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Simulated Nitrogen Deposition Causes a Decline of Intra- and Extraradical Abundance of Arbuscular Mycorrhizal Fungi and Changes in Microbial Community Structure in Northern Hardwood Forests

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Abstract

Increased nitrogen (N) deposition caused by human activities has altered ecosystem functioning and biodiversity. To understand the effects of altered N availability, we measured the abundance of arbuscular mycorrhizal fungi (AMF) and the microbial community in northern hardwood forests exposed to long-term (12 years) simulated N deposition (30 kg N ha^{-1} y^{-1}) using phospholipid fatty acid (PLFA) analysis and hyphal in-growth bags. Intra- and extraradical AMF biomass and total microbial biomass were significantly decreased by simulated N deposition by 36, 41, and 24%, respectively. Both methods of extraradical AMF biomass estimation (soil PLFA 16:1ω5c and hyphal in-growth bags) showed comparable treatment responses, and extraradical biomass represented the majority of total (intra-plus extraradical) AMF biomass. N deposition also significantly affected the microbial community structure, leading to a 10% decrease in fungal to bacterial biomass ratios. Our observed decline in AMF and total microbial biomass together with changes in microbial community structure could have substantial impacts on the nutrient and carbon cycling within northern hardwood forest ecosystems.

Key words: arbuscular mycorrhizal fungi; phospholipid fatty acid; nitrogen; mycelium; microbial community; sugar maple (*Acer saccharum*); forest; in-growth bags.

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Introduction

Nitrogen (N) plays a critical biological and biogeochemical role in the biosphere. Increased anthropogenic atmospheric deposition of reactive nitrogen causes unintended and often damaging changes in terrestrial ecosystems, including acidification and eutrophication, which can in turn drive changes in NPP, biodiversity, and community structure of many groups of organisms (Vitousek and others 1997). Mycorrhizal fungi and saprotrophs have been found to be sensitive to N deposition in many ecosystem types (Wallenda and Kottke 1998; Cunha and others 2002; Treseder 2004, 2008). Changes in these groups have consequences not only for biodiversity, but also for plant function and biogeochemical cycles of nutrients and carbon (for example, van der Heijden and others 1998).

Of the different classes of mycorrhizal fungi, arbuscular mycorrhizal fungi (AMF) are most common (Smith and Read 2008). Most plants, including the majority of agricultural crops and tree species of tropical origin, have a symbiotic relationship with AMF. However, in northern high-latitude forests ectomycorrhizal fungi (EMF) predominate (Smith and Read 2008). However, even in these ecosystems, some AMF tree species can be dominants with a wide geographic extent, for example, sugar maple (*Acer saccharum* Marshall) (Godman and others 1990). Yet few studies within these typically N-limited boreal and higher-latitude temperate forests have focused on the response of AMF to N deposition, and all have been short-term (<2 years) and/or had unrealistically high fertilization rates.

Mycorrhizal fungi play a pivotal role in plant carbon and nutrient balance, supplying nutrients to the plant in exchange for carbon they receive from their host (Smith and Read 2008). Thus, mycorrhizal fungi are sensitive to changes in soil and host nitrogen status. The effects of N-addition on AMF abundance have been studied in several different ecosystems and are generally negative (Treseder 2004 and references therein; van Diepen and others 2007). However, most of these N-addition studies have focused on the intraradical (within-root) abundance of AMF, are short-term, and have been performed in a lab or greenhouse setting.

Intraradical hyphae are responsible for exchange of resources with the host plant, whereas extraradical (outside of root) mycelium is responsible for the uptake of nutrients from the soil. Extraradical AMF can constitute up to 30% of the total microbial biomass in the soil (Leake and others 2004), but have not been as extensively studied as intraradical AMF, mainly because of the difficulty of estimating the abundance of extraradical mycelium within the soil.

Nitrogen effects on soil microbial communities could also have major impacts on forest ecosystems via shifts in C and nutrient cycling in the soils. In field studies, N-addition has been found to decrease total microbial biomass on average by 15% (Treseder 2008) and in some cases, changed microbial community structure (Compton and others 2004; Frey and others 2004). For example, Compton and others (2004) and DeForest and others (2004) found a decrease in total microbial biomass after N-addition for 11 and 7 years, respectively. However, only Compton and others (2004) found changes in microbial community structure. DeForest and others (2004) and the present study were conducted at the same study sites, providing the opportunity to compare medium and longer-term impacts of N inputs on microbial communities.

Phospholipid fatty acid (PLFAs) analysis and hyphal in-growth bags have recently been used to estimate AMF abundance. The PLFA 16:1ω5c has been successfully used as an indicator of AMF abundance for both roots and soil (for example, Olsson 1999; Olsson and Johansen 2000; Balser and others 2005; Gryndler and others 2006; van Diepen and others 2007). Hyphal in-growth bags containing sand are primarily colonized by mycorrhizal fungi and exclude roots using a fine mesh size and a matrix with no organic matter (Wallander and others 2001). Provided the bags are retrieved from the field prior to the accumulation of hyphal residues, their colonization by saprotrophic fungi is minimized (Wallander and others 2001).

In addition to estimating extraradical AMF abundance, PLFA analyses can simultaneously provide insight into the total microbial biomass and structure of the rest of the microbial community. PLFA analysis has been proven to successfully identify and estimate the biomass of groups of bacteria and fungi within the soil environment (Tunlid and White 1992; Frostegård and Bååth 1996). This analysis has also been applied to better understand changes in soil microbial communities caused by various disturbances (for example, Zak and others 1996; Waldrop and others 2004; Allison and others 2005).

In this study, we took advantage of a long-term simulated N deposition study in sugar maple dominated northern hardwood forests, the Michigan Gradient Study. In this study system, we examined the effects of long-term simulated N

deposition on the intra- and extraradical abundance of AMF using PLFA analyses and hyphal in-growth bags, and compared the two techniques. To our knowledge, this is the first study to address the intra- and extraradical AMF biomass simultaneously using the same measurement technique, giving us the opportunity to calculate their proportions at an ecosystem level. We also used marker PLFAs to determine soil microbial community responses to the imposed N deposition treatments. We hypothesized that both intra- and extraradical AMF and total microbial biomass would decrease and that we would observe changes in soil microbial community structure after 12 years of simulated N deposition.

Materials and Methods

Study Sites

Four sugar maple (*Acer saccharum* Marshall) dominated forest sites throughout Michigan, USA (Figure 1) were studied. Each site consisted of three untreated and three N-amended 30 × 30 m plots. To experimentally simulate atmospheric nitrogen (N) deposition, N has been applied annually since 1994 in six equal increments of $NaNO_3$ during the growing season, for a total of 30 kg N ha^{-1} y^{-1}. Ambient wet N deposition at the sites ranged from 3.0 kg N ha^{-1} y^{-1} near site A to 6.8 kg N ha^{-1} y^{-1} near site D in 2006 (NADP 2006). All sites have similar soil development (sandy spodosols), stand age, and plant composition, but differ in mean annual temperature and growing season length. More detailed information about the sites can be found by Burton and others (1991).

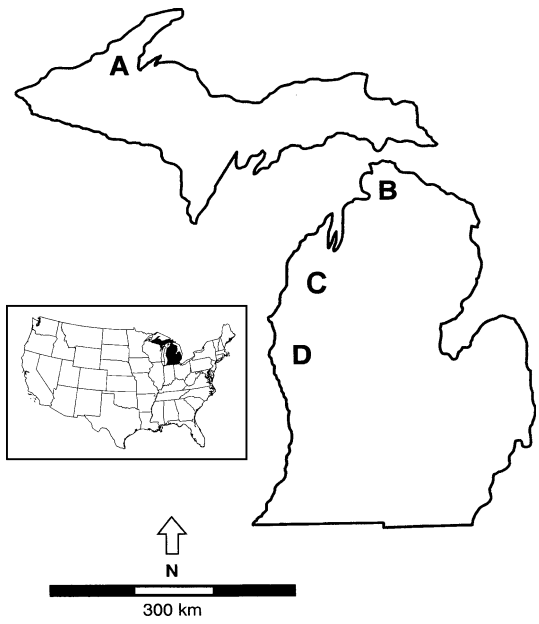

Figure 1. Locations of the study sites (*A–D*) in Michigan. The *inset* shows the location of Michigan (*filled area*) within the USA.

Soil and Root Sampling

For soil and root sampling, all the plots were divided into six equally sized subplots (10 × 15 m). Two paired soil cores, 2-cm diameter and 10-cm deep after removal of the litter layer, were taken randomly in each subplot in early October 2006. The first set of soil cores was washed through a 2- and 1-mm screen until most soil particles were removed. Maple (*Acer spp.*) roots, easily recognized by their unique beaded structure (Pregitzer and others 2002), were handpicked from the screens, cleaned more thoroughly with DI water, and sonicated for 5 min. The maple roots were divided up into two diameter classes, fine roots (<0.5 mm) and larger roots (>0.5 mm), and weights of both the classes were recorded. For each core, the fine maple roots were divided into two equal subsamples. One subsample was frozen and freeze-dried (Labconco, Freezone 4.5) for DNA-based community analysis (not discussed in this article), and the other subsample was composited at the plot level, frozen, freeze-dried and ground (SPEX Certiprep Mill, 8000 M), and used for PLFA analysis to estimate AMF intra-radical abundance.

The second set of soil cores was composited at the plot level, frozen, freeze-dried (Labconco, Freezone 4.5), sieved through a 2-mm sieve, and the smaller-than-2-mm fraction was mixed thoroughly. Subsequently, roots and litter particles were manually removed from a subsample of approximately 10-g mixed soil sample (<2 mm), and the remaining soil was used for phospholipid fatty acid (PLFA) analysis to estimate abundance of extraradical AMF and other members of the microbial community.

Hyphal In-Growth Bags

Hyphal in-growth bags were developed after Wallander and others (2001) using 50-μm mesh (Sefar Filtration Inc., Depew, NY, USA) filled with 130 g of Flint silica sand (>250 μm) (Faulks Brother Construction Inc., Waupaca, WI, USA). Combustion of a subsample of the sand in a muffle furnace (6 h at 600°C) confirmed that there was no measurable organic matter. The bags had a cylindrical shape, with an average length of 9.1 (±0.2) cm, diameter of 3.3 (±0.3) cm, and volume of 75 cm^3.

The in-growth bags were buried horizontally in the soil at the interface of the organic (Oa-horizon) and mineral (E horizon) layer at the beginning of

the growing season (May 2008) and harvested in September 2008. Each plot had five in-growth bags, one at each corner and the center of a 10 × 10 square in the center of the 30 × 30 m plot. At harvest, bags were placed in a zip-lock bag, stored on ice for transport, and frozen at −20°C within 12 h after harvest.

Wallander and others (2001) have shown that the mycelium of hyphal in-growth bags in a coniferous forest originated from EM fungi and not from saprotrophic fungi. We performed an additional experiment to check for potential saprotrophic mycelium growth within our hyphal in-growth bags. Trenched plots were created at one of our study sites by inserting a PVC tube (30-cm long and 20 cm in diameter) 25 cm into the soil, and consequently completely severing roots inside the tube from the plants, creating a non-mycorrhizal environment inside the tube. As an extra measure against plant roots, the opening at the bottom of the PVC tube was covered with 50-μm mesh. In November 2004, four paired bags were placed per plot, consisting of one hyphal in-growth bag in trenched soil (PVC tube), paired with one bag outside of the tube, totaling eight bags per plot. All bags were harvested in October 2005 and measured for hyphal biomass by the extraction method described below. Furthermore, PLFA analysis was performed on a subset of mycelia extracted from the in-growth bags outside of the tubes.

Hyphal Extraction from In-Growth Bags

The frozen in-growth bags were thawed, emptied into a large beaker to which approximately 600 ml of water was added, stirred, and decanted through a sieve with 50 μm mesh. This process was repeated 6 times, after which the hyphae were washed into a Petri-dish. The hyphae were further cleaned by multiple washings with DI water and handpicking of sand particles under a dissecting microscope. Cleaned hyphae were frozen and freeze-dried to obtain the dry weight. To check for mineral contamination after extraction, plot level composite samples were analyzed for C on a ThermoFinnigan Delta^{plus} Continuous-Flow Stable Isotope Ratio Mass Spectrometer. Hyphal biomass was further calculated on a volume basis using the volume of 75 cm^3 of the in-growth bags.

Lipid Extraction and Analysis

Lipids were extracted from 2.5 g of cleaned freeze-dried soil (<2 mm), 15 mg of freeze-dried ground roots, or 4 mg of freeze-dried ground hyphae using the protocol of van Diepen and others (2007).

The fatty acid 16:1ω5c was used as an indicator for AM fungi (Olsson 1999; Olsson and Johansen 2000; Allison and others 2005). PLFAs representing bacteria were 14:0, a15:0, i17:0, a17:0, cy19:0a, i15:0 (gram+), i16:0 (gram+), 16:1ω7 (gram−), cy17:0 (gram−), 18:1ω7 (gram−) (Allison and others 2005). For actinomycetes the PLFAs 10me16:0 and 10me18:0 were used, and total (non-AM) fungi were defined using phospho- and neutral lipid fatty acids 18:2ω6, 18:1ω9, and 20:4ω6 (Allison and others 2005). Total soil non-AM fungi will be referred to as saprotrophic fungi from here on. Although ectomycorrhizal fungi were likely present at our sites, standing biomass of trees associated with ectomycorrhizal fungi was on average only 6.2% (±2.7) of total standing biomass per site, and contribution of ectomycorrhizal fungal hyphae to soil fungal PLFA would therefore be relatively small. Total PLFA was calculated using all PLFAs values representing the different microbial groups including 15:0, 16:0, 18:0, 20:0, 20:1ω9c, 22:0, 24:0. Furthermore the ratio of cyclopropyl PLFAs (cy17:0 plus cy19:0) to their precursors (16:1ω7c plus 18:1ω7c) was calculated and used as an indicator for the physiological status of gram-negative bacterial communities with an increase indicating higher stress and carbon limitation (Allison and others 2005).

Estimation of AMF biomass in soil using PLFAs is complicated by soil bacteria which may also contain small amounts of PLFA 16:1ω5c (Olsson 1999). Phospholipids are a major constituent of the cell membrane, present in relatively stable ratios within microbial biomass, and are degraded quickly after cell death. This makes them suitable markers for live biomass where total PLFA concentration in soil is a direct measure for microbial biomass. In contrast, neutral lipid fatty acids (NLFA) are storage lipids, which can greatly vary in abundance over time, and are therefore better indicators of proportional allocation to storage (van Diepen and others 2007). Bacteria tend not to use lipids as energy reserves, and therefore have very low NLFA 16:1ω5c storage lipid content. In soils with an NLFA to PLFA 16:1ω5c ratio greater than one, PLFA 16:1ω5c can be assumed to be mainly derived from AMF (Olsson and others 1997; Olsson 1999; Olsson and Wilhelmsson 2000).

Calculations of AMF Intra- and Extraradical Biomass

To understand the effects of elevated N deposition on AMF biomass, the responses were calculated using three different metrics: (1) colonization

intensity is expressed as nmol of fatty acid 16:1ω5c per biomass of root or soil; (2) stand-level AMF biomass is expressed as nmol of fatty acid 16:1ω5c per volume of soil or hyphal biomass (from in-growth bags) per volume of sand; and (3) proportional allocation to AMF as nanomole of fatty acid 16:1ω5c or gram of hyphal biomass (from in-growth bags) per maple aboveground biomass. For calculation details, see van Diepen and others (2007). For the calculations of the proportional allocation metric, only maple aboveground biomass was used. At our sites, the trees black cherry (*Prunus serotina*) and white ash (*Fraxinus americana*) are also associated with AMF, and may have contributed to the extraradical AMF hyphal biomass. However, on average these two species represented $<5\%$ of the total standing aboveground biomass at our study sites in 2006, and statistical analysis including aboveground biomass of all AMF trees showed similar results when compared to using only maple aboveground biomass.

Statistical Analysis

Differences in dependent variables (all PLFA/NLFA values, AMF biomass from in-growth bags) between treatments were determined using a two-way ANOVA with N treatment ($n = 2$) and site location ($n = 4$) as factors. Transformations (natural logarithm, cosine, sine, and arctangent) were applied as appropriate to ensure a normal distribution and equal variances.

Differences in microbial community structure were analyzed using permutational multivariate analysis of variance (PERMANOVA, Anderson 2005) based on the Bray–Curtis distance measure. PERMANOVA does not provide a graphical data display, so the community data were visualized using biplots of Canonical Analysis of Principal Coordinates (CAP, Anderson 2004) output. CAP was performed with each treatment at each site as a separate group (total of eight groups).

Results

Verification of AMF in Hyphal In-Growth Bags

The 2005 pilot study showed that hyphal biomass from in-growth bags inside the PVC tube, assumed to be of saprotrophic origin, ranged from 5 to 29% (mean 16%) of the biomass of mixed mycorrhizal and saprotroph origin extracted from bags outside the tube. PLFA analyses on mycelia extracted from the in-growth bags outside the tubes indicated that 92% (±3.2) of total fungi (PLFA 16:1ω5c and 18:2 ω6,9) in untrenched bags was AMF (PLFA 16:1ω5c). We can therefore assume that our mesh bags were strongly dominated by AMF rather than saprotrophic fungi. In-growth bags from 2008 containing greater than 90% AMF hyphae as a percent of total fungal PLFA (PLFA 16:1ω5c and 18:2 ω6,9) had an average PLFA 16:1ω5c concentration of 1.2 (±0.1) nmol mg^{-1} dry hyphae. PLFA 16:1ω5c represented on average 32.5% (±1.4) of total PLFA (3.1 ± 0.2 nmol mg^{-1} dry hyphae), and AMF biomarker NLFA-to-PLFA ratio averaged 4.2 (±0.2).

Furthermore, the %C of the hyphal samples averaged 41.4% (±0.3), which is comparable with values found for fungal sporocarps found in other studies (for example, Hart and others 2006) and at our sites (L. T. A. van Diepen, unpublished data). The low variability in %C of the AMF hyphae extracted from the bags in combination with the comparable %C with fungal sporocarps confirmed that all mineral particles were removed and the total weight represented fungal mycelium only.

AMF Intra- and Extraradical Biomass

NLFA-to-PLFA ratios of 16:1ω5c fatty acid in roots (intraradical) and soil (extraradical) were greater than 1 on all plots (mean of 20 and 2.7, respectively), and the ratio did not differ among treatment ($P = 0.37$ and $P = 0.56$, respectively). Therefore, PLFA 16:1ω5c could be used as an estimator for AMF live biomass. NLFA-to-PLFA ratio of extraradical hyphae from the in-growth bags (4.2 ± 0.2) was closer to the soil than the root ratio.

Using indicator PLFA 16:1ω5c, AMF biomass was found to be significantly reduced by N-addition in maple fine roots and in soil and total biomass (intra- plus extraradical) for all three metrics (Table 1; Figure 2A–C, Appendix 1 in Supplementary material). The mean decrease differed among metrics for both intra- and extraradical AMF biomass, and ranged from 36 to 51% (Table 1).

Measurement of net extraradical hyphal biomass production using the in-growth bags also showed a significant mean decrease of 41% with chronic N-addition at the stand level and expressed as maple proportional allocation ($P = 0.019$ and $P = 0.008$, respectively) (Figure 2D). Mean extraradical hyphal biomass in the in-growth bags for control plots was 16.5 (±0.4) μg g^{-1} sand or 28.6 (±0.7) μg cm^{-3} sand compared to 9.7 (±2.4) μg g^{-1} sand or 16.9 (±4.1) μg cm^{-3} sand for N-amended plots.

Both the methods of estimating extraradical hyphal biomass showed an identical mean decline

Table 1. Nitrogen and Site Effects on Arbuscular Mycorrhizal Fungal Abundance

Metric	Measurement	*P* values						Mean N-effect (%)	
		N-addition		Site		N-addition × site			
		PLFA	NLFA	PLFA	NLFA	PLFA	NLFA	PLFA	NLFA
AMF colonization intensity[1]	Intraradical	0.003	0.002	0.946	0.929	0.02	0.01	−36	−28
	Extraradical	<0.001	0.05	0.09	0.01	0.02	0.05	−41	−28
Stand level AMF abundance[1]	Intraradical	0.01	<0.001	0.59	0.17	0.25	0.01	−47	−43
	Extraradical	<0.001	0.06	0.15	0.01	0.05	0.13	−41	−29
	Intra- and extraradical	<0.001	0.003	0.15	0.02	0.04	0.03	−42	−34
Proportional allocation to AMF[1]	Intraradical	0.003	<0.001	0.64	0.68	0.29	0.01	−51	−46
	Extraradical	<0.001	0.03	0.24	0.02	0.10	0.16	−44	−34
	Intra- and extraradical	<0.001	0.001	0.24	0.03	0.07	0.04	−45	−38
Proportion of intraradical AMF to total AMF		0.39	0.33	0.42	0.03	0.90	0.47	−13	−16

PLFA phospholipid fatty acid 16:1ω5c, NLFA neutral lipid fatty acid 16:1ω5c, AMF arbuscular mycorrhizal fungal, Mean N-effect negative numbers indicate a decrease with N-addition and positive numbers an increase.
[1]See "Methods" for a description of the metrics.

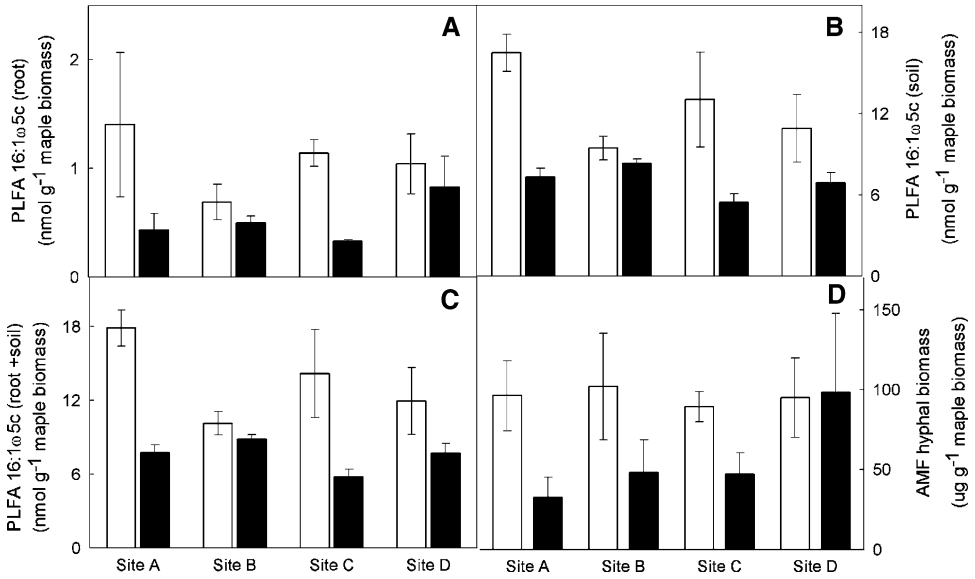

Figure 2. Mean maple proportional allocation to phospholipid fatty acid (PLFA) 16:1ω5c in maple (*Acer spp.*) fine roots (**A**), soil (**B**), and roots plus soil (**C**), and extraradical hyphal biomass in in-growth bags (**D**) for the four study sites by treatment (*open bars* control; *closed bars* N-amended). *Error bars* indicate 1 SE of the mean. Overall N treatment effect was significant in all cases (see Table 1 for details).

of 41% with simulated N-deposition. When plotting the values of soil PLFA 16:1ω5c from 2006 against hyphal biomass from the in-growth bags from 2008 expressed as maple proportional allocation, a significant ($P = 0.012$) positive linear relationship was found, but with a low R^2 of 0.25. This low R^2 was mainly caused by two extremely high values of the hyphal in-growth bags, and after removal of these two outliers, a much stronger and significant relationship was found ($P < 0.001$, $R^2 = 0.47$).

In both root and soil samples, the storage NLFA 16:1ω5c showed trends similar to PLFA 16:1ω5c, with the exception of stand level maple AMF extraradical abundance which had only a marginal decrease with N-addition (Table 1). N-addition on average reduced NLFA 16:1ω5c by 28% for both root and soil colonization intensity and by 38% for maple proportional allocation to total AMF biomass (Table 1).

For soil and root AMF abundance, there were some different treatment responses among sites as indicated by significant treatment by site interactions for PLFA and NLFA 16:1ω5c values for all three metrics (Table 1). In general, AMF biomass was more affected by N-addition at sites A and C when compared to sites B and D. Significant site effects were only observed for the neutral lipid

measurements of AMF extraradical C storage, and total (soil + root) AMF biomass (Table 1).

The percentage of intraradical AMF versus intra- plus extraradical AMF averaged 7.5% (±2.1%) for phospholipids and 35% (±5.3%) for neutral lipids. The major part of total AMF biomass within the ecosystem was thus found in the soil. No treatment effects were found on the proportion of intraradical AMF when compared to total AMF biomass (Table 1), thus both intra- and extraradical AMF biomass estimates were equally negatively affected by N-addition.

Microbial Biomass and Community

Total microbial biomass, represented by total soil PLFA, decreased significantly (24%) with simulated N deposition, with only a marginal treatment by site interaction (Table 2). Among the different microbial groups, as defined by PLFA analysis, all had significant negative responses to simulated N-deposition, except for gram-positive bacterial phospholipids, and fungal (not including AMF) neutral lipids which showed no significant treatment effect (Table 2; Appendix 1 in Supplementary material: all soil PLFA values). Total bacterial PLFA and AMF biomarker 16:1ω5c represented on average 65 and 3.0% of total PLFA, respectively, and 15 and 17% of total NLFA, respectively. This confirmed the fact that bacteria have low levels of NLFA, whereas AMF represented a large portion of total NLFA. The ratio of cyclopropyl PLFAs (cy17:0 and cy19:0) to their precursors (16:1ω7c and 18:1ω7c) increased significantly by a mean of 25% with simulated N deposition (Table 2) indicating higher stress and carbon limitation of the gram-negative bacteria.

The structure of the microbial community was also significantly affected by treatment ($P = 0.0007$), and differed among sites ($P = 0.0001$) (Figure 3), largely because of a decrease in the fungal to bacterial ratio (Figures 3, 4A; Table 2). The decline was mostly driven by declines in the AMF to bacterial biomass ratio (Table 2; Figure 4B). Chronic N deposition only marginally affected the saprotrophic fungal to bacterial biomass ratio (Table 2; Figure 4C) and the saprotrophic to AM fungal ratio increased significantly with N-addition (Table 2).

Discussion

Decline in AMF Intra- and Extraradical Biomass

In the present study, simulated N deposition suppressed both intra- and extraradical AMF biomass as measured by PLFA 16:1ω5c and hyphal in-growth bags. We found similar results for intraradical AMF biomass at these sites in 2005 (van Diepen and others 2007). In 2001, extraradical AMF biomass measured as soil PLFA 16:1ω5c was not found to be significantly reduced by N-addition ($P = 0.121$), but there was a trend in this direction (DeForest and others 2004).

Table 2. Nitrogen and Site Effects on Abundance of Different Soil Microbial Groups

Microbial group	Fatty acid[1]	*P* values			Mean N-effect (%)
		N-addition	Site	N-addition × site	
Total microbial biomass	Phospholipid	0.02	0.43	0.05	−24
	Neutral lipid	0.02	0.33	0.06	−14
All bacteria	Phospholipid	0.03	0.58	0.08	−22
Gram+ bacteria	Phospholipid	0.42	0.64	0.20	−22
Gram− bacteria	Phospholipid	0.01	0.24	0.12	−29
Actinomycetes	Phospholipid	0.03	0.88	0.18	−21
Fungi (no AMF)	Phospholipid	0.009	0.05	0.02	−27
	Neutral lipid	0.34	0.20	0.16	−24
Ratio TOT_F:B	Phospholipid	<0.001	0.003	0.07	−10
Ratio AMF:B	Phospholipid	<0.001	0.003	0.07	−24
Ratio SapF:B	Phospholipid	0.07	<0.001	0.08	−7
Ratio SapF:AMF	Phospholipid	0.001	0.74	0.27	+25
	Neutral lipid	0.99	0.06	0.66	+0.2
Cyclopropyl:precursors	Phospholipid	0.002	0.02	0.91	+24

TOT_F:B total fungal to bacterial biomass, AMF:B arbuscular mycorrhizal fungal to bacterial biomass, SapF:B, saprotrophic fungal to bacterial biomass, SapF:AMF saprotrophic fungal to arbuscular mycorrhizal fungal biomass. Mean N-effect: negative numbers indicate a decrease with N-addition and positive numbers an increase.
[1]For overview values all fatty acids, see Appendix 1 in Supplementary material.

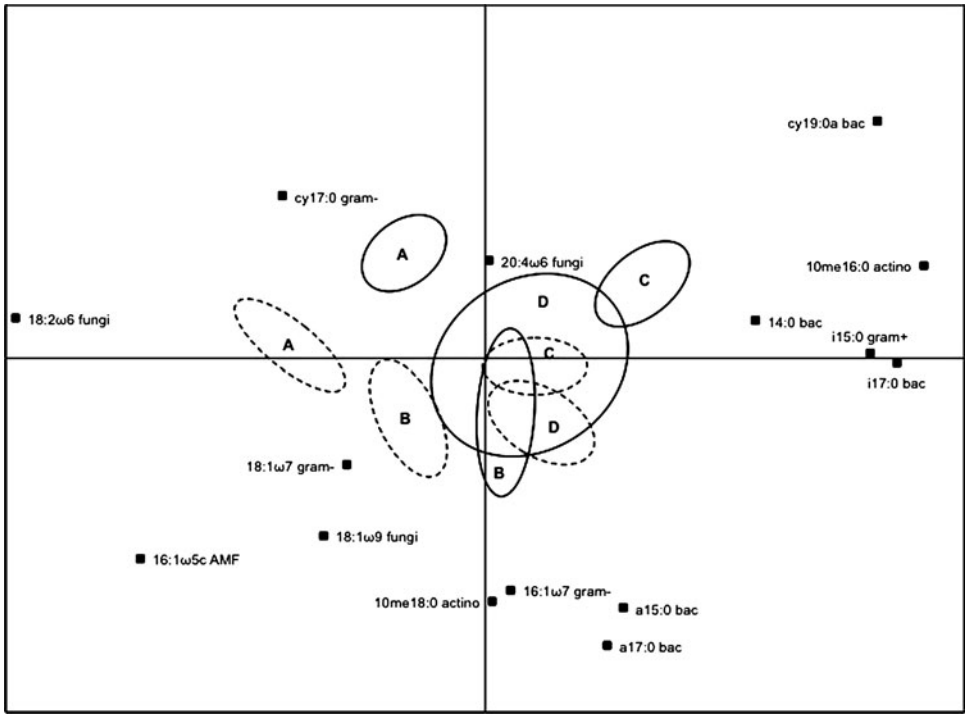

Figure 3. Canonical analysis of principal coordinates (CAP) biplots based on Bray–Curtis distance measure of microbial community structure of plots at treatment by site level based on phospholipid fatty acids (PLFAs) (*dashed circles* control, *solid circles* N-amended, sites denoted by *capital letters*). First two axes explain 74% of total variation among plots.

This disparity in extraradical AMF biomass response to N-addition between 2001 and 2006 could be caused by a lag-period in the effect of N-addition or cumulative dose effect, as was also observed for soil respiration at our sites (Burton and others 2004).

Effects on mycorrhizal abundance might increase with the duration of N addition or total dose. In contrast to our long-term study, the short-term field studies that analyzed both intra- and extraradical AMF biomass showed no decline in AMF (Table 3). Garcia and others (2008) and Treseder and others (2007) found an increase in intraradical AMF colonization and no effect on extraradical AMF mycelium in a temperate forest (1 year of 100 kg N ha^{-1} y^{-1}) and in a boreal forest (2 years of 100 kg N ha^{-1} y^{-1}), respectively (Table 3).

The effects of long-term N-addition are more likely to be negative, but can vary widely among studies. This variation might be related to the type of ecosystem that was studied in interaction with length of N-addition and cumulative dose (Table 3). For example, a tallgrass prairie had increased biomass production of intra- and extraradical AM fungal mycelium after 9 years of 100 kg ha^{-1} y^{-1} N-addition (dose of 900 kg N, Table 3; Eom and others 1999). In contrast, in a desert Johnson and others (2003) found significantly decreased AMF hyphal lengths in the soil after only 3 years of N-addition (100 kg ha^{-1} y^{-1} N, dose of 300 kg N) (Table 3). In an ecosystem similar to our study sites Phillip and Fahey (2007) showed a reduction in intraradical AMF abundance with 2 years of 167 kg ha^{-1} y^{-1} N-addition (Table 3). Their cumulative N dose (334 kg) was comparable to the dose in our study (360 kg N).

One reason for the variety of responses could be differences in absolute and relative availability of different nutrients at the beginning of the experiment in combination with the cumulative N dose. The combination of these two factors could influence temporal dynamics of plant nutrient limitation, which in turn affect allocation to mycorrhizal fungi (Treseder and Allen 2002). For example, the increased AMF biomass that Eom and others (1999) still observed after 9 years of N-addition could be explained by lower soil inorganic phosphorus (P) concentration in the tall grass-prairie driving P limitation and consequent increased belowground allocation. Experimental P limitation has been found to increase allocation to mycorrhizal fungi (for example, Wallander and Nylund 1992; Treseder and Allen 2002).

In our study, no shortage of P in the foliage of N-amended plots was found (L. T. A. van Diepen, unpublished data), and therefore no increased allocation to AMF would be expected. Soil pH can also affect nutrient availability, but at our sites no significant changes of soil pH have been found after 14 years of N addition (D. R. Zak, unpublished data), and in addition no significant changes in other foliar nutrients, for example, Ca, K, Mg, Na, Al, Cu, Zn were found (L. T. A. van Diepen,

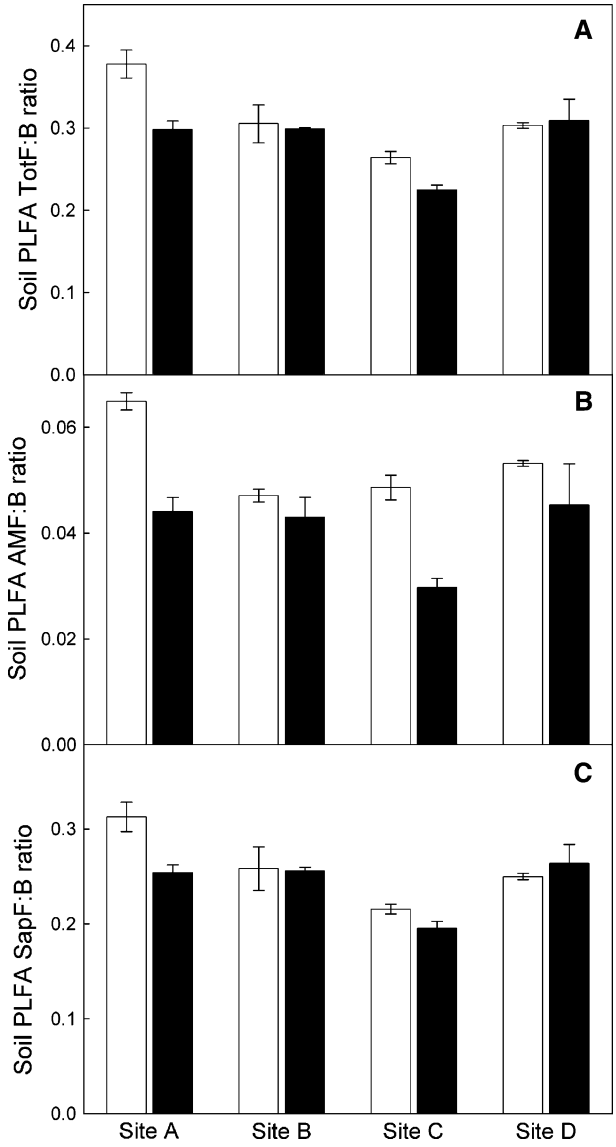

Figure 4. Mean **A** total fungal to bacterial biomass ratio, **B** arbuscular mycorrhizal fungal (AMF) to bacterial biomass ratio, and **C** saprotrophic to bacterial biomass ratio within soil for the four study sites by treatment as measured by phospholipid fatty acids (PLFAs) (*open bars* control, *closed bars* N-amended). *Error bars* indicate 1 SE of the mean.

unpublished data). Consistent with the nutrient limitation hypothesis, we found that elevated N deposition in our sites, combined with sufficient P and other nutrients, has led to reduced proportional allocation of C to AMF. Decreased soil respiration (Burton and others 2004) together with increased foliar N and tree growth (Pregitzer and others 2008) in response to elevated N deposition appear to confirm lower belowground carbon investment across our study sites.

Effect of Methods on AMF Abundance Measurements

Soil PLFA 16:1ω5c and in-growth bags gave comparable treatment responses, but different estimates of AMF biomass. Using the average PLFA 16:1ω5c content of AMF extraradical hyphae (1.2 ± 0.1 nmol mg^{-1} dry hyphae) and the soil PLFA 16:1ω5c concentrations, calculations of hyphal concentration (g hyphae m^{-3} soil) were on average 120 times (ranging from 24 to 284) higher with soil PLFA 16:1ω5c measurements than concentrations found with hyphal in-growth bags. Our conversion factor of 1.2-nmol PLFA 16:1ω5c mg^{-1} dry hyphae was comparable with other studies (Olsson and Johansen 2000; Olsson and Wilhelmsson 2000). A possible reason for this disparity could be the simulated soil environment of the bags. The bags contained sand without any organic matter and nutrients and therefore would attract less hyphal colonization when compared to normal soil. However, because some soil bacteria contain small amounts of PLFA 16:1ω5c, this could possibly cause an increased soil PLFA 16:1ω5c concentration. Better estimates of 16:1ω5c values in soil bacteria are needed to know the contribution of bacteria to 16:1ω5c concentrations in soil (Joergensen and Wichern 2008). Our soil PLFA 16:1ω5c values were comparable with soil PLFA 16:1ω5c values found in other studies from prairie and agricultural soils (Allison and others 2005; Gryndler and others 2006, respectively), but 50× higher than measurements done in a Hawaiian forest soil (Balser and others 2005). Furthermore, the contribution of AMF biomarker 16:1ω5c to total soil PLFA at our study sites was comparable with results found in other forest soils (3 ± 0.6 and $2.9 \pm 1.1\%$ (SD), respectively) (Joergensen and Wichern 2008).

Intra- Versus Extraradical AMF Biomass

At the stand level most of the live AMF biomass ($92.5 \pm 2.1\%$ of phospholipid 16:1ω5c) was extraradical, whereas stored energy (neutral lipid 16:1ω5c) was more equally divided over intra- and extraradical AMF biomass ($65 \pm 5.3\%$ extraradical). No comparisons could be made with other studies, because they used different measurements methods and units for assessing intra- versus extraradical AMF biomass. In a review, Leake and others (2004) found that most of the photosynthetically derived carbon of the host plant is allocated to the extraradical hyphae of associated mycorrhizae.

Table 3. Comparison of Different Studies of N-addition Effects on AMF Intra- and/or Extraradical Biomass

Study	Ecosystem	Application rate (kg N ha^{-1} y^{-1})	Length of study (year)	Cumulative dose (kg N ha^{-1})	N-addition effect on AMF biomass	
					Intraradical	Extraradical
Garcia and others (2008)	Temperate forest	100	1	100	↑	↔
DeForest and others (2004)[1]	Temperate forest	30	7	210	Not measured	(↓)
Treseder and others (2007)	Boreal forest	100 (200 in 1st year)	2	300	↑	↔
Johnson and others (2003)	Desert	100	3	300	Not measured	↓
Van Diepen and others (2007)	Temperate forest	30	11	330	↓	Not measured
Phillips and Fahey (2007)	Temperate forest	167	2	334	↓	Not measured
This study	Temperate forest	30	12	360	↓	↓
Eom and others (1999)	Tallgrass prairie	100	9	900	↑	↑
Johnson and others (2003)	Agricultural	120	9	1080	Not measured	↓
	Agricultural	170	10	1700	Not measured	↓
Gryndler and others (2006)	Agriculture	63–91	48	3024–4368	↓	↓

↓ Significant decrease, ↔ no significant change, ↑ significant increase, (↓) downward non-significant trend.
[1]The same study sites as in this study, but sampled in 2001.

The significantly reduced proportional allocation of C to extraradical AMF we found with N deposition represented AMF from all AMF-associated plants present at our sites, including any understory plant species. We realize that our calculations for C allocation to intraradical AMF may not reflect the complete intraradical AMF community, because only maple roots were sampled. On average 90% of standing biomass at our sites is represented by maple trees (sugar and red maple). Although a multi-aged understory has developed with a small percentage of AMF associated plants species other than maple, maple is the dominant tree species in all size classes of tree stems. No quantitative analysis of understory species has been performed to understand their contribution to root biomass, but our sites have a sparse understory dominated by maple seedling regeneration. Furthermore, no significant effects of N on total and fine root biomass have been observed at our study sites (Burton and others 1991). By not including all roots from AMF-associated plant species in our analysis, we could have underestimated the stand level AMF biomass and proportional allocation to AMF. However, we can conclude that simulated N deposition significantly reduced AMF root colonization of maple trees, which represented the majority of the aboveground, and most likely also belowground, biomass at our sites.

Microbial Community

The decrease in total microbial biomass and lower fungal to bacteria ratio that we observed with N-addition has been found in several other studies (Treseder 2008). Our reduction in total microbial biomass was very similar to the mean response found for temperate forests by Treseder (2008) (mean of treatment divided by mean of control = 0.76 and 0.80, respectively). Wallenstein and others (2006) also showed a significant decrease in total soil microbial biomass and fungal to bacterial activity ratio in three different long-term N-addition experiments in forest ecosystems. In contrast, two studies in a boreal forest ecosystem did not find an effect of short-term N fertilization on microbial biomass and fungi to bacteria ratio (Allison and others 2008), or extraradical AMF biomass (Treseder and others 2007) indicating that long-term N-addition might be needed to observe some of the effects of increased N deposition. This interpretation is supported at our study sites, where N-effects seen by DeForest and others (2004) after 7 years were of a smaller magnitude than those seen after 12 years. Treseder (2008) also found that microbial biomass decreased with increasing N-addition and duration within the 29 studies of her meta-analysis.

All the different groups of the microbial community were negatively affected by simulated N deposition, with fungi the most, followed by gram-negative bacteria. The effect on the latter was also observed by the higher ratio of cyclopropyl (cy17:0 plus cy19:0) to their precursors (16:1ω7c plus 18:1ω7c) in the N-amended plots, indicating that the gram-negative bacteria were in the stationary growth phase, possibly because of stress caused by C limitation (Allison and others 2005).

Reduced saprotrophic fungal biomass could contribute to changes in decomposition. In our study system, the reduction in saprotrophic fungal biomass is paralleled by decline in lignolytic enzyme activity (DeForest and others 2004) and increases in both DOC export (Pregitzer and others 2004) and soil C storage (Pregitzer and others 2008). Lignolytic activity is primarily associated with saprotrophic fungi. Berg and Matzner (1997) found that in later stages of litter decomposition, when the decomposition rate is more dependent on lignin concentration, increased N deposition and litter N content retard decomposition. The increase in lignin concentration together with a decrease in lignolytic enzyme activity can thus reduce decomposition rates in the later stages, and our data suggest that this response could be driven at least in part by changes in saprotrophic fungal biomass rather than by decreased enzyme activity alone.

Ecosystem Implications of AMF and Microbial Community Change

Reductions in mycorrhizal extraradical mycelial length could lead to reduced soil resource supply to hosts. In particular, reduced hyphal length could lead to increases in water stress in hosts (Stahl and others 1998; Augé 2004), reduced protection against root pathogen attack and uptake of toxic metals (Leake and others 2004), and shortage of phosphorus and other nutrients leading to reduced plant health (Smith and Read 2008). In addition to their role in soil resource acquisition, AMF might also affect SOM cycling. AMF can make up a large part of the microbial biomass, especially the extraradical mycelium (Leake and others 2004). AMF hyphal walls also contain a glycoprotein, glomalin, which has a very long residence time in soil, and can represent between 3 and 8% of soil organic carbon (SOC) (Rillig and others 2001) and is also believed to be important in soil aggregate formation, leading to physical protection of additional SOC (Rillig 2004). A decrease in AMF extraradical biomass could therefore have large consequences for soil organic matter content. However, the relationship between the amount of hyphae and the glomalin production is not straightforward, differing among AMF species and the nutrient status of the soil (Treseder and Turner 2007). Also, in the present study soil C is increasing (Pregitzer and others 2008), suggesting that other factors, such as a decrease in the activity of lignolytic saprotrophic fungi (DeForest and others 2004) might more than counteract effects of AMF and glomalin production on SOM stabilization.

Soil microbes play a critical role in nutrient cycling by mineralizing organic matter into inorganic nutrients which are then available for uptake by plants. The observed decline in microbial biomass could therefore affect the decomposition of organic matter negatively and alter nutrient and carbon cycling within these forest ecosystems. This decline is consistent with Zak and others (2008) who reported an increase in organic matter with no changes in organic matter input, indicating slower decomposition.

In conclusion, after 12 years of simulated N deposition both the intra- and the extraradical AMF biomass and total microbial biomass decreased significantly in sugar-maple-dominated hardwood forests. PLFA analysis and hyphal in-growth bags provided similar estimates of the effect of N-addition on AMF extraradical biomass and are thus comparable methods for measurements of AMF extraradical response to N. The microbial community structure was also different under N-addition and was dominated by a decrease in fungal to bacterial biomass ratios. The difference in results found for microbial community structure after 7 and 12 years of N-addition suggests that a lag or cumulative dose effect exists in community response to N addition. Our observed declines in fungal biomass and fungal to bacterial ratio could have significant implications for both nutrient and carbon cycling within sugar-maple-dominated forest ecosystems.

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