THE ROLE AND DIVERSITY OF ARBUSCULAR MYCORRHIZAL FUNGI IN *ACER SACCHARUM* DOMINATED FOREST ECOSYSTEMS UNDER NATURAL AND N-AMENDED CONDITIONS

By
Linda van Diepen

A DISSERTATION

Submitted in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

(Forest Science)

MICHIGAN TECHNOLOGICAL UNIVERSITY
2008

This dissertation, "The role and diversity of arbuscular mycorrhizal fungi in *Acer saccharum* dominated forest ecosystems under natural and N-amended conditions," is hereby approved in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in the field of Forest Science.

School of Forest Resources and Environmental Science-Forest Science

Signatures:

Dissertation Advisor _____

Erik Lilleskov

Dissertation Co-advisor _____

Kurt Pregitzer

Dean _____

Margaret Gale

Date _____

Table of contents

Chapter 1

Van Diepen, L.T.A., Lilleskov, E.A., Pregitzer, K.S, Miller, R.M., 2007. Decline of arbuscular mycorrhizal fungi in northern hardwood forests exposed to chronic nitrogen additions. New Phytologist 176: 175-183.

Published paper, permitted to use for other publications of own work (see appendix 1).

Chapter 2

Effects of chronic nitrogen additions on diversity and community composition of arbuscular mycorrhizal fungi in northern hardwood forests.

Manuscript, in progress

Chapter 3

Chronic nitrogen addition causes a decline of intra- and extraradical abundance of arbuscular mycorrhizal fungi and changes in microbial community composition in northern hardwood forests.

Manuscript, in progress

Chapter 4

Effects of chronic nitrogen deposition on respiration of extraradical mycelium of arbuscular mycorrhizal fungi in northern hardwood forests.

Manuscript, in progress

Summary

Human activities have altered global nitrogen (N) deposition and fixation through fossil fuel combustion, the use of fertilizers, and increases in N-fixing agricultural crops. Mycorrhizal fungi make up a large part of the microbial biomass in terrestrial ecosystems and play a pivotal role in plant carbon and nutrient balance, supplying nutrients, including nitrogen, to host plants in exchange for carbon. This mycorrhizal symbiosis could therefore be influenced by changes in nitrogen deposition, which in turn could alter not only nutrient cycling, but also carbon cycling within an ecosystem.

The main goal of my study was to assess changes in the functioning of arbuscular mycorrhizal fungi (AMF) in an ecosystem exposed to elevated levels of nitrogen. This was addressed using four established long term research sites looking at the effects of altered N-availability in northern hardwood forests dominated by sugar maple (*Acer saccharum*). Sugar maple belongs to one of the few dominant north temperate tree genera that form a symbiotic relationship with arbuscular mycorrhizal fungi (AMF), and forms extensive stands in northern temperate biomes.

The first objective was to gain basic knowledge of changes in AMF presence with N addition in northern hardwood forests. This was addressed by estimating the AMF abundance within the roots of the dominant tree species, sugar maple. The abundance was measured using a traditional root staining technique with ocular estimation, as well as analyzing roots for the AMF indicator fatty acid 16:1ω5c in phospholipid (biomass indicator) and neutral lipid (lipid storage indicator) fractions. AM fungal biomass, storage structures and lipid storage declined in response to N addition measured by both methods. This pattern was found when AM response was characterized as colonization intensity, on an areal basis and in proportion to maple aboveground biomass. The phospholipid fraction of the fatty acid 16:1ω5c was positively correlated with total AMF colonization and the neutral lipid fraction with vesicle colonization. The fatty acid (phospho- and neutral lipid fraction) 16:1ω5c was found to be a good indicator for AMF active biomass and stored energy, respectively.

The observed decrease in AMF abundance in roots could be associated with 1) a decrease in all species present, 2) a change in relative abundance of the associated species, or 3) a complete change in community composition. My second objective therefore was to identify the AMF species present within the roots and their diversity among treatments. I performed molecular DNA-based AMF community analyses on maple roots targeting the 18S rDNA region using fungal specific primers. Changes in the AMF community composition were observed with N-amendment, and effects on AMF species diversity differed among sites. Over 80% of all the AMF clones present in the roots were represented by seven dominant OTU's (Operational Taxonomic Units). Some of the OTU's declined in response to N-amendment, some increased and a few were unaffected by N-amendment. Other studies on AMF species have found that some AMF taxa that are abundant at high N-levels are less beneficial or even detrimental to the host plant, which is reflected through decreased nutrient uptake efficiency or an increased carbon cost for the host plant.

The actual nutrient uptake in a mycorrhizal system takes place in the extraradical mycelium of AMF. Hence, when studying nutrient cycling within ecosystems, the function of both the intra- and extraradical AMF are of importance. My third objective therefore was to study the effects of N-amendment on both intra- and extraradical AMF biomass simultaneously including the rest of the microbial biomass within the soil. Root and soil samples, taken as paired samples, were analyzed for phospho- and neutral lipid fatty acids (PLFA and NLFA). Intra- and extraradical AMF biomass were decreased equally by N-amendment as measured by AMF indicator fatty acid 16:1ω5c. Furthermore, total microbial biomass decreased and a change in microbial community composition was found under N-amendment. The composition change was dominated by a decrease in fungal to bacterial biomass ratios. The largest portion of AMF biomass within our system was represented by extraradical mycelium (ERM). Other studies have estimated ERM provides up to 30% of the total microbial biomass. ERM have a substantial amount of photosynthetically derived carbon flowing through them, thus the observed changes in ERM biomass could greatly influence soil respiration.

Therefore my fourth and last objective was to estimate the production and respiration rate of AMF extraradical mycelium biomass. Hyphal in-growth bags were designed that would allow ERM but no root colonization and minimize ERM of saprotrophic fungi, by using mesh of 50μm and sand without organic matter content, respectively. Bags were buried in the soil for one growing season to allow colonization of ERM and measured for CO_2 flux at harvest at the end of the growing season. A similar mean reduction of AMF extra-radical biomass with N-amendment was found using the in-growth bags compared to the soil PLFA 16:1ω5c measurements. Hyphal in-growth bag CO_2 flux was not significantly decreased by N-amendment, but a trend was seen at two sites, and an average decrease of 7% was found. However, hyphal in-growth bags CO_2 flux was positively related to hyphal biomass, which suggests that AMF hyphal CO_2 flux is mainly controlled by the biomass of AMF mycelium.

In conclusion, increased chronic nitrogen deposition as simulated by long term N-amendment had a negative effect on both intra- and extraradical AMF biomass, total microbial biomass, fungal to bacterial biomass ratio, and AMF CO_2 efflux in a northern hardwood forest dominated by sugar maple. The observed decrease in AMF abundance correlated with a decrease in AMF CO_2 flux suggests reduced carbon (C) allocation to these fungi or a direct soil N-mediated decline. The decrease in fungal to bacterial biomass ratio could have been caused by a change in organic matter quality, specifically a decrease in C:N ratio, which has been observed in the litter at our sites. The decrease in overall microbial biomass could have negative effects on soil organic matter decomposition. Consistent with this, a decreased rate of organic matter decomposition has been observed at our study sites. Furthermore N-amendment changed intraradical AMF community composition, with some taxa increasing while others decreased in abundance with N-amendment. Given that functional diversity exists among AMF species, this change in community composition could have implications for the functioning of this type of ecosystem. Our observed decrease in AMF and microbial biomass together with changes in intraradical AMF and soil microbial community composition with N-amendment thus has the potential to substantially change both nutrient and carbon cycling within northern hardwood forests. Further investigations

should focus on effects of additional environmental variables on the AMF biomass and community composition, such as foliar nutrient content. These analyses could reveal some indirect effects of increased N-amendment and could be useful for the creation of models predicting changes in fungal biomass and communities and plant-fungal relationships.

Chapter 1 Decline of arbuscular mycorrhizal fungi in northern hardwood forests exposed to chronic nitrogen additions

Summary

Arbuscular mycorrhizal (AM) fungi are important belowground carbon (C) sinks that can be sensitive to increased nitrogen (N) availability. The abundance of AM fungi was estimated in maple (*Acer spp.*) fine roots following more than a decade of experimental additions of N designed to simulate chronic atmospheric N deposition. Abundance of AM fungi was measured by staining and ocular estimation, as well as by analyzing for the AMF indicator fatty acid 16:1ω5c in phospholipid (biomass indicator) and neutral lipid (lipid storage indicator) fractions. AM fungal biomass, storage structures and lipid storage declined in response to N addition measured by both methods. This pattern was found when AM response was characterized as colonization intensity, on an areal basis and in proportion to maple aboveground biomass. The phospholipid fraction of the fatty acid 16:1ω5c was positively correlated with total AMF colonization and the neutral lipid fraction with vesicle colonization. Decreased AMF abundance with simulated N deposition suggests reduced C allocation to these fungi or a direct soil N-mediated decline. The fatty acid (phospho- and neutral lipid fraction) 16:1ω5c was found to be a good indicator for AMF active biomass and stored energy, respectively.

