000	0.000
S × N	0.084	NS
S × D	NS	0.018
N × D	0.000	NS
S × N × D	0.004	NS

Results of three-way ANOVA using O and A horizon data.

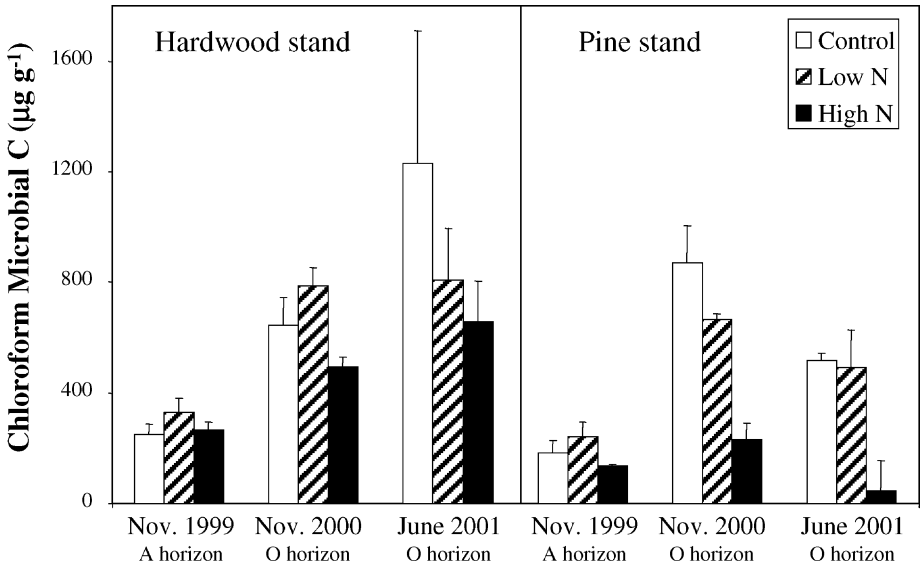

Fig. 1. Microbial biomass C determined by chloroform fumigation-extraction. The date of sampling and the horizon sampled are shown on the *x*-axis. Error bars are ±1 S.E.

N additions did not have a strong effect on microbial biomass in the A horizon. Microbial biomass was generally higher in the hardwood stand than the pine stand, although this pattern varied by date.

Extractable DOC was similar between stands, but was influenced by N addition (Tables 1 and 2). However, the effect of N addition is somewhat difficult to interpret, because this factor interacted with all other factors. In the A horizon soil (November 1999), extractable DOC increased strongly with N addition level. Extractable DOC in the O horizon was slightly higher in June than in November and was not consistently influenced by N additions. In both November sampling dates, microbial biomass and extractable DOC were positively correlated ($r^2 = 0.41$ and 0.95 in 1999 and 2000, respectively). In June 2001, however, chloroform microbial C and extractable DOC were not correlated.

Table 2
Extractable dissolved organic carbon (DOC)

Stand	N level	Extractable DOC (μg g^{-1} soil)		
		November 1999, A horizon	November 2000, O horizon	June 2001, O horizon
Hardwood	Control	234 ± 8	287 ± 72	460 ± 79
	Low N	258 ± 13	326 ± 48	320 ± 52
	High N	324 ± 53	255 ± 27	348 ± 101
Pine	Control	277 ± 38	383 ± 43	394 ± 56
	Low N	347 ± 43	286 ± 26	312 ± 10
	High N	388 ± 19	218 ± 30	404 ± 55

Values presented are means for each sampling period ± standard error.

3.2. Fungi and bacteria determined by direct microscopy and culture techniques

Direct microscopy estimates of fungal and bacterial biomass were influenced by stand and horizon, but we found no effect of N load (Table 3). Fungal counts were generally higher in the hardwood stand than the pine stand in September 1999. Both bacterial and fungal counts generally decreased from the A to the B horizon. Fungal biomass dominated the microbial counts, but there were large differences between the ratios of fungi to bacteria in September 1999 (4–6) and in September 2000 (9–35). The abundance of fungi drove this relationship, since fungal counts were much higher in September 2000 than September 1999, perhaps a consequence of the intense summer drought of 1999 (Savage and Davidson, 2001).

Culturable fungi and bacteria counts also were not influenced by N additions, and decreased with increasing soil depth (Tables 4 and 5). Spore-forming bacteria

Table 3
Direct microscopy estimates of fungal and bacterial biomass

Stand	N added	Soil horizon	September 1999			September 2000		
			Fungi ($\mu g\ g^{-1}$)	Bacteria ($\mu g\ g^{-1}$)	Ratio fungi:bacteria	Fungi ($\mu g\ g^{-1}$)	Bacteria ($\mu g\ g^{-1}$)	Ratio fungi:bacteria
Hardwood	Control	A	803	197	4.1	6060	174	35.2
		B	575	133	4.4	2105	201	11.2
	Low	A	1094	175	6.2	4742	205	24.1
		B	751	130	5.9	1933	158	12.1
	High	A	789	139	5.5	3823	215	17.5
		B	988	130	7.7	1755	190	9.3
Pine	Control	A	849	149	5.4	3539	228	15.7
		B	675	126	5.4	1902	193	9.7
	Low	A	428	146	3.0	2810	213	13.3
		B	690	113	6.2	2845	171	16.7
	High	A	629	105	6.2	3841	203	18.6
		B	641	120	5.3	2063	170	12.1
Significant factors			S	S, H	NS[a]	H, SH	H	H, SN, NH

Significant factors ($P < 0.05$) determined using multi-way ANOVA by stand type (S), horizon (H), N level (N) and interactions (multiple letters).
[a] No significant factors identified.

were greater in the N addition plots in November 2000, but not affected by N additions in June 2001. The spore-forming bacteria represent Gram-positive bacteria, such as *Bacillus* sp. and actinomycetes. The spore-forming bacteria represented approximately two-thirds of the total bacterial CFUs in June 2001 (the only date when both were measured). Culturable bacteria decreased with depth for all sampling dates except one, and the hardwood stand had more culturable bacteria in September and November 1999.

3.3. Substrate utilization

In the pine stand, utilization of all N-containing substrates tended to be lower in the high N than the control plots (Fig. 2b). Variability among samples was high, such that the only significant effect of N addition was for the pine treatments utilization of tyramine and *N*-acetyl-D-mannosamine (data not shown). The pine low N treatment generally had a utilization rate intermediate between the pine control and high N. In the hardwood stand, substrate utilization was not consistently influenced by N additions, and was generally higher than in the pine stand. Only one sample period was used for the substrate utilization work, due to the limited availability of the EN plates. However, these initial data suggest that microbial utilization of N-containing substrates was lower in the N-treated pine plots, while substrate utilization was not strongly influenced by N additions the hardwood stand.

3.4. Restriction analysis of the PCR products

The *amoA* gene for ammonia oxidation was detected in only 4% of the control samples examined, but was detected in 71% of the high N samples (Table 6). The fingerprints contained three distinct and dominant restriction fragment length polymorphisms (RFLP) patterns. Pattern A1 (142, 87, 70, 66, 61, and 41 bp) was dominant in 10/12 high N samples in the O horizon (Fig. 3A, lanes 4–6 and 10–12). Two additional patterns A2 and A3 were detected in the high N hardwood A horizon samples (Table 6). The detected *amoA* RFLP patterns were compared to Genbank sequences of other *amoA* containing microbes. The RFLP of *Nitrosospira* sp. AHB1 (Genbank X90821) was the same as pattern A1 and the RFLP of *Nitrosospira briensis* (Genbank U76553) was the same as pattern A2. The similar RFLPs suggest the presence of those microbes in our soil samples.

Table 4
Culturable fungi

Stand	N level	Soil horizon	Culturable fungi (CFU g^{-1} soil)			
			November 1999	September 2000	November 2000	June 2001
Hardwood	Control	O	3.44×10^6	ND[a]	9.84×10^5	1.17×10^7
		A	5.57×10^6	1.35×10^6	1.02×10^6	2.52×10^6
		B	1.56×10^6	6.73×10^4	1.11×10^6	9.40×10^4
	Low	O	1.26×10^7	ND	2.03×10^6	1.42×10^7
		A	1.53×10^6	1.60×10^6	8.97×10^5	1.37×10^6
		B	4.23×10^6	1.29×10^5	4.72×10^5	1.04×10^5
	High	O	5.73×10^6	ND	1.50×10^6	1.17×10^7
		A	2.33×10^6	1.53×10^6	1.24×10^6	1.74×10^6
		B	6.43×10^6	7.94×10^4	1.62×10^6	5.05×10^5
Pine	Control	O	5.86×10^6	ND	8.32×10^5	8.84×10^6
		A	2.42×10^6	9.19×10^5	1.31×10^6	6.71×10^5
		B	1.50×10^6	5.34×10^4	5.98×10^5	1.87×10^5
	Low	O	4.00×10^6	ND	1.09×10^6	6.98×10^6
		A	8.98×10^5	7.83×10^5	1.72×10^6	6.77×10^5
		B	1.76×10^6	5.25×10^4	9.23×10^5	2.08×10^5
	High	O	8.02×10^6	ND	1.08×10^6	8.99×10^6
		A	1.38×10^6	9.52×10^5	1.10×10^6	4.60×10^5
		B	4.04×10^6	6.20×10^4	7.36×10^5	7.76×10^4
Significant factors			H	S, H	NS	H

Significant factors as in Table 3.
[a] Not determined.

The 16S rDNA gene for *Nitrobacter* containing the *NOR* gene for nitrite oxidation was detected in 100% of the samples examined (Table 6). The fingerprints contained four distinct and dominant RFLP patterns. Patterns N1, N2, N3, and N4 contained two fragments (188 and 43 bp) with 1–3 additional bands at 102, 87 and 73 bp (Fig. 3C). No other bands were observed in the *Nitrobacter* 16S rDNA fingerprints. Dominant RFLP differences were observed between tree stands (Fig. 3D, lanes 1–9 for hardwood and lanes 10–18 for pine) and horizons (Fig. 3D for A horizon and Fig. 3E for B horizon). Even so, effects of N level were observed by the presence, absence, and dominance of the 102, 87 and 73 bp fragments in the gene pool, especially in the pine B horizon samples from September 2000 (Fig. 3E, lanes 10–12, 13–15, and 16–18). The fragment differences are also shown as phylotype differences in Table 6 (N1, N2, N3, and N4). The dominant phylotype patterns in the B horizon pine samples were N1 and N2 for the controls (Fig. 3E, lanes 10–12), N2 and N3 for the low N samples (Fig. 3E, lanes 13–15), and N1 and N2 for the high N samples (Fig. 3E, lanes 16–18). Nitrogen additions did not influence *Nitrobacter* in the hardwood stand. The detected 16S *Nitrobacter* RFLP patterns were compared to five retrieved Genbank 16S *Nitrobacter* sequences. The RFLPs of *Nitrobacter hamburgensis* (Genbank L11663) and *Nitrobacter winogradskyi* (Genbank L35507) were similar to the N3 experimental pattern. The similar RFLP suggests the presence of those microbes in the environmental samples. The presence of patterns N1, N2, and N4 in the fingerprints suggests the presence of yet other *Nitrobacter* phylotypes in the samples that were not contained in Genbank.

The *nifH* gene for N_2-fixation was detected in 100% of the O and A horizon samples. However in 25% of the A horizon samples, the gene was extremely weak or very difficult to obtain, particularly in the pine stand (Table 6). The fingerprints contained at least six distinct and dominant RFLP patterns. Generally, the dominant patterns (Table 6, patterns NH1, NH2, and

Table 5
Culturable bacteria and spore-forming bacteria (CFU g^{-1} soil)

Stand	N level	Soil horizon	Culturable bacteria				Spore-forming Bacteria	
			September 1999	November 1999	September 2000	June 2001	November 2000	June 2001
Hardwood	Control	O	ND[a]	5.40×10^6	ND	1.37×10^8	2.00×10^5	2.28×10^5
		A	9.87×10^8	2.13×10^6	1.06×10^7	2.01×10^7	2.23×10^5	3.78×10^5
		B	6.63×10^8	3.23×10^5	5.66×10^6	4.90×10^6	2.19×10^5	4.05×10^5
	Low	O	ND	7.89×10^6	ND	3.15×10^8	2.45×10^5	3.60×10^5
		A	8.77×10^8	2.59×10^6	1.34×10^7	9.17×10^6	2.73×10^5	6.73×10^5
		B	6.52×10^8	3.21×10^4	3.56×10^6	2.71×10^6	2.54×10^5	4.13×10^5
	High	O	ND	9.47×10^6	ND	2.31×10^8	6.16×10^5	1.80×10^5
		A	6.96×10^8	1.57×10^6	9.73×10^6	1.11×10^7	6.11×10^5	1.16×10^6
		B	6.49×10^8	2.29×10^4	1.82×10^6	1.58×10^7	2.69×10^5	9.08×10^5
Pine	Control	O	ND	5.93×10^6	ND	4.18×10^8	4.17×10^5	2.70×10^5
		A	7.46×10^8	1.96×10^6	7.62×10^6	7.95×10^6	2.45×10^5	1.59×10^6
		B	6.31×10^8	2.44×10^4	2.06×10^6	5.79×10^6	1.3×10^5	8.11×10^5
	Low	O	ND	1.16×10^6	ND	1.06×10^8	3.65×10^5	8.18×10^4
		A	7.28×10^8	3.08×10^6	3.13×10^6	5.04×10^6	4.61×10^5	3.63×10^5
		B	5.65×10^8	2.11×10^4	2.25×10^6	3.42×10^6	2.41×10^5	7.26×10^5
	High	O	ND	1.11×10^6	ND	3.73×10^8	4.39×10^5	4.66×10^5
		A	5.24×10^8	2.93×10^6	1.23×10^7	4.09×10^6	3.09×10^5	5.37×10^5
		B	6.01×10^8	3.54×10^4	2.01×10^6	3.13×10^6	4.57×10^5	5.83×10^5
Significant factors			S, H	S, H, SN, SH, SNH	H	H, SN, SNH	N	H, SN, SNH

Significant factors as in Table 3.
[a] Not determined.

NH3) for hardwood and pine control O horizon (Fig. 3B, lanes 1–3 and 7–9) were different from patterns (NH2 and NH4) for high N O horizon samples (Fig. 3B, lanes 4–6 and 10–12). Differences were observed in the triplicate fingerprints for the A horizon samples with some of the fingerprints containing only two dominant RFLP patterns. RFLP patterns NH1, NH3, and NH5 were frequently present in the A horizon control samples and patterns NH1, NH3, NH5, and NH6 were observed in the A horizon high N samples. RFLP patterns of the *nifH* gene pool from this study were compared to RFLP patterns of cloned *nifH* fragments from a Douglas fir forest site and positive *nifH* controls (Widmer et al., 1999). Pattern NH1 from this study was similar to pattern XIV (Genbank AF099792) from the Douglas fir forest study that was shown to phylogenetically cluster with microbes representative of *Sinorhizobium*, *Azospirillum*, and *Rhizobium*. The similar RFLP suggests the presence of at least one of those microbes in the samples. Neither the Douglas fir *nifH* clones nor *nifH* Genbank controls were helpful in identifying the remaining five *nifH* patterns (NH2, NH3, NH4, NH5, and NH6) from this study.

4. Discussion

4.1. Comparison of methods for assessing microbial biomass

While long-term N additions appeared to reduce microbial biomass as determined by chloroform fumigation-extraction, there was no discernable effect of N addition on total fungi, total bacteria or ratios of fungi to bacteria as determined by culture or direct count approaches. We cannot easily explain the disparate responses of the different methods. The culture and count techniques generally produced more variable data than chloroform fumigation-extraction. Because

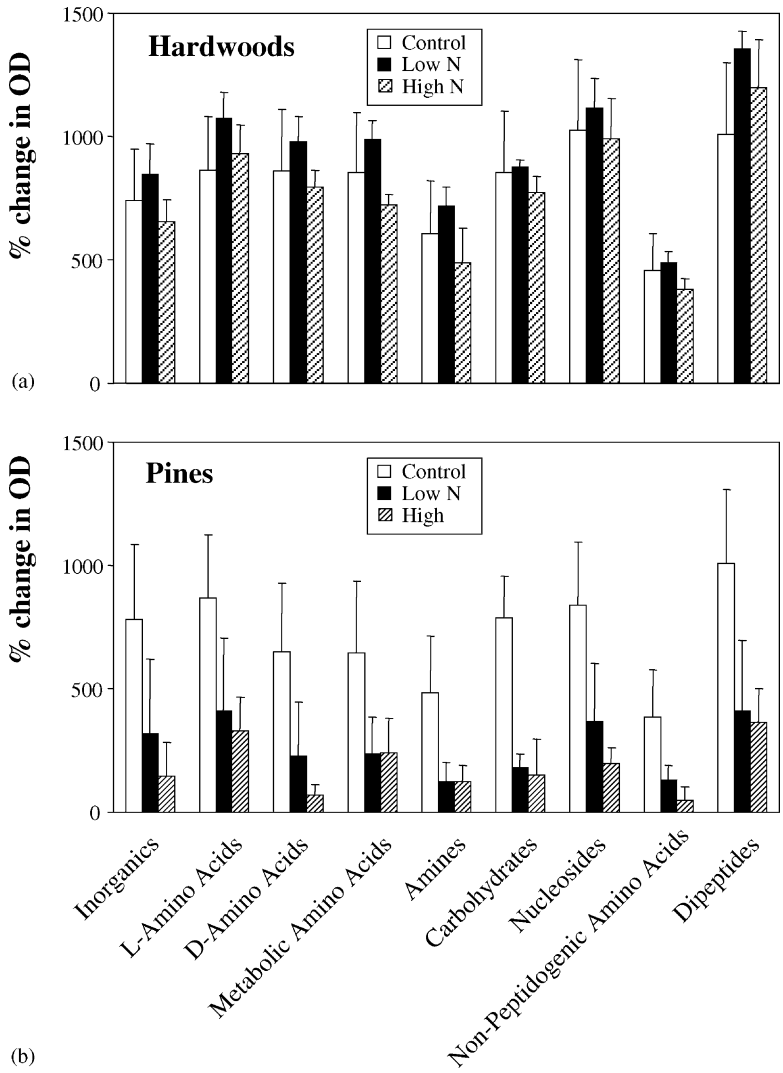

Fig. 2. Substrate utilization as determined using BIOLOG EN plates, A horizon, November 1999 (standardized as % change in optical density, OD). Error bars are ±1 S.E.

chloroform fumigation is a chemical technique, however, changes in soil chemistry associated with the chronic N amendments could influence the solubility of microbial C. Using very similar techniques at the same plots, Frey et al. (2004) also found that total fungi were not affected by N addition, and chloroform microbial biomass was lower in N amended plots. In a recent comparison of several methods, chloroform fumigation-extraction was the best predictor of total microbial phospholipid fatty acids ($r^2 = 0.77$ for forest and agricultural soils; Bailey et al., 2002). The phospholipid fatty acid approach is an increasingly common tool in assessing microbial communities.

Other studies have shown a variation in response when comparing different techniques for assessing microbial populations. Biederbeck et al. (1996) found contradictory patterns when examining the microbial response to N additions: plate counts for fungi and bacteria were positively related to N addition rates, while chloroform-released microbial biomass decreased with increasing N level. Culture techniques access only a small proportion of expected microbial

Table 6
Phylotype patterns that appeared as combinations of dominant fragments and were extrapolated from the PCR-RFLP community fingerprints that reflect the diversity of microbial populations in the samples

Stand	N level	Horizon	*amoA*[a]		16S *Nitrobacter*[b]		*nifH*[c]	
			September 2000	June 2001	September 2000	June 2001	September 2000	June 2001
Hardwood	Control	O	Negative	Negative	N2, N3	N2, N3	NH1, NH4	NH1, NH3
		A	Negative	Negative	N1, N2	N1	NH5	NH1, NH3, NH5
		B	ND	ND	N2, N3	N3	ND	ND
	Low	O	ND	ND	N2, N3	N2, N3	ND	ND
		A	ND	ND	N2	N1	ND	ND
		B	ND	ND	N3	N3	ND	ND
	High	O	A1	A1	N1, N2	N2, N3	NH2	NH2
		A	A2, A3	A1, A2	N1, N2	N1	NH1, NH3, NH5	NH1, NH3
		B	ND	ND	N3	N3	ND	ND
Pine	Control	O	Negative	Negative	N1, N3	N2	NH1, NH2, NH3	NH1, NH2
		A	A1	Negative	N1	N1	NH1, NH5	NH1
		B	ND	ND	N1, N2	N3	ND	ND
	Low	O	ND	ND	N3, N4	N2, N4	ND	ND
		A	ND	ND	N1	N1	ND	ND
		B	ND	ND	N3	N3	ND	ND
	High	O	A1	A1	N2, N4	N1, N4	NH2	NH1, NH2, NH3
		A	A1	Negative	N1	N4	NH3, NH6	NH1, NH3
		B	ND	ND	N1	N4	ND	ND

ND: not determined. Negative: no PCR products detected.
[a] *amoA* phylotypes: A1 (142, 87, 70, 66, 61, and 41 bp); A2 (259, 85, 80, and 68 bp); A3 (143, 143, 111, 60, and 40 bp).
[b] 16S *Nitrobacter* phylotypes: N1 (188, 87, 73, and 43 bp); N2 (188, 102, 87, 73, and 43 bp); N3 (188, 102, 73, and 43 bp); N4 (188, 87, 87, and 43 bp).
[c] *nifH* phylotypes: NH1 (189, 168 bp); NH2 (168, 74, 72 bp); NH3 (370 bp); NH4 (236, 140 bp); NH5 (189, 112, 72 bp); NH6 (165, 165, 50 bp).

biomass and diversity (Torsvik et al., 1990), and thus are far from comprehensive. The individual limitations of both culture and non-culture approaches suggest that it may be best to compare a variety of techniques to broadly assess microbial response to changing environmental conditions. We show that that chloroform fumigation-extraction assessment of the microbial response was consistent with the general trend in substrate utilization patterns and with the decrease in root-free soil respiration after N treatment (Bowden et al., 2004).

4.2. Effects of chronic N amendment on microbial biomass and substrate utilization

After approximately 10 years of chronic N additions, O horizon microbial biomass was much lower in the N amended than in the control plots. Addition of inorganic N fertilizers has decreased microbial biomass in forest (Söderström et al., 1983; Arnebrandt et al., 1996), prairie (Ajwa et al., 1999) and agricultural soils (McAndrew and Malhi, 1992; Ladd et al., 1994). While the reduction in microbial biomass in response to N additions may be a common finding, the mechanisms behind this reduction have not been clearly identified (Arnebrandt et al., 1990).

Nitrogen additions may alter soil microbial communities via direct N effects or indirectly by decreasing soil pH or by altering the supply and quality of organic matter. Nitrogen additions can alter fruiting body abundance, hyphal networks, formation of mycorrhizae and community composition in ectomycorrhizal fungi (Wallenda and Kottke, 1998; Lilleskov et al., 2002; Frey et al., 2004). Although we did not examine mycorrhizal fungi directly, it is possible that a reduction in the biomass of mycorrhizal fungi could

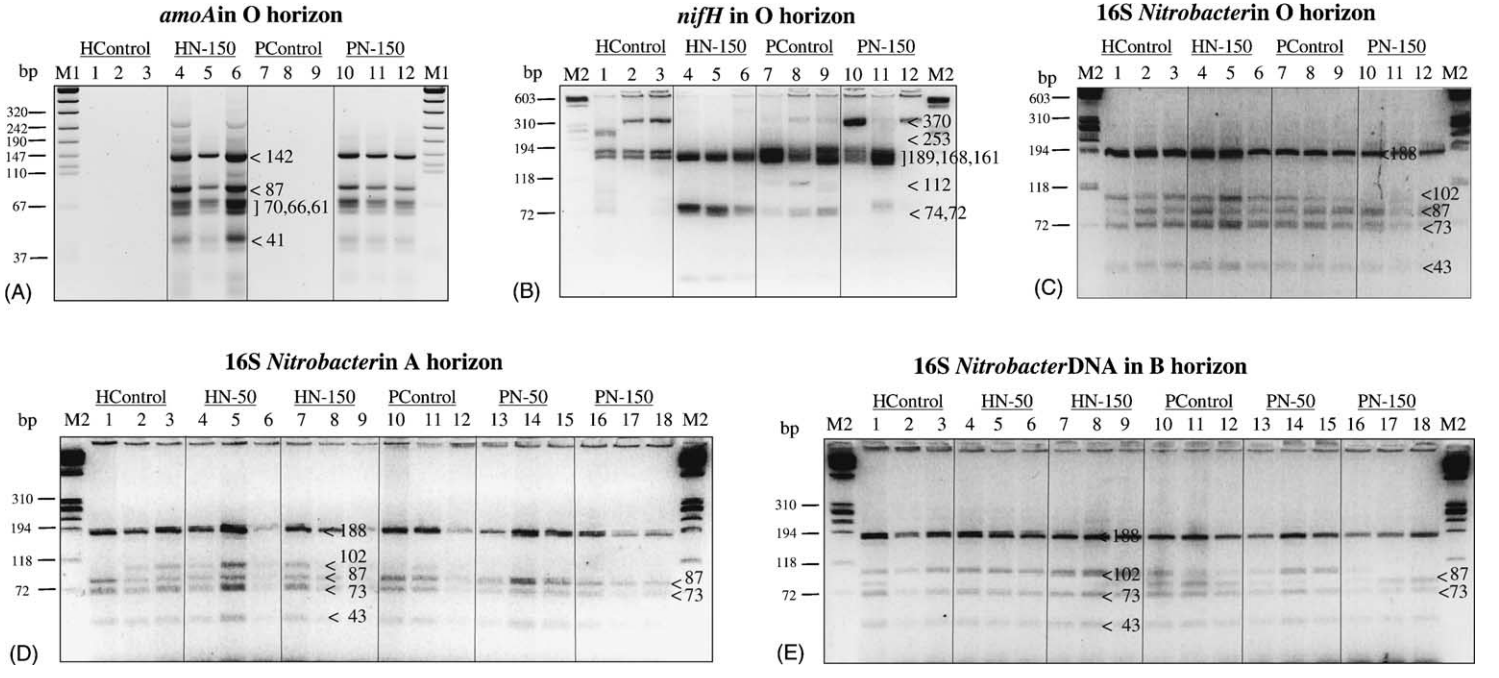

Fig. 3. Community DNA fingerprints of *amoA* (A), *nifH* (B), and 16S *Nitrobacter* (C)–(E) gene pools. Numbers and arrows indicate molecular weights of dominant fragments detected from hardwood (H) and pine (P) stands for June 2001 (panels (A)–(C)) and September 2000 (panels (D) and (E)). M1 and M2 are electrophoresis gel markers.

contribute to our observed decrease in microbial biomass. However, we did not find an effect of N additions on fungal biomass through direct counts or culture techniques. Soil pH declines are common with increased N loading, and could also influence microbial communities. The pH of the N-treated surface soils has decreased by approximately 1–1.5 units in the Harvard Forest Chronic N Amendment Study (Magill et al., 2004). The consequences of this decrease in pH for soil biological activity may be significant, in particular for the pine soil.

The microbial response to N additions may be strongly linked to changes in plant production and carbon supply. Plant biomass and litterfall increased in response to N additions early in the chronic N experiment, but over time this trend reversed. After 11 years of chronic N addition, aboveground plant production is lower in the pine and hardwood high N plots respectively, relative to the controls (Magill et al., 2004). Microbial communities in the pine stand also were more responsive to N additions than those of the hardwood stand (e.g., chloroform microbial C, substrate utilization patterns, *Nitrobacter* populations). This pattern parallels some of the ecosystem-level effects, where the pine stand has responded more quickly and dramatically to N amendment (Magill et al., 1997). It is very likely that a decrease in the supply of organic matter to soil microbes, and in turn the microbial response to this change in supply, are important long-term effects of chronic N addition.

Nitrogen additions stimulated cellulase activity in decaying leaf litter of hardwood species (Carreiro et al., 2000), suggesting that N addition may initially increase microbial decomposition of organic matter. However, some fungi produce lignin-degrading enzymes only when their growth is N-limited (Kirk, 1987). Inorganic N therefore may suppress microbial production of lignin-degrading enzymes (Berg, 1986), illustrating a possible negative feedback of N addition on the long-term decomposition of organic matter. Lignolytic enzyme production was reduced by field chronic N additions in central New York (Carreiro et al., 2000) and the Harvard Forest Chronic N Amendment Study (Frey et al., 2004). We also found that N additions decreased utilization of N-containing substrates, which suggests that microbial breakdown of organic compounds may be inhibited under excess N supply.

Field measurements indicate that slower breakdown of organic matter in response to N additions may already be occurring in these soils. Bowden et al. (2004) determined that soil respiration was lower in the N-treated plots several years after chronic N addition. Lab experiments with root-free and bulk soils indicated that chronic N additions have reduced the rate of microbial activity, thus contributing to the decrease in CO_2 production measured in the N-treated sites in the field. Soil C storage may increase and soil respiration could decrease, however the balance between plant and soil processes will determine the overall effect on net ecosystem C exchange. Clearly the effects of increased N on microbial biomass and activity will have important consequences at ecosystem and larger scales, if these changes lead to a decrease in soil C turnover.

4.3. Influence of N amendments on DNA community profiles of N cycling organisms

A variety of PCR techniques were used in this study to detect three distinct gene pools in the samples, and these techniques have different levels of sensitivity. For ammonia-oxidizing beta proteobacteria detection, Webster et al. (2002) showed that 16S rDNA PCR primer sets yielded two to three times more PCR product than the functional *amoA* amplification product using the same environmental DNA templates. Therefore, the functional *amoA* and *nifH* techniques may in fact be less sensitive than the 16S *Nitrobacter* method. To increase sensitivity, nested PCR was utilized with the *nifH* primers and may have improved the detection of *amoA* sequences from the DNA templates if it had been used. Even so, Webster et al. (2002) concluded that *amoA* recovery was generally lower in unimproved grasslands than in N improved grasslands. Our work indicates that the presence of *amoA* using our specific primers was considerably lower or not detected in the control samples, indicating absence of these organisms or shifts in populations from control to N amended plots.

Two related species carrying the *amoA* gene were detected and distinguished in the ammonia-oxidizing microbial populations from the high N soils using the PCR-RFLP method. The A1 pattern similar to *Nitrosospira* sp. AHB1, was affiliated with *Nitrosospira* cluster 2 subgroup, and has been reported to favor acid

(pH 4.2) soils (Stephen et al., 1998). The A2 pattern detected in the A horizon and similar to *N. briensis*, was associated with *Nitrosospira* cluster 3 subgroup that favored more neutral (pH 7) conditions, and has been reported to dominate tilled and nitrogen amended soils (Stephen et al., 1998; Bruns et al., 1999). Other researchers have also shown that populations of *Nitrosospira* sp. may be limited to microsites: monospecific clusters of either *Nitrosospira* or *Nitrosomonas* have been found to form close associations with similar monospecies clusters of nitrite oxidizers (Schramm et al., 1998) such as *Nitrobacter*. Ammonia-oxidizing populations have also been shown to exhibit a great degree of temporal and spatial stability (Bruns et al., 1999). The monospecies groups of *Nitrosospira* sp. (A1) detected in the O horizon hardwood and pine soils for high N showed temporal reproducibility over the two sampling dates and spatial stability within the replicate and different samples. However, even though *Nitrosospira* sp. (A2) was detected consistently in the A horizon hardwood soils, the presence of at least two other species (A1 and A3) created multi species clusters that were observed at both samplings. It has been reported that *Nitrosospira* can dominate the *amoA* microbes over *Nitrosomonas* when subjected to increased anthropogenic N loads (Oved et al., 2001). Results of our study indicate that similar A2 RFLP *Nitrosospira* dominated the A horizon samples and that A1 RFLP *Nitrosospira* occupied the O horizon samples.

Samples collected from control plots generally did not amplify for the ammonium oxidizing *amoA* gene under the conditions of this study. Amplification may have not occurred in the controls due to non-specificity or lack of sensitivity of the PCR primers. The *amoA* primers were based on the sequences of known autotrophic ammonia-oxidizing beta proteobacteria. It is possible that the target *amoA* populations were below the level of detection of the method or that different *amoA* species lacking the described primer specific sites were present but unable to anneal with the primers. Under either of these conditions the presence and absence of PCR products and community fingerprints suggests there were actual population shifts in the samples. The lack of positive *amoA* control results made it impossible to determine whether there was greater microbial diversity in the control than the treated system, as others have recently reported (Webster et al., 2002). The consistent detection of *Nitrosospira* in this study confirms the importance of this microbe in the ammonia-oxidizing process as others have reported (Stephen et al., 1998; Mendum et al., 1999). However, the *amoA* gene was found almost exclusively in the N-treated soils (Fig. 3), which suggests that ammonia oxidation is much less important when N availability is lower. Our work also indicates that ammonium oxidizers are not present or have very different populations in control soils as compared to the N-treated soils. This represents an important functional shift in response to increased N supply, and may explain the pattern of nitrification in N poor and N rich soils.

Although the *amoA* gene was only found in the N-treated soils, nitrite-oxidizing *Nitrobacter* were found in all soils. The consistent detection of *Nitrobacter* patterns in all the samples suggests that *Nitrobacter* appears to be the important and dominant microbe in soil nitrite-oxidizing processes, and not *Nitrospira* (Grundmann and Normand, 2000). We could not distinguish different closely related species, such as *N. hamburgensis* and *N. winogradskyi*. Nonetheless, using the PCR-RFLP method, the presence of other different *Nitrobacter* types was confirmed in the gene pool. Since ammonia oxidizers provide an important source of nitrite for soil microbes, we were surprised that although nitrite-oxidizing *Nitrobacter* was present in 100% of the samples, there was little evidence of ammonia-oxidizing microbes in the control samples. Our findings suggest a link between microbial community structure and function: that the absence of ammonia oxidizers, or shifts in ammonia oxidizer community composition, may proximally limit autotrophic nitrification in these soils. This suggestion is consistent with the pattern of much higher nitrate production and leaching in the N-treated plots (Magill et al., 1997).

The *nifH* PCR protocol can detect a number of free-living N_2-fixing microbes carrying the *nifH* gene (e.g., *Bradyrhizobium*, *Frankia*, *Rhizobium*, and *Cyanobacteria*) and when followed by *Hae*III restriction analysis shows considerable resolution to distinguish differences in the RFLP patterns. One dominant RFLP (*nifH*1) was similar to RFLP patterns of *Bradyrhizobium* and *Sinorhizobium*, microbes that are highly associated with plant rhizospheres (Fenchel et al., 1998). The diversity was difficult to estimate since many of the control and high N fingerprints contained

the same few fragments that often appeared as a single dominant phylotype pattern identified in each of the samples. Kolb and Martin (1988) found that N addition decreased the populations of free-living N_2-fixers. The *nifH* gene was difficult to amplify, even after using the nested method, especially in the pine N-treated soils rather than the controls, suggesting the potential for N fixation was depressed after chronic N additions. In the hardwood and forest floor samples, there was no effect of N addition on *nifH*. We found less of the *nifH* gene in the pine high N-treated A horizon soils, indicating a negative feedback between N availability and N_2-fixation by free-living microbes in these soils.

Acknowledgements

The authors thank Alison Magill and Glen Berntson for guidance and for providing samples from the Chronic N experiment. We are grateful to Tamotsu Shiroyama, Genie Coleman and Christine Redmond for sample processing and culture work. Brenda Shaffer, Bonnie Smith and Uyen Nguyen assisted with the molecular work, and Ephraim Govere and Rachel Wahl conducted the carbon analyses. This work was funded by the U.S. Environmental Protection Agency, and approved for publication after EPA review. Approval does not signify that the contents reflect the views of EPA, and mention of trade names or commercial products does not imply endorsement.

References

Aber, J.D., McDowell, W.H., Nadelhoffer, K.J., Magill, A.H., Berntson, G., Kamekea, M., McNalty, S.G., Currie, W., Rustad, L., Fernandez, I., 1998. Nitrogen saturation in temperatate forest ecosystems: hypotheses revisited. Bioscience 48, 921–934.

Ågren, G., Bosatta, E., Magill, A.H., 2001. Combining theory and experiment to understand effects of inorganic nitrogen on litter decomposition. Oecologia 128, 94–98.

Ajwa, H.A., Dell, C.J., Rice, C.W., 1999. Changes in enzyme activities and microbial biomass of tallgrass prairie soil as related to burning and nitrogen fertilization. Soil Biol. Biochem. 31, 769–777.

Arnebrandt, K., Bååth, E., Söderström, B., 1990. Changes in microfungal community structure after fertilization of scots pine forest soils with ammonium nitrate or urea. Soil Biol. Biochem. 22, 309–312.

Arnebrandt, K., Bååth, E., Söderström, B., Nohrstedt, H.Ö., 1996. Soil microbial activity in eleven Swedish coniferous forests in relation to site fertility and nitrogen fertilization. Scand. J. For. Res. 11, 1–6.

Babiuk, L.A., Paul, E.A., 1970. The use of flourescein isothiocyanate in the determination of the bacterial biomass of grassland soil. Can. J. Microbiol. 16, 57–62.

Bailey, V.L., Peacock, A.D., Smith, J.L., Bolton Jr., H., 2002. Relationships between soil microbial biomass determined by chloroform fumigation-extraction, substrate-induced respiration and phospholipid fatty acid analysis. Soil Biol. Biochem. 34, 1385–1389.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Rapp, B.A., Wheeler, D.L., 2002. Genbank. Nucl. Acids Res. 30, 17–20.

Berg, B., 1986. Nutrient release from litter and humus in coniferous forest soils—a mini review. Scand. J. For. Res. 1, 359–369.

Biederbeck, V.O., Campbell, C.A., Ukrainetz, H., Curtin, D., Bouman, O.T., 1996. Soil microbial and biochemical properties after ten years of fertilization with urea and anhydrous ammonia. Can. J. Soil Sci. 76, 7–14.

Bock, E., Koops, H.P., Harms, H., 1989. Nitrifying bacteria. In: Schlegel, H.G., Bowien, B. (Eds.), Autotrophic Bacteria. Science Tech Publishers, Madison, WI, pp. 80–96.

Bowden, R.D., Davidson, E., Savage, K., Arabia, C., Steudler, P., 2004. Chronic nitrogen additions reduce total soil respiration and microbial respiration in temperate forest soils at the Harvard Forest. For. Ecol. Manage. 196, 43–56.

Bruns, M.A., Stephen, J.R., Kowalchuk, G.A., Prosser, J.I., Paul, E.A., 1999. Comparative diversity of ammonia oxidizer 16S rRNA gene sequences in native, tilled, and successional soils. Appl. Environ. Microbiol. 65, 2994–3000.

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.

Degrange, V., Bardin, R., 1995. Detection and counting of *Nitrobacter* populations in soil by PCR. Appl. Environ. Microbiol. 61, 2093–2098.

Di Giovanni, G.D., Watrud, L.S., Seidler, R.J., Widmer, F., 1999. Comparison of parental and transgenic alfalfa rhizosphere communities using Biolog GN metabolic fingerprinting and enterobacterial repetitive intergeneric consensus sequence-PCR (ERIC-PCR). Microb. Ecol. 37, 129–139.

Eady, R.R., 1991. The dinitrogen-fixing bacteria. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., Schleifer, K.-H. (Eds.), The Prokaryotes. Springer-Verlag, New York, NY, pp. 534–553.

Fenchel, T., King, G.M., Blackburn, T.H., 1998. Bacterial Biogeochemistry: The Ecophysiology of Mineral Cycling. Academic Press, San Diego, CA.

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

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

Galloway, J.N., Cowling, E.B., 2002. Reactive nitrogen and the world: 200 years of change. Ambio 31, 64–71.

Grundmann, G.L., Normand, P., 2000. Microscale diversity of the genus *Nitrobacter* in soil on the basis of analysis of genes encoding rRNA. Appl. Environ. Microbiol. 66, 4543–4546.

Hermansson, A., Lindgren, P.-E., 2001. Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR. Appl. Environ. Microbiol. 67, 972–976.

Hobbie, S.E., Vitousek, P.M., 2000. Nutrient limitation of decomposition in Hawaiian forests. Ecology 81, 1867–1877.

Ingham, E.R., Griffiths, R.P., Cromack Jr., K., Entry, J.A., 1991. Comparison of direct vs. fumigation incubation microbial biomass estimates from ectomycorrhizal mat and non-mat soils. Soil Biol. Biochem. 23, 465–471.

Ingham, E., Klein, D., 1984. Soil fungi: measurement of hyphal length. Soil Biol. Biochem. 16, 279–280.

Jackson, R.B., Linder, C.R., Lynch, M., Purugganan, M., Somerville, S., Thayer, S.S., 2002. Linking molecular insight and ecological research. Trends Ecol. Evol. 17, 409–414.

Kaye, J.P., Hart, S.C., 1997. Competition for nitrogen between plants and soil microorganisms. Trends Ecol. Evol. 12, 139–143.

Kirk, T.K., 1987. Enzymatic combustion: the microbial degradation of lignin. Annu. Rev. Microbiol. 41, 465–505.

Kolb, W., Martin, P., 1988. Influence of nitrogen on the number of N_2-fixing and total bacteria in the rhizosphere. Soil Biol. Biochem. 20, 221–225.

Ladd, J.N., Amato, M., Li-Kai, Z., Schultz, J.E., 1994. Differential effects of rotation, plant residue and nitrogen fertilizer on microbial biomass and organic matter in and Australian alfisol. Soil Biol. Biochem. 26, 821–831.

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

Lodge, D.J., Ingham, E.R., 1991. A comparison of agar film techniques for estimating fungal biovolumes in litter and soil. Agric. Ecosyst. Environ. 34, 131–144.

Magill, A.H., Aber, J.D., Hendricks, J.J., Bowden, R.D., Melillo, J.M., Steudler, P.A., 1997. Biogeochemical response of forest ecosystems to simulated chronic nitrogen deposition. Ecol. Appl. 7, 402–415.

Magill, A.H., Aber, J.D., Currie, W.S., Nadelhoffer, K.J., Martin, M.E., McDowell, W.H., Melillo, J.M., Steudler, P., 2004. Ecosystem response to 15 years of chronic nitrogen additions at the Harvard Forest LTER, Massachusetts, USA. For Ecol. Manage. 196, 7–28.

McAndrew, D.W., Malhi, S.S., 1992. Long-term N fertilization of a solonetzic soil: effects on chemical and biological properties. Soil Biol. Biochem. 24, 619–623.

Mendum, T.A., Sockett, R.E., Hirsch, P.R., 1999. Use of molecular and isotopic techniques to monitor the response of autotrophic ammonia-oxidizing populations of the β subdivision of the class *Proteobacteria* in arable soils to nitrogen fertilizer. Appl. Environ. Microbiol. 65, 4155–4162.

Micks, P., Aber, J.D., Boone, R.D., Davidson, E.A., 2004. Short-term soil respiration and nitrogen immobilization response to nitrogen applications in control and nitrogen-enriched temperate forests. For. Ecol. Manage. 196, 57–70.

Oved, T., Shaviv, A., Goldrath, T., Mandelbaum, R.T., Minz, D., 2001. Influence of effluent irrigation on community composition and function of ammonia-oxidizing bacteria in soil. Appl. Environ. Microbiol. 67, 3426–3433.

Porteous, L.A., Seidler, R.J., Watrud, L.S., 1997. An improved method for purifying DNA from soil for polymerase chain reaction amplification and molecular ecology applications. Mol. Ecol. 6, 787–791.

Purkhold, U., Pommerening-Rösser, A., Juretschko, S., Schmid, M.C., Koops, H.-P., Wagner, M., 2000. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: implications for molecular diversity survey. Appl. Environ. Microbiol. 66, 5368–5382.

Rotthauwe, J.-H., Witzel, K.-P., Liesack, W., 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. 63, 4704–4712.

Savage, K.E., Davidson, E.A., 2001. Interannual variation of soil respiration in two New England forests. Glob. Biogeochem. Cycl. 15, 337–351.

Schramm, A., de Beer, D., Wagner, M., Amann, R., 1998. Identification and activities in situ of *Nitrosospira* and *Nitrospira* spp. as dominant populations in a nitrifying fluidized bed reactor. Appl. Environ. Microbiol. 64, 3480–3485.

Shaffer, B.T., Widmer, F., Porteous, L.A., Seidler, R.J., 2000. Temporal and spatial distribution of the *nifH* gene of N_2 fixing bacteria in forests and clearcuts in western Oregon. Microb. Ecol. 39, 12–21.

Söderström, B., Bååth, E., Lundgren, B., 1983. Decrease in soil microbial activity owing to nitrogen amendments. Can. J. Microbiol. 29, 1500–1506.

Stephen, J.R., Kowalchuk, G.A., Bruns, M.-A.V., McCaig, A.E., Phillips, C.J., Embley, T.M., Prosser, J.I., 1998. Analysis of β-subgroup proteobacterial ammonia oxidizer populations in soil by denaturing gradient gel electrophoresis analysis and hierarchical phylogenetic probing. Appl. Environ. Microbiol. 64, 2958–2965.

Torsvik, V., Goksøyr, J., Daae, F.L., 1990. High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. 56, 782–787.

Ueda, T., Suga, Y., Yahiro, N., Matsuguchi, T., 1995. Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of nifH gene sequences. J. Bacteriol. 177, 1414–1417.

Vance, E., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass C. Soil Biol. Biochem. 19, 703–707.

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.

Vitousek, P.M., Howarth, R.W., 1991. Nitrogen limitation on land and in the sea. How can it occur? Biogeochemistry 13, 87–115.

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

Webster, G., Embley, T.M., Prosser, J.I., 2002. Grassland management regimens reduce small-scale heterogeneity and species diversity of α-proteobacterial ammonia oxidizer populations. Appl. Environ. Microbiol. 68, 20–30.

Widmer, F., Shaffer, B.T., Porteous, L.A., Seidler, R.J., 1999. Analysis of *nifH* gene pool complexity in soil and litter at a Douglas fir forest site in the Oregon Cascade mountain range. Appl. Environ. Microbiol. 65, 374–380.