Introduction

Human activities have dramatically increased atmospheric nitrogen (N) deposition across large regions of the world, and rates of atmospheric N deposition are still increasing (Galloway *et al.*, 1994; Vitousek *et al.*, 1997). Deposition rates can exceed 75 kg N ha^{-1} yr^{-1} in regions downwind of large industrial complexes or intensive agriculture in the U.S. and Europe (Dise & Wright, 1995; Cunha *et al.*, 2002). Within the U.S., the northeastern region is the area with the highest rates of N deposition, with rates

routinely exceeding 10 kg N ha^{-1} yr^{-1} (NADP, 2006). Atmospheric N deposition can significantly impact natural ecosystems by increasing acidification and eutrophication, leading to a decrease in biodiversity and altered ecosystem function (Vitousek *et al.*, 1997).

Mycorrhizal fungi make up a large part of the microbial biomass in terrestrial ecosystems and are important suppliers of nutrients to plants, especially in nutrient-poor environments such as boreal and temperate forests (Smith & Read, 1997). Increasing amounts of N deposition could therefore have a potential effect on the presence and functioning of mycorrhizal fungi. Increased atmospheric N deposition generally has a negative effect on the abundance of mycorrhizal fungi (Wallenda & Kottke, 1998; Treseder, 2004). However, most studies in temperate forests have involved ectomycorrhizal fungi. Arbuscular mycorrhizal (AM) fungal abundance in temperate deciduous forests has been examined in only two multi-year N-fertilization studies (Hutchinson *et al.*, 1998; Lansing, 2003) with a range of results reported for different tree species. Both of these studies used N addition levels much higher than anthropogenic atmospheric N deposition and were carried out for a maximum of four years, so there is a need for investigations covering longer periods at realistic deposition levels.

The Michigan Gradient Study offers a rare opportunity to study the long-term effects of altered N-availability on AM fungi (AMF) in the roots of maple trees (*Acer spp.*). This study has applied experimentally simulated N deposition since 1994 to *Acer saccharum* (sugar maple) dominated hardwood forests. Sugar maple belongs to a dominant temperate tree genus forming a symbiotic relationship solely with AM fungi. Previous results in this study have shown a change in stand-level C allocation across sites. Of particular interest in the present context, N-amended plots showed decreased soil respiration (Burton *et al.*, 2004) not explained by changes in root biomass or root respiration (Burton *et al.*, 2004) or microbial biomass or microbial respiration in the mineral soil (Zak *et al.*, 2006). Therefore the present study focuses on the intraradical response of AM fungi, which can be direct recipients of a significant fraction of NPP and are known to be responsive to N inputs. The abundance of extraradical hyphae, the other active pool, will be addressed in a subsequent study.

The abundance of AM fungi has traditionally been quantified microscopically using stained roots (% root colonization), estimation of extraradical hyphal length and/or spore counts. These techniques are time consuming and can be subjective; they also do not provide reliable estimates of living biomass (Allison & Miller, 2004). Fatty acid (phospho- and neutral lipids) analysis has recently been used to quantify AM fungi. AM fungi have a much higher level of the fatty acid 16:1ω5c compared to other fungi and this fatty acid is not present in plant cells. It has been successfully used as an indicator of AMF abundance within both soil and plant roots (Olsson, 1999; Olsson & Johansen, 2000). The phospholipid (PLFA) 16:1ω5c has been found to be a good measure of live AMF biomass (hyphae, arbuscules, coils & vesicles) and the neutral lipid (NLFA) 16:1ω5c measures stored lipids within AMF storage structures such as vesicles and spores (Olsson, 1999).

The first objective of the present study was to examine the potential effects of realistic levels of simulated N deposition over 12 years on the abundance of AM fungi in the roots of maples. We hypothesized that the abundance and energy storage of AM fungi and host proportional allocation to intraradical AM fungi would decline in response to N-addition, which could explain the observed decline in soil respiration across the sites. The second objective was to perform a comparison between traditional staining and lipid analysis techniques. Specifically, we compared the percentage of roots colonized by stained AM fungi and the concentration of PLFA 16:1ω5c as estimators of total AM fungal root colonization; and number of vesicles vs. NLFA 16:1ω5c as estimators of AMF stored energy.

Materials and methods

Site description and sampling

The Michigan Gradient Study consists of four study sites located in sugar maple dominated forests throughout Michigan, USA (Fig. 1). Sugar maple accounts for more than 80% of the total stand basal area at these sites (Pregitzer *et al.*, 2004). Each site consists of six 30x30 m plots, with three plots at each study site receiving N amendment

and the other three untreated. N-additions have been applied annually as six 5 kg N increments of $NaNO_3$ during the growing season since 1994, for a total of 30 kg N ha^{-1} yr^{-1}. Ambient N deposition at these sites in 2004 ranged from 4.8 kg N ha^{-1} yr^{-1} at the northernmost site to 8.3 kg N ha^{-1} yr^{-1} at the southernmost sites (NADP, 2006). From north to south along the gradient the mean annual temperature increases from 4.8 ºC to 7.6 ºC, with a concurrent increase in the length of the growing season. Soil development, stand age and plant composition are similar among the sites. More detailed information on the sites can be found in Burton *et al.* (1991).

All the plots were divided into six equally sized subplots (10x15m) for soil sampling. One soil core, 2 cm diameter and 10 cm deep, was taken randomly in each subplot in July and October 2005. Each soil core was washed through a 2mm and 1mm screen until most soil particles were washed out. The remaining roots in both screens were handpicked for maple roots, easily recognized by their unique beaded structure (Pregitzer *et al.*, 2002). Because the distinction between red maple (*Acer rubrum*) and sugar maple roots can not be made easily, some samples might contain minor quantities of red maple roots. However, the percentage of red maple in the plots averaged only 7% of the total basal area and there were no differences between treatments in the percentage of red maple.

After sorting, the roots were cleaned from residual soil particles by sonication for 5 minutes. After cleaning, all maple roots were scanned (HP Scanjet ADT 6300C series) for further specific root length analysis (cm g^{-1} root; WinRhizo Pro 2005b, Regent instruments Inc.). The maple roots were divided into two diameter classes, fine roots (≤ 0.5 mm) and larger roots (> 0.5mm), and root weights were recorded for both diameter classes. In each core the maple fine roots were divided into three subsamples. One subsample was frozen and freeze-dried (Labconco, freezone 4.5) for molecular community analysis (not discussed in this paper), and the other two subsamples were each composited at the plot level to be used in AM fungal colonization and lipid analyses.

AM fungal colonization

The first composite sample was used to measure the AMF colonization of maple roots using a trypan blue (TB) staining technique after Koske & Gemma (1989). Approximately 0.25 gram of fresh roots were placed in a 10 % KOH solution and heated to 90 °C using a water bath until sufficiently cleared. To increase uptake of the staining solution, the cleared roots were slightly acidified by submerging them in an HCl (0.5N) solution for 5 min. The roots were then stained overnight in a TB solution (0.6 gram TB in 1l of 1:2:2 (v:v:v) lactic acid: glycerol: deionized water). To remove excess stain the roots were first rinsed in deionized water and destained in a 1:2:2 (v:v:v) lactic acid: glycerol: deionized water solution.

Stained roots were permanently mounted on microscope slides in three parallel rows with polyvinyl alcohol-lactoglycerol (PVLG) and colonization was measured at 200x magnification using the line intersect method of McGonigle *et al.* (1990). Three slides were analyzed per sample measuring AM fungal colonization at 50 intersects per slide by presence of hyphae, vesicles, spores, arbuscules and/or coils. Total AMF colonization is defined as occurrence of AM intraradical hyphae, arbuscules, coils, vesicles and intraradical spores. Spores and vesicles had similar morphology, so their occurrences were summed and will be referred to as vesicles.

Lipid extraction and analysis

The second composite fine root sample was frozen, freeze-dried (Labconco, Freezone 4.5), weighed and ground (SPEX Certiprep Mill, 8000M). Lipids were extracted from approximately 15 mg of ground roots vortexed in a one-phase mixture of methanol, chloroform and phosphate buffer (pH 7.4) with a volume ratio of 2:1:0.8, using the Frostegård *et al.* (1991) modification of the Bligh and Dyer (1959) method. The extracted lipids were separated into neutral-, glyco-, and phospholipid fractions by silicic acid column chromatography by eluting with chloroform, acetone and methanol, respectively. The neutral- and phospholipids were methylated to free fatty acid methyl esters (FAME's) using a mild alkaline solution. The FAME's were then dissolved in hexane and analyzed by gas chromatography using a 6890N GC (gas chromatograph; Agilent

Technologies, Palo Alto, CA) with a Ultra 2 column (30m, 0.2mm ID, 0.33µm film), an FID detector and hydrogen as carrier gas. Conditions (temperature and time) for the GC analyses are set by the Microbial Identification System (Sherlock version 4.5, MIDI Inc., Newark, DE). The FAME 19:0 (Matreya Inc., State College, PA) was used as internal quantitative standard and the FAME's were identified by a Microbial Identification System (Sherlock version 4.5, MIDI Inc., Newark, DE) using a eukaryotic mix (MIDI Inc., Newark, DE) as qualitative standards. To confirm MIDI software identification several samples were also analyzed using a GC-MS (gas chromatograph-mass spectrometer) at Argonne National laboratory (R.M. Miller laboratory) in parallel to the analysis using the GC with MIDI software. The fatty acid 16:1ω5c was used as an indicator for AM fungi (Olsson, 1999; Olsson & Johansen, 2000).

Calculations of AMF abundance

AMF root colonization can be calculated on different bases each of which sheds light on different aspects of response to treatments. The most commonly used metric is colonization as a percentage of root length (staining method) or fatty acid biomass as a proportion of root biomass (fatty acid method), which shed light on treatment effects on the colonization intensity of AM fungi in roots, but not on the net changes in fungal abundance at the stand level, or proportional allocation to mycorrhizae. Fungal colonization on an area or soil volume basis sheds light on cumulative treatment effects on fungal abundance mediated by both colonization intensity and variations in length and biomass of host roots in response to treatments; these methods are useful for defining treatment response of fungal biomass at the stand level. Expression of fungal colonization as a proportion of host aboveground (woody and litter) biomass shed light on the proportional biomass allocation of maples to AM fungi, which is of interest to ecosystem modelers.

For calculation of maple AMF abundance per cm^3 of soil, referred to as stand-level maple AMF colonization, the following equations were used:
for ocular (root staining) data,
(cm AMF root length cm^{-3} soil) = (% AMF colonization) x (cm root length g^{-1} root) x (g root cm^{-3} soil);
and for fatty acid data,
(nmol 16:1ω5c cm^{-3}) = (nmol 16:1ω5c g^{-1} root) x (g root cm^{-3} soil).
To determine the effect of treatments on maple proportional allocation to AMF biomass, the AMF abundance was expressed in relation to maple aboveground biomass:
for ocular data,
(cm AMF root length g^{-1} maple biomass) = (cm AMF root length m^{-2} soil) / (g maple biomass m^{-2} soil);
and for fatty acid data,
(nmol 16:1ω5c g^{-1} maple biomass) = (nmol 16:1ω5c m^{-2} soil) / (g maple biomass m^{-2} soil).

The (cm AMF root length m^{-2} soil) and (nmol 16:1ω5c m^{-2} soil) represent the values per m^2 obtained from the top 10 cm soil. Maple aboveground biomass (woody plus litter) have been measured annually since 1988 at all sites using the methods described in Reed *et al.* (1994) and Burton *et al.* (1991).

Statistical analysis

Differences in dependent variables (root colonization, vesicle colonization, PLFA 16:1ω5c and NLFA 16:1ω5) between treatments were determined using a two-way repeated measures ANOVA with N-treatment ($n = 2$) and site ($n = 4$) as factors and the sample date ($n = 2$) as repeated measures. Transformations (square root, natural logarithm and arcsin) were applied as appropriate to ensure a normal distribution and equal variances. Given significant time effects, site effects and (in some cases) treatment x site interaction terms, we examined site-level effects using univariate ANOVA with N-treatment as a fixed factor. For each date, the relationships between total AMF

colonization and PLFA 16:1ω5, and between vesicle colonization and NLFA 16:1ω5, were analyzed using linear regression analysis (n = 24 per sample date).

Results

Root staining

N addition led to significantly reduced total AM fungal structures (Fig. 2a,b) and exchange structures (arbuscules + coils) (Fig. 2e,f) for all three metrics (colonization intensity, stand-level maple AMF abundance, and proportional allocation to AM fungi) (Table 1). Other individual structures (hyphae, vesicles) showed similar, but weaker trends of decline in response to N addition that were significant for only a subset of metrics (Table 1, Fig. 2c,d,g,h).

Changes from July to October were common for multiple metrics and structures (Table 1). For total AMF structures significant growing season increases occurred for colonization intensity and stand-level abundance (Table 1). For hyphae (Fig. 2c,d) and exchange structures (Fig. 2e,f) the seasonal effect was significant for stand-level abundance and proportional allocation with various directions of change. For vesicles a significant growing season increase was observed only using the colonization intensity metric (Table 1).

Significant site effects were seen for all AMF structures using all metrics, with the main trend showing a decrease in total AMF abundance and vesicles in both treatments from site A to C (most metrics) or site A to D (Fig. 2a,b,g,h) (Table 1). This decrease was most apparent for vesicles (Fig. 2g,h), and not observed with hyphae or exchange structures (Fig. 2c,d and 2e,f, respectively).

There were also some significant treatment x site and site x time interactions. Treatment x site interactions were strongest and most consistent across metrics for exchange structures and to a lesser extent for vesicles and total AMF structures (Table 1). Variation in the strength of the response to N addition was evident at the site level, with strongest effects at site C and weakest effects at site D (Fig. 2). Site x time interactions were seen in total AMF structures, exchange structures and hyphae for all three metrics,

with site D again diverging from other sites for total AMF structures (Fig. 2a,b) and exchange structures (Fig. 2e,f) and site B diverging for hyphae (Fig. 2c,d) (Table 1). There were no significant treatment x time or treatment x site x time interactions for any variables (data not shown).

Lipid analysis

N addition led to a decrease in AMF indicator PLFAs and NLFAs for all metrics (Table 1, Fig. 3). A significant growing season increase was evident for PLFA 16:1ω5c only for the colonization intensity metric (Fig. 3a,b), but for NLFAs the increase was evident with all metrics (Table 1, Fig. 3e,f,g,h). Differences in PLFA 16:1ω5c among sites only became apparent when PLFA 16:1ω5c was expressed at the stand level or as proportional allocation (Table 1, Fig. 3c,d), whereas differences among sites in NLFA 16:1ω5c were evident for all metrics (Table 1, Fig. 3e,f,g,h). As for staining, there was some variation in the strength of the treatment effect among sites, although no significant interaction terms were found for PLFAs or NLFAs (Table 1, Fig. 3).

Relationship of root staining and lipid analysis

PLFA 16:1ω5c content had a significant positive linear relationship with total AMF colonization in October ($R^2 = 0.39$, $P = 0.001$) and a similar trend in July ($R^2 = 0.14$, $P = 0.07$) (Fig. 4a). NLFA 16:1ω5c showed a strong positive linear relationship with the percentage vesicle colonization ($P < 0.0001$) on both sampling dates ($R^2 = 0.59$ (July) and $R^2 = 0.67$ (October), Fig. 4c). Although the percentage vesicle colonization had an equal range on both sampling dates, the amount of NLFAs for a particular percentage of vesicle colonization was much higher in October ($y = 1.47 + 0.41x$) compared to the amount of NLFAs in July ($y = 0.67 + 0.09x$) (Fig. 4c). This large increase in NLFA 16:1ω5c content with a similar percentage of vesicles is very apparent in the almost five-fold increase of the October regression line slope compared to the July slope (0.41 and 0.09, respectively). When expressed on a maple stand level both the relationships between PLFA 16:1ω5c vs. AMF root colonization and NLFA 16:1ω5c vs. vesicle colonization became stronger (Fig. 4b,d).

Discussion

N effects on temperate forest AMF

The decrease in AMF root colonization with N addition is very apparent from the results using both techniques. This is the first study to demonstrate such a response in temperate hardwood forests treated for so long with realistic levels of simulated N deposition. Hutchinson *et al.* (1998) found a significant decrease in percent AMF colonization of sugar maple roots at one site after three years of 1000 kg ha^{-1} yr^{-1} N addition, while another site showed no difference after two years of N addition. Lansing (2003) also found a reduction in AMF colonization levels for sugar maple after four years of 100 kg ha^{-1} yr^{-1} N addition. Interestingly Lansing's reduction in AMF colonization for sugar maple in Michigan was similar to the reduction found in our study (R of 0.88 and 0.80, respectively) where R is the response ratio (R = mean of treatment divided by mean of control) (Treseder, 2004). Our total N addition over 12 years (360 kg ha^{-1}) was comparable to their total N addition over four years (400 kg ha^{-1}).

Several factors could be causing the reduction of the arbuscular mycorrhizal symbiont in an N-amended environment. One hypothesis is that N addition reduces host C allocation to AM fungi. This is consistent with the significant results found in the analyses of proportional allocation to AM fungi by the maples (aboveground and litter biomass), which is lower in the N-amended plots. The high N deposition sites (site C and D) also had a lower proportional allocation to AMF independent of treatment, which might be the result of long-term differences in ambient N deposition. If less C is being allocated to the fungal symbiont this could also explain some of the reduced soil respiration found in the N-amended plots. This decline in soil respiration has not been explained by other factors, i.e. root respiration or microbial respiration in mineral soil (Burton *et al.*, 2004; Zak *et al.*, 2006). Furthermore the N-amended plots have shown increased tree growth (K.S. Pregitzer *et al.*, unpublished), which suggests that more C is invested in aboveground biomass.

Another hypothesis for reduced AM fungal biomass with N addition could be that the mycorrhizae are directly affected by the higher amounts of N in the soil. Wallander (1995) suggested that reduced fungal growth was not caused by reduced C flow to

ectomycorrhizal fungi (EMF), but that the increased amount of N supply caused the mycorrhizal fungi to use more C in the costly process of N assimilation instead of using the C for growth. This hypothesis is consistent with the increased N content of the foliage and leaf litter of the N-amended plots at our study sites (K.S. Pregitzer, unpublished). Fungal growth response can also differ among species depending on their capacity for N assimilation and the pathway of N assimilation (Wallander, 1995), and this might also explain some of the differences in treatment response among sites. However, N uptake costs may be lower for AMF than EMF, because of the difference in their N-assimilation pathways. In AMF symbioses studied so far, N is transferred to the host plant as ammonium and not, as in EM symbiosis, as an amino acid (Govindarajulu *et al.*, 2005). Therefore AM fungi retain most of the C from the amino acids, while EM fungi lose the C in the transfer of N as amino acids to the host plant (Govindarajulu *et al.*, 2005). However, the N-uptake by AM fungi still has energetic and C costs that could affect AMF growth.

Sites varied in the strength of the reduction in AMF colonization with N addition. Site D showed only a marginal decline in AMF abundance with N addition in July and a trend toward an increase in October. Although the ambient N deposition of site D is about the same level as site C, the lack of strong reduction of AM fungal root abundance with N addition could possibly be caused by site-level differences in C allocation to, or N-assimilation by AMF caused by variation in mean annual temperature, precipitation, tree growth, N-mineralization rates, C:N ratio in litter or phosphorus availability. Alternatively, site-level differences in AMF abundance could be driven by changes in AM fungal community structure. Functional diversity, e.g. variation in C demand vs. nutrient supply, exists among AM fungi, and compositional and functional community responses have been found in previous studies of AM fungal response to N (e.g., Johnson 1993; Corkidi *et al.*, 2002). For example, Johnson (1993) found a change in AM fungal community with N (and other nutrients) fertilization and suggested that the AM fungal species dominant at the fertilized sites were more parasitic than those dominant at low N sites. We will address these alternative hypotheses in a future paper.

Staining vs. fatty acid methods

The positive linear relationship of the fatty acid 16:1ω5c with percent AMF colonization in stained roots found in this study is consistent with findings from other studies which have performed both staining and fatty acid analysis (Olsson *et al.*, 1997; Van Aarle & Olsson, 2003; R.M. Miller, unpublished). In a controlled greenhouse study with cucumber plants, inoculated with a single AMF species, very strong relationships were found between colonized root length and both PLFA and NLFA 16:1ω5c (R^2 = 0.92 and 0.95, respectively) (Olsson *et al.*, 1997). In another greenhouse study, Van Aarle & Olsson (2003) found weaker significant relationships between both PLFA and NLFA 16:1ω5c and percent AMF colonization (R^2 = 0.44 and 0.57, respectively). The higher R^2 values within the Olsson *et al.* (1997) and the Van Aarle & Olsson (2003) study compared to our study could be caused by 1) the much more controlled environment vs. a field study, 2) a single AMF species vs. greater AMF diversity combined with differences in fatty acid composition and amounts between AMF species (Bentivenga & Morton, 1996; Olsson & Johansen, 2000) and/or 3) a bigger range and better distribution of the values of root colonization.

The relationship of NLFA 16:1ω5c with the amount of storage structures (Fig. 4c) was stronger than that of PLFA 16:1ω5c with the percent total AMF colonization (Fig. 4a). It is unclear exactly why this is, but possibilities include 1) the inability to distinguish live and dead hyphae using staining methods, 2) poor staining of some AMF species (Morton & Redecker, 2001), 3) vesicles' larger size and distinctive shape compared with hyphae which minimizes error in counting, and 4) the larger potential for variability in hyphal density compared to vesicle density at an intersect.

The steeper slope of the relationship between NLFA 16:1ω5c and vesicle colonization in October compared to July is indicative of vesicle filling, i.e. the accumulation of storage lipids through the growing season. This suggests that most of the AMF storage structures (vesicles) are already present earlier on in the colonization process of the roots, and more lipids are added to these vesicles during the growing season for storage and use for the next year. A similar observation was made by Van Aarle & Olsson (2003) in their greenhouse study. NLFA 16:1ω5c is therefore perhaps a

better indicator of the amount of stored energy than the numbers of vesicles present in the roots.

We saw a similar, but weaker effect of season on the relationship of PLFA 16:1ω5c and percent total AMF colonization. The distinction between the two regression lines in this relationship (Fig. 4a) is less obvious than for NLFA 16:1ω5c vs. vesicle colonization (Fig. 4c). The 3-fold steeper slope in October compared to July is a much smaller relative increase compared to the 5-fold steeper slope for the neutral lipids vs. percentage vesicles. PLFA 16:1ω5c also appeared to be a more sensitive biomass indicator than our frequency-based ocular measurements of AMF colonization, probably because the ocular method does not take colonization intensity into account. As a result, when only ocular measurements are performed, changes in biomass could be overlooked or underestimated.

The improvement of the relationship of lipid and ocular estimates after rescaling to a volumetric (cm^3 soil) basis was striking, indicating that the strength of the relationship of the two metrics depends on the form of their expression. Since mycorrhizae and roots exploit space rather than mass, the stand-level values (Fig. 4b,d), which show the actual mycorrhizal biomass in a volume of soil, are perhaps more relevant to use than concentration values (Fig. 4a,c). Both root biomass and specific root length, which were used to calculate AM fungal biomass on a stand-level basis, were not affected by treatment. However, the percent colonization decreased with an increase in specific root length ($R^2 = 0.47$, $P < 0.0001$) and root biomass decreased at all sites from July to October ($P = 0.001$). By expressing the AMF abundance on a volumetric basis, these length and biomass differences were taken into account, and improved the relationships between ocular measurements and fatty acid 16:1ω5c.

In conclusion, after 12 years of simulated N-addition, the abundance of AM fungi within the active fine root system of maples and proportional investment in AM fungi decreased significantly as estimated by both lipid analysis and staining. Positive linear relationships were found between the fatty acid 16:1ω5c and the percent total AMF colonization and number of storage structures. The phospholipid fraction seems to be a good indicator of active AMF biomass and NLFA 16:1ω5c was found to be a better indicator of AMF stored energy than the number of vesicles present. The fatty acids

analyses gave better insight into changes in AMF total biomass and stored energy over time compared to the staining method, and avoided possible under- or overestimation of the total AM fungal abundance. However, the staining method can elucidate changes in specific fungal structures (arbuscules, coils, etc), which is not possible with fatty acid analyses. The observed decrease in AMF abundance and investment could suggest either reduced C allocation to these fungi or a direct soil N-mediated decline. The observed reduction in the abundance of and investment in AM fungi belowground is consistent with the reduction in soil respiration reported earlier for this study (Burton *et al.*, 2004). Future research will focus on the effects of increased N inputs on AMF extraradical hyphae and community analyses designed to understand if N-deposition is altering AMF community composition, structure and function.

Acknowledgements

We thank the NSF for their continued financial support of this project. We also thank Kate Bradley, Rasmus Kjøller and two anonymous reviewers for comments on a previous version of this manuscript. Furthermore we are grateful to Zhanna Yermakov for her helpful advice and training in lipid extraction, and to Carrie Andrew, Christa Luokkala, John Hribljan, Eric Koronka, Alan Talhelm and Kate Bradley for their assistance with the sampling and root sorting.

References

Allison VJ, Miller RM. 2004. Using fatty acids to quantify arbuscular mycorrhizal fungi. In: Podila G, Varma A, eds. Basic Research and Applications of Mycorrhizae. New Delhi: I.K International Pvt. Ltd., 141-161.

Bentivenga SP, Morton JB. 1996. Congruence of fatty acid methyl ester profiles and morphological characters of arbuscular mycorrhizal fungi in Gigasporaceae. Proceedings of the National Academy of Sciences 93: 5659-5662.

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37: 911-917.

Burton AJ, Pregitzer KS, Crawford JN, Zogg GP, Zak DR. 2004. Simulated chronic NO_3^- addition reduces soil respiration in northern hardwood forests. Global Change Biology 10:1080-1091.

Burton AJ, Pregitzer KS, Reed DD. 1991. Leaf area and foliar biomass relationships in northern hardwood forests located along an 800 km acid deposition gradient. Forest Science 37: 1041-1059.

Corkidi L, Rowland DL, Johnson NC, Allen EB. 2002. Nitrogen fertilization alters the functioning of arbuscular mycorrhizas at two semiarid grasslands. Plant and Soil 240: 299-310.

Cunha A, Power SA, Ashmore MR, Green PRS, Haworth BJ, Bobbink R. 2002. Whole ecosystem nitrogen manipulation experiments: An updated review. JNCC report.

Dise NB, Wright RF. 1995. Nitrogen leaching in European forests in relation to nitrogen deposition. Forest Ecology and Management 71: 153-162.

Frostegård A, Tunlid A, Bååth E. 1991. Microbial biomass measured as total lipid phosphate in soils of different organic content. Journal of Microbiological Methods 14: 151-163.

Galloway JN, Levy IIH, Kasibhatla PS. 1994. Year 2020: Consequences of population growth and development on deposition of oxidized nitrogen. AMBIO 23: 120-123.

Govindarajulu M, Pfeffer PE, Jin H, Abubaker J, Douds DD, Allen JW, Bücking H, Lammers PJ, Shachar-Hill Y. 2005. Nitrogen transfer in the arbuscular mycorrhizal symbiosis. Nature 435: 819-823.

Johnson NC. 1993. Can Fertilization of soil select less mutualistic mycorrhizae? Ecological Applications 3: 749-757.

Hutchinson TC, Watmough SA, Sager EPS, Karagatzides JD. 1998. Effects of nitrogen deposition and soil acidification on sugar maple (*Acer saccharum*) in Ontario, Canada: an experimental study. Canadian Journal of Forest Research 28: 299-310.

Koske RE, Gemma JN. 1989. A modified procedure for staining roots to detect VA mycorrhizas. Mycological Research 92: 486-505.

Lansing JL. 2003. Comparing arbuscular and ectomycorrhizal fungal communities in seven North American forests and their response to nitrogen fertilization. PhD thesis. University of California, Davis, CA, USA.

McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA. 1990. A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytologist 115: 495-501.

Morton JB, Redecker D. 2001 Two new families of Glomales, Archaeosporaceae and Paraglomaceae, with two new genera Archaeospora and Paraglomus, based on concordant molecular and morphological characters. Mycologia 93: 181-195.

NADP. 2006. National Atmospheric Deposition Program (NRSP-3). NADP Program Office, Illinois State Water Survey, IL, USA.

Olsson PA. 1999. Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. FEMS Microbiology Ecology 29: 303-310.

Olsson PA, Bååth E, Jakobsen I. 1997. Phosphorus effects on the mycelium and storage structures of an arbuscular mycorrhizal fungus as studied in the soil and roots by analysis of fatty acid signatures. Applied and Environmental Microbiology 63: 3531-3538.

Olsson PA, Johansen A. 2000. Lipid and fatty acid composition of hyphae and spores of arbuscular mycorrhizal fungi at different growth stages. Mycological Research 104: 429-434.

Pregitzer KS, DeForest JL, Burton AJ, Allen MF, Ruess RW, Hendrick RL. 2002. Fine root architecture of nine North American trees. Ecological Monographs 72: 293-309.

Pregitzer KS, Zak DR, Burton AJ, Ashby JA, MacDonald NW. 2004. Chronic nitrate additions dramatically increase the export of carbon and nitrogen from northern hardwood ecosystems. Biogeochemistry 68: 179-197.

Reed DD, Pregitzer KS, Liechty HO, Burton AJ, Mroz GD. 1994. Productivity and growth efficiency in sugar maple forests. Forest Ecology and Management 70: 319-327.

Smith SE, Read DJ. 1997. Mycorrhizal Symbiosis. New York, USA: Academic Press.

Treseder KK. 2004. A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric CO2 in field studies. New Phytologist 164: 347-355.

Van Aarle IM, Olsson PA. 2003. Fungal lipid accumulation and development of mycelial structures by two arbuscular mycorrhizal fungi. Applied and Environmental Microbiology 69: 6762-6767.

Vitousek PM, Aber JD, Howarth RW, Likens GE, Matson PA, Schindler DW, Schlesinger WH, Tilman DG. 1997. Human alteration of the global nitrogen cycle: sources and consequences. Ecological applications 7: 737-750.

Wallander H. 1995. A new hypothesis to explain allocation of dry matter between mycorrhizal fungi and pine seedlings in relation to nutrient supply. Plant and Soil 168-169: 243-248.

Wallenda T, Kottke I. 1998. Nitrogen deposition and ectomycorrhizas. New Phytologist 139: 169-187.

Zak DR, Holmes WE, Tomlinson MJ, Pregitzer KS, Burton AJ. 2006. Microbial cycling of C and N in northern hardwood forests receiving chronic atmospheric NO_3^- deposition. Ecosystems 9: 242-253.

Table 1 Comparison of P-values for all quantification methods and metrics of estimation of mycorrhizal fungal colonization of sugar maple roots across a nitrogen deposition gradient in Michigan.

Metric	Method	Structures	Treatment	Site	Time	Treatment x Site	Site x Time
AMF root colonization intensity	Staining	Total AMF	0.002	0.001	0.001	0.26	0.003
		Hyphae	0.05	0.03	0.08	0.18	<0.001
		Exchange structures	<0.001	0.008	0.08	0.01	0.004
		Vesicles	0.03	<0.001	<0.001	0.04	0.81
	Fatty acid 16:1ω5c	Phospholipid	0.005	0.55	<0.001	0.43	0.97
		Neutral lipid	0.007	0.005	<0.001	0.27	0.85
Stand-level maple AMF abundance	Staining	Total AMF	0.02	<0.001	0.03	0.05	0.001
		Hyphae	0.06	0.01	0.03	0.10	<0.001
		Exchange structures	0.001	0.007	0.003	0.006	0.006
		Vesicles	0.06	<0.001	0.93	0.03	0.06
	Fatty acid 16:1ω5c	Phospholipid	0.01	0.001	0.24	0.14	0.07
		Neutral lipid	0.02	<0.001	<0.001	0.07	0.22
Proportional allocation to AMF	Staining	Total AMF	0.007	<0.001	0.11	0.08	0.001
		Hyphae	0.02	0.007	0.03	0.19	<0.001
		Exchange structures	<0.001	0.009	0.003	0.01	0.006
		Vesicles	0.05	<0.001	0.93	0.07	0.06
	Fatty acid 16:1ω5c	Phospholipid	0.004	0.001	0.27	0.21	0.07
		Neutral lipid	0.01	<0.001	<0.001	0.12	0.22

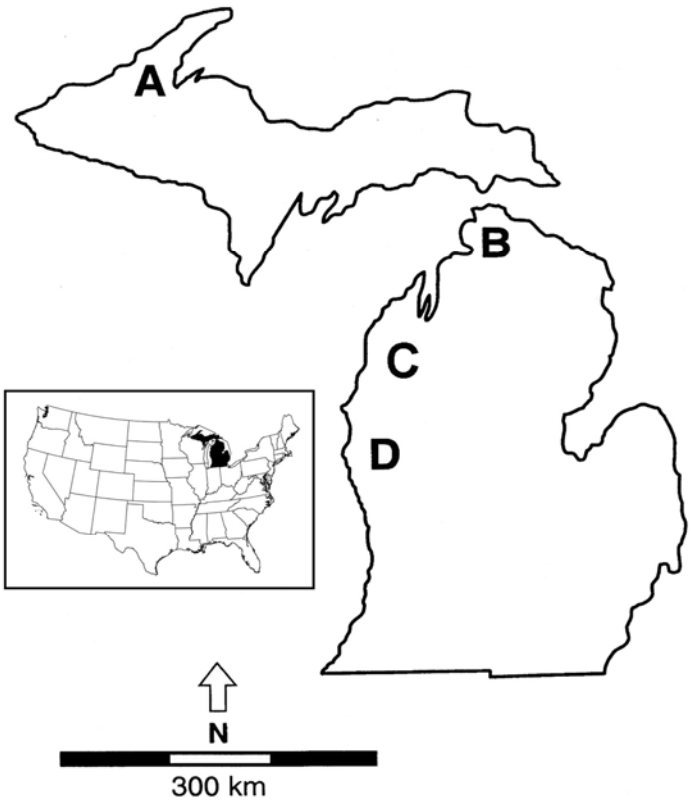

Fig. 1 Locations of the study sites (A-D) in Michigan. The inset shows the location of Michigan (filled area) within the USA.

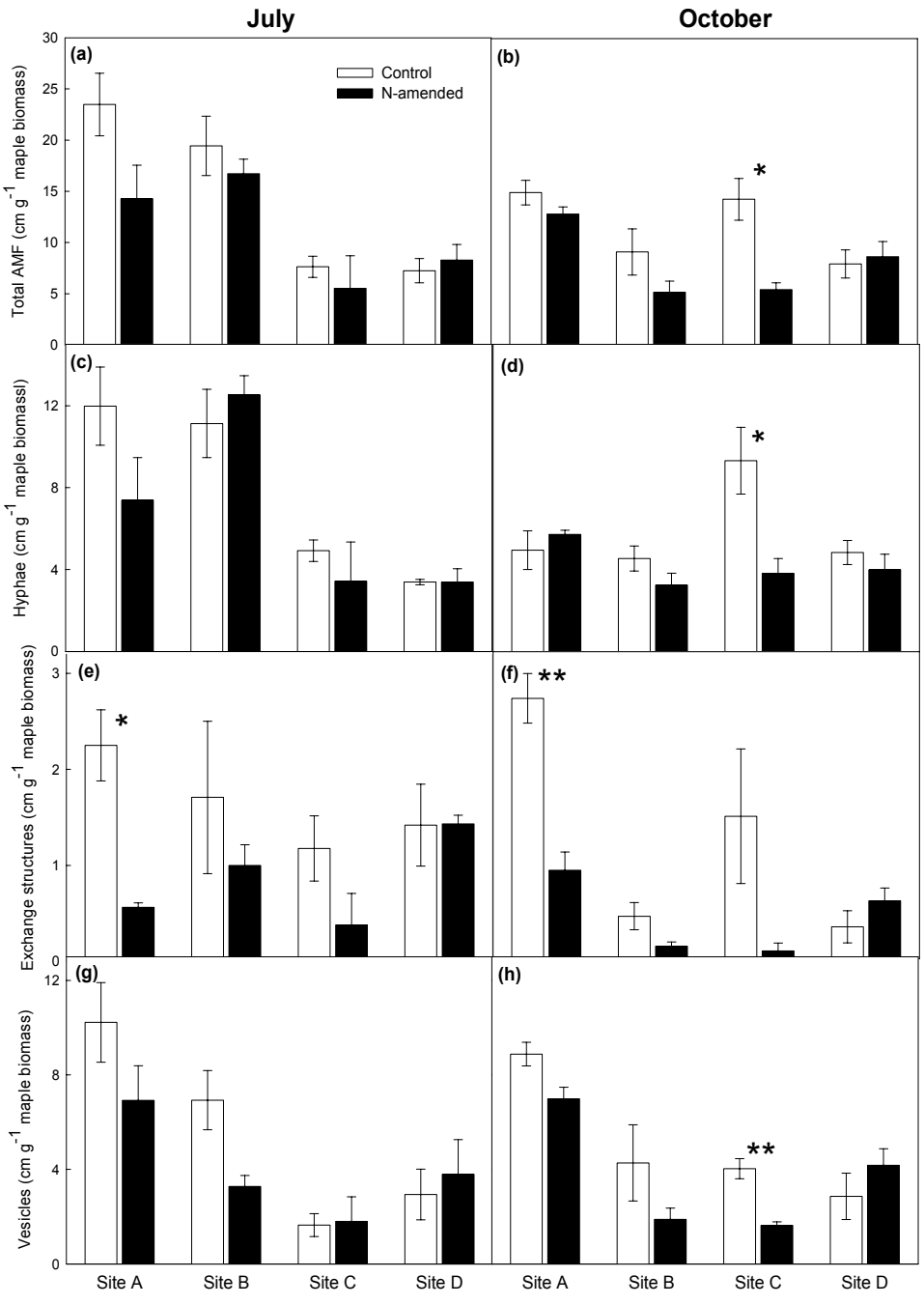

Fig. 2 Mean proportional allocation to AMF in maple fine roots for the four study sites by treatment for July and October 2005: total (a,b), hyphal (c,d), exchange structures (e,f) and vesicles (g,h). Error bars indicate one standard error of the mean. All AMF structures showed a significant overall N treatment and site effect (see Table 1 for details). * Means of N treatments differ significantly at site (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

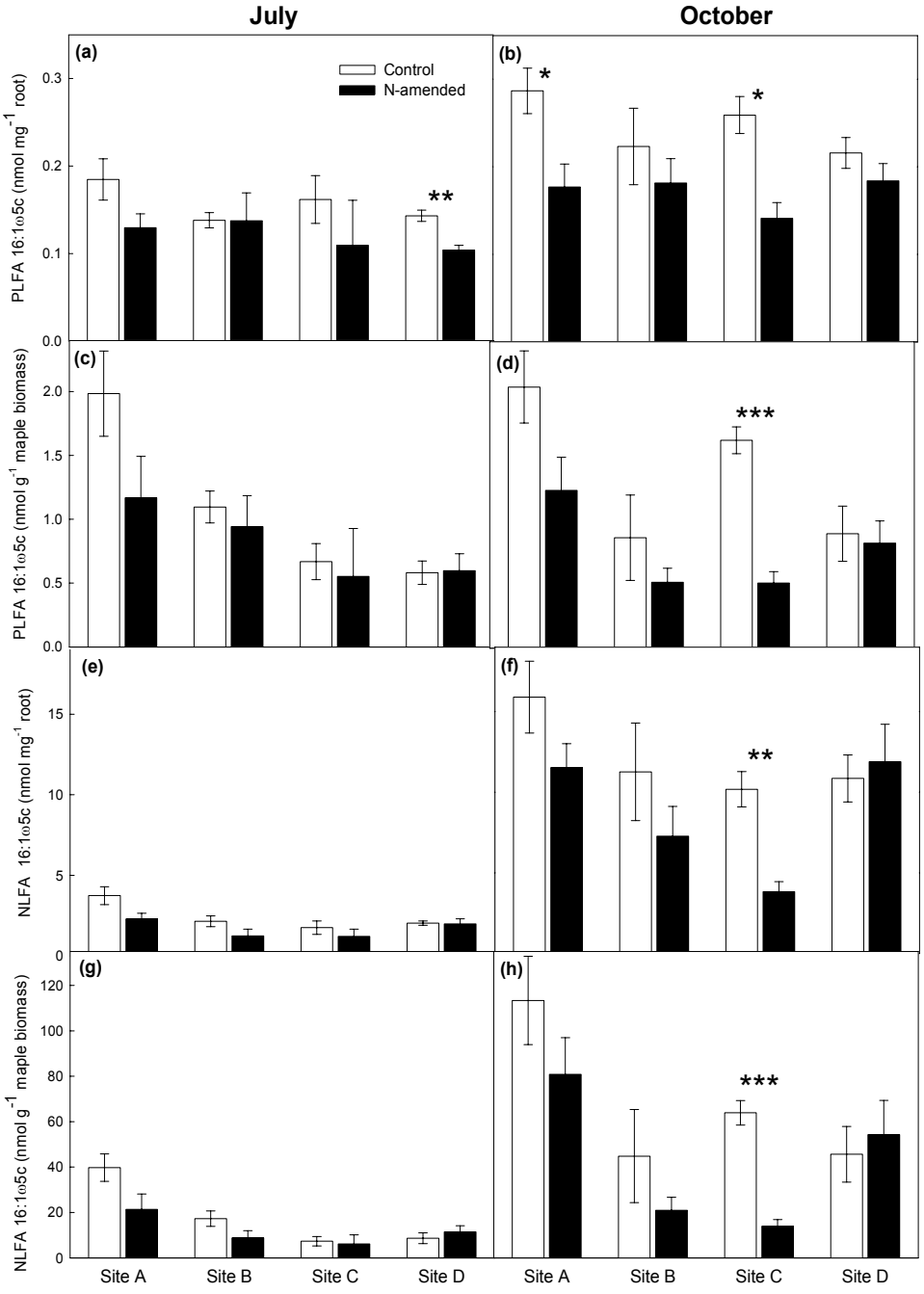

Fig. 3 Mean PLFA (a,b) and NLFA 16:1ω5c (e,f) concentrations in maple fine roots, and maple proportional allocation to PLFA (c,d) and NLFA 16:1ω5c (g,h), for the four study sites by treatment for July and October 2005. Error bars indicate one standard error of the mean. Overall N treatment effect was significant in all cases (see Table 1 for details). * Means of N treatments differ significantly at site (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

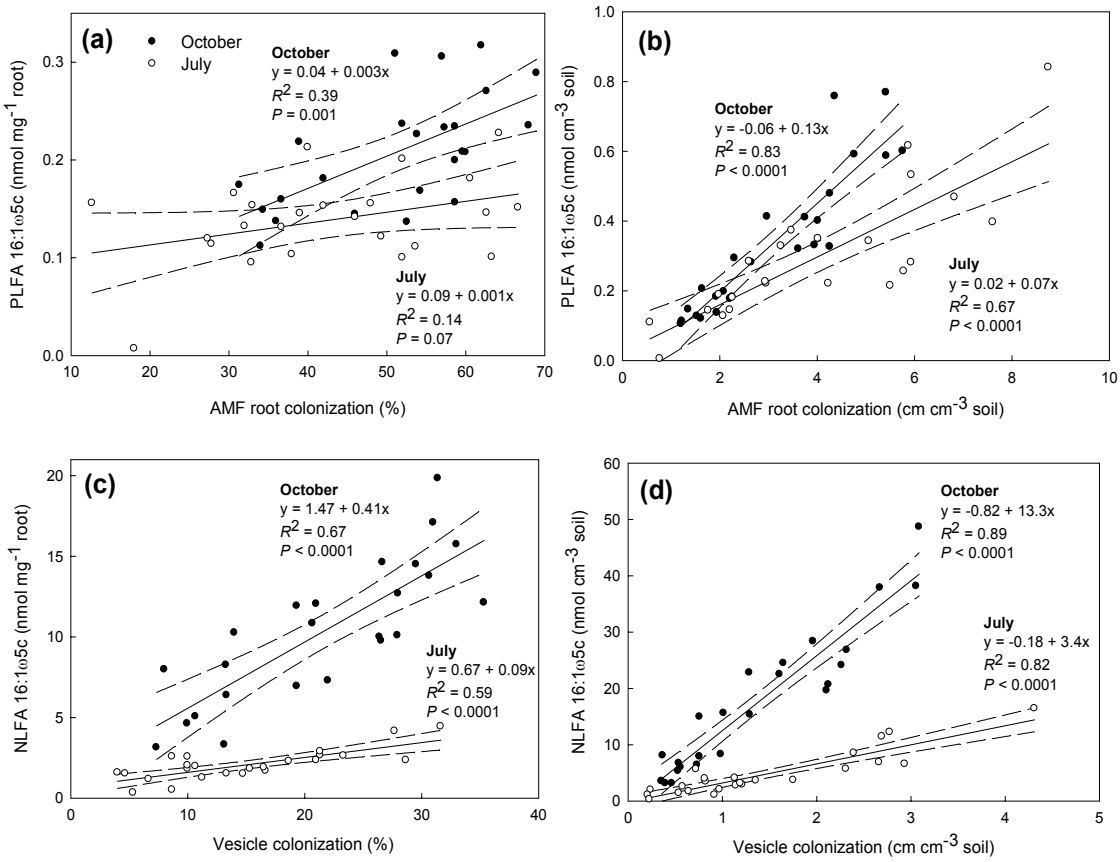

Fig. 4 Relationship between PLFA 16:1ω5c and total AMF colonization and between NLFA 16:1ω5c and vesicle colonization (vesicles and intraradical spores) in maple fine roots for the four study sites as colonization intensity (a,c) and on maple stand-level basis (b,d). Regression line (solid) and 95% confidence interval (dashed) are plotted.

Chapter 2 Effects of chronic nitrogen additions on diversity and community composition of arbuscular mycorrhizal fungi in northern hardwood forests

Summary

Arbuscular mycorrhizal fungi are in general negatively affected by increased nitrogen (N) deposition, but little is known about how nitrogen affects the AMF community composition within forest ecosystems. Therefore we studied the diversity and community composition of arbuscular mycorrhizal fungi (AMF) in northern hardwood forests after more than 12 years of nitrogen addition. We performed molecular analyses on maple (*Acer* spp.) roots targeting the 18S rDNA region using the fungal specific primers AM1 and NS31. PCR products were cloned and identified using restriction fragment length polymorphism (RFLP) and sequencing. The effect of nitrogen on AMF species diversity varied among sites. N addition also affected the AMF community composition significantly. Eighty percent of all clones were represented by seven dominant operational taxonomic units (OTU's), of which over 90% belonged to the genus *Glomus* and the remainder to the genus *Acaulospora*. The observed effect on community composition implies that AMF species associated with maples differ in their response to elevated nitrogen. Knowing that functional diversity exists among AMF species, this change in community composition could have implications for the functioning of this type of ecosystem.

Introduction

Arbuscular mycorrhizal fungi are ubiquitous and have a very broad host range (Smith and Read, 1997). However, it is clear that species can vary in abundance in response to different hosts (e.g. Helgason et al., 2002; Helgason et al., 1998) and environmental conditions, (e.g. Wirsel, 2004; Helgason et al., 2002; Treseder & Allen, 2002; Helgason et al., 1998). It is also clear that both AMF diversity (van der Heijden, 1998) and the identity of mycorrhizal fungal symbionts can have a large impact on host function (e.g.

Corkidi et al., 2002; Helgason et al., 2002; Johnson, 1993). With the current changing environmental conditions (increased CO_2, N-deposition, warming), it is therefore of interest to study how these plant-fungal relationships are changing. To do this we will first need to identify the AMF species present in a broad range of ecosystem types and across a variety of environmental gradients to be able to understand the distribution and function of mycorrhizal fungal species and communities (Lilleskov and Parrent, 2007).

Increased nitrogen (N) deposition has generally been found to negatively affect the abundance of arbuscular mycorrhizal fungi (AMF) (Treseder, 2004; van Diepen et al. 2007). Decrease in AMF abundance could be associated with 1) a decrease in all species present, 2) a change in relative abundance of the associated species, or 3) a complete change in community composition.

Studies of AMF community response to N additions have had varying results often depending on ecosystem type and amount of nitrogen addition combined with the years of fertilizer application. Several have shown increased nitrogen deposition leading to a change in AMF community composition via analysis of asexual spores (e.g. Egerton-Warburton and Allen, 2000; Eom et al., 1999 ; Johnson, 1993), and more recently community changes have been detected via molecular techniques (Porras-Alfaro et al. 2007, Jumpponen et al. 2005). However, most of the N deposition studies looking at AMF community composition in plant roots have been performed in grassland or chaparral ecosystems. To our knowledge, no published studies have examined changes in forest ecosystems, so the generality of N deposition effects on AMF community composition across ecosystem types is yet to be determined.

Identifying AMF species is a prerequisite for elucidating the functional significance of changes in AMF species composition and abundance. We performed molecular analyses on maple roots from northern temperate hardwood forests, targeting the 18S rDNA region using fungal specific primers. Our specific objectives within this study were to 1) estimate AMF species diversity and identify AMF community composition in a northern hardwood forest, and 2) determine how both are affected by increased chronic nitrogen deposition.

Materials and Methods

Site description and sampling

Roots were sampled from four sugar maple (*Acer saccharum* Marshall) dominated forest sites throughout Michigan, USA (Fig. 1). Three untreated and three N-amended plots (30 x 30 m) are located at each site. Nitrogen has been applied annually in six equal increments of $NaNO_3$ during the growing season since 1994, for a total of 30 kg N ha^{-1} yr^{-1}. Ambient wet N deposition at the sites ranged from 3.0 kg N ha^{-1} yr^{-1} near site A to 6.8 kg N ha^{-1} yr^{-1} near site D in 2006 (NADP, 2006). All sites have similar soil development, stand age and plant composition. More detailed information about the sites can be found in Burton et al. (1991).

For root sampling, each plot was divided in to 6 equal (10 x 15 m) subplots, and one soil core (2 cm in diameter and 10cm deep) was taken randomly in each subplot in October 2006. Each soil core was washed through a 2mm and 1mm screen until most soil particles were washed out. Maple roots were handpicked from the screens and cleaned more thoroughly with DI water and by sonication for 5 min. The maple roots were divided up into two diameter classes, fine roots (<0.5mm) and larger roots (>0.5mm), and weights of both classes were recorded. For each core, the fine maple roots were divided into two equal subsamples. One subsample was composited at the plot level and used for PLFA analysis to estimate AMF intra-radical abundance (Chapter 3), similarly to the previous year (van Diepen et al., 2007). The other subsample was frozen, freeze dried and used for molecular analyses. Each subsample (144 in total) was analyzed separately in all the steps of the molecular analyses to ensure maximum sensitivity to infrequent and rare taxa.

Molecular analysis

DNA was extracted from 5mg lyophilized fine sugar maple roots per sample using a DNeasy 96 Plant Kit (Qiagen, USA) according to manufacturer's instructions. Extracted DNA was amplified according to Helgason et al. (1999) PCR cycling parameters, in a 25µl reaction mixture of 0.5µl Easy-A high fidelity PCR cloning enzyme (Stratagene), 2.5µl manufacturer's Easy-A reaction buffer, 2.5µl dNTP's (2mM), 0.25 µl (20µM)

eukaryotic primer AM1 (Simon et al., 1992) and 0.25 µl (20µM) fungal primer NS31 (Helgason et al., 1998). This combination preferentially amplifies AM fungi, although certain other fungal taxa are amplified. PCR products were cloned into TOPO TA pCR2.1 vector (Invitrogen) and transformed into *Escherichia coli* (One Shot TOP10 Chemically competent) according to manufacturer's instructions.

From each sample 15 putative clones were randomly selected and amplified using the same reaction mixture as in the first PCR, but replacing the cloning enzyme with Paq500 DNA polymerase (Stratagene). Up to 12 positive PCR products per sample were digested with restriction enzymes *Nla*III and *Dpn*II (New England Biolabs Inc.). Representatives of each RFLP (restriction fragment length polymorphism) type were then re-amplified, cleaned with StrataPrep PCR purification kit (Stratagene) according to manufacturer's instructions, and sequenced. Sequencing was done by Nevada Genomics Center (Reno, Nevada, USA) on a ABI Prism 3730 DNA analyzer using primers NS31 and AM1.

Sequence analyses

In order to identify the obtained sequences, the sequences were compared with all known sequences in GenBank using BLAST. Only sequences matching confirmed AMF taxa from GenBank were used for alignment in Bioedit (Hall, 1999), using ClustalW (Thompson et al., 1994). The ClustalW alignment was checked and improved manually where needed.

To define OTU's (Operational Taxonomic Units) a distance matrix was computed of our sequences using DNADIST version 3.5c (J. Felsenstein, University of Washington, Seattle, WA). To understand the effects of N amendment at different taxonomic levels OTU's were defined at three different levels of RFLP type sequence similarity: 100%, 97% and 95%.

Sample-based rarefaction curves (species accumulation curves) were calculated per treatment and site, and for all pooled data in EstimateS version 7.5 (Colwell, 2005) using the analytical formulas of Colwell et al. (2004). Furthermore the estimated total species

richness by functional extrapolation was calculated in EstimateS using the Michaelis-Menten function (Colwell and Coddington, 1994).

Shannon diversity index was calculated at each OTU level, and effects of treatments were determined using a two-way ANOVA with N treatment (n = 2) and site (n = 4) as factors.

The effects of N-amendment on AMF community composition for all three levels of OTU's were examined using permutational multivariate analysis of variance (PERMANOVA, Anderson, 2005) based on the Bray–Curtis distance measure. PERMANOVA does not provide graphical data display, so the community data were visualized using biplots of CAP (Canonical Analysis of Principal Coordinates, Anderson 2004) output. CAP was performed with each treatment at each site as a separate group (total of eight groups). Furthermore effects of treatment on the most abundant OTU's were analyzed using a two way ANOVA with N treatment (n = 2) and site (n = 4) as factors.

An additional sequence alignment was made in BioEdit combining our sequences, AMF taxa from GenBank with high similarity (>97%) with our sequences, and known AMF species. The full alignment was used to create phylogenetic trees in PAUP 4.0b10 (Swofford, 1998), using maximum parsimony and 1000 bootstrap replicates to check support for the tree. The tree was rooted with a *Paraglomus occultum* sequence obtained from GenBank. Phylogenetic trees were visualized using Treeview Win32 version 1.6.6 (R.D.M. Page, 2001).

Results

AMF taxa and diversity

Within the study a total of 2160 clones (15 clones per sample) were amplified and resulted in an average of 12 positive PCR products of ~550 bp per sample. Digestion of the ~550 bp positive PCR products resulted in 45 different RFLP patterns of which the sequences of 27 were matched with confirmed AMF taxa, four with Basidiomycetes (1 clone in 4 different samples), and six with Ascomycetes from GenBank using BLAST.

Five of the Ascomycetes found only represented 1-2 clones within 6 samples, however Ascomycete *Menispora tortuosa* was found in 32 of the 144 samples (3.3% of clones). The remainder of the sequenced RFLP types did not match with any fungal species. However, over 94% of all clones analyzed within this study represented AMF taxa. Using the 27 AMF sequences, a total of 12 (95% similarity) and 17 (97% similarity) unique OTU's (Operational Taxonomic Units) were found (Table 1). The different OTU's are evident in the maximum parsimony phylogenetic tree (consensus tree), and showed AMF clades similar to OTU's defined at 97% and 95% sequence similarity (Fig. 2, Table 2). The names of the OTU's as defined in Table 2 were used for further analyses and discussion of the data.

Rarefaction curves of the pooled data showed a saturation of number of taxa found in our samples for both OTU groupings (95% and 97% similarity), but at the taxonomic level of RFLP types the rarefaction curve did not level off completely, suggesting that more samples could have increased the number of RFLP types found in our maple roots (Fig. 3a). When observing taxa accumulation curves at the treatment by site level, no complete saturation was observed at the taxonomic level of RFLP types. At the taxonomic level of 97% sequence similarity it only showed saturation of the taxa accumulation curve for control plots at site C (Fig. 3b), and at 95% sequence similarity for control plots of site A and C, and N-amended plots of site A and D. However, the estimated total taxa richness by functional extrapolation was less than 3 RFLP types or 2 OTU's (97% and 95% similarity) greater than our observed number of taxa, indicating that we were very close to finding all taxa present within our system.

AMF taxa Shannon's diversity was not significantly affected by treatment or site, and varied from an average of 1.94 (based on RFLP types) to 1.64 (95% similarity) (Table 1). An average of 10.3 (±0.4), 9.0 (±0.3), and 7.3 (±0.2) RFLP types or OTU's were found overall at our study sites at the different taxonomic levels of RFLP type, 97% similarity and 95% similarity, respectively. There was a significant site by treatment interaction at the taxonomic levels of RFLP type and 97% similarity (Table 1, Fig. 4). Site A showed a slight increase of taxon diversity with N-amendment, Site B showed no difference, and site C and D had a slight decrease (Fig. 4).

AMF community composition

Increased N-addition led to a significant change in AMF community composition at all taxonomic levels (Table 1, Fig. 5). The significance of the chronic N-addition effect on AMF community composition increased slightly from RFLP type taxonomic level to 95% sequence similarity (Table 1). All taxonomic levels also showed a significant site effect (Table 1). The first two axes of the CAP biplot explained 51%, 53% and 60% of the total variation in community composition among plots at the taxonomic level of RFLP type, 97% (Fig. 5) and 95% sequence similarity, respectively.

All OTU's belonged to only two families of the Glomeromycota, with over 90 % of all analyzed clones from the genus *Glomus*, and the remainder from the Acaulosporaceae (Acau1) (Table 2). Overall the most abundant OTU's were (in order of decreasing abundance); Glo2, Glo5, Glo6b, Glo8, Glo6, Glo1 (all belong to Glomus group A (Schüßler et al., 2001)), and Acau1, which all formed distinct clades within the phylogenic analysis (Fig. 2) and represented on average more than 80% of all analyzed clones for each treatment (Table 2).

Of the most abundant OTU's, only Acau1 showed a significant N treatment effect ($p < 0.001$), and Glo2, Glo5 and Glo8 a marginal treatment effect ($p < 0.1$) (Table 3). Acau1 had increased abundance at the N-amended plots compared to the control plots and it comprised ~20 % of all analyzed clones of the N-amended plots of site C (Table 2). Acau1 also showed a significant site effect and site by treatment interaction (Table 3). N-amendment affected Glo5 and Glo8 in opposite directions. Glo5 had a trend towards increased abundance with N-amendment, whereas Glo8 decreased with N-amendment (Table 2 and 3). For Glo2 the marginal negative N treatment effect was paralleled by a strong significant site effect (Table 3), with Glo2 decreasing in abundance going south (to higher N sites) along the gradient (Table 2). Glo2 was present at all sites and treatments and it was highly abundant at the control plots of site A (lowest N site) where it represented just over 50% of the clones analyzed, while in the N-amended plots it represented ~25% (Table 2, Fig 5b).

Two of the most abundant OTU's, Glo6b and Glo6, did not show any significant N-treatment effect, but did have a significant site effect (Table 3), suggesting that their

abundance was affected by the natural nitrogen gradient or by other site differences. Glo6b increased in abundance from site A (~6% of analyzed clones) to site D (~20% of analyzed clones) (Table 2). Besides the significant site effect, Glo6 also had a significant site by treatment interaction (Table 3). In response to N amendment Glo6 showed a slight increase at site A and B and a marginal decrease at sites C and D (Table 2). Site B had the highest abundance of Glo6.

No treatment or site effects were found for Glo1 (Table 3) and the OTU's that comprised the remainder of the clones (20%) found at our study sites. Glo1 was present in all the plots, while most of the rest of the OTU's were only present at some of the plots (Table 2).

Discussion

While sequence matches of 97-100% similarity with AMF taxa in GenBank were found for all our AMF sequences, most were to unidentified environmental isolates, with only a few sequences matching known AMF species within the GenBank database. This could indicate that our forests contain a large number of new species that have not been cultured before, or that the GenBank database is insufficient. The sequences that were matched with AMF taxa from GenBank were mostly from the genus *Glomus* and a few from the genus *Acaulospora*. The primer set we used within this study, NS31 and AM1, is known to adequately amplify the families Glomeraceae, Gigasporaceae and Acaulosporaceae (Helgason et al., 1998), but AM1 has been found to not properly target the Archaeosporaceae and Paraglomaceae (Redecker et al., 2000). It is therefore possible that we haven't identified the complete AMF taxa community composition of our study sites and we can only draw conclusions of the effects of N-amendment on the families of Glomeraceae, Gigasporaceae and Acaulosporaceae. However, of all the known AMF spores studies done in sugar maple (*Acer saccharum* Marshall) forests, no spores of the Archaeosporaceae or Paraglomaceae families were found (e.g. Lansing, 2003; Lerat, 2003; Coughlan et al., 2000; Moutoglis and Widden, 1995).

Chronic N-addition had different effects on AMF taxa diversity per site, indicated by a significant treatment by site interaction. Diversity at site A had a trend towards a positive response to N-amendment, while the N-amended plots at site C and D showed a trend towards a negative response by decreasing in taxa diversity. Porras-Alfaro et al. (2007) also found higher taxa diversity with N-amendment in a semiarid grassland. The fact that site C and D had a trend towards the reverse response of taxa diversity to N-amendment might be explained by the higher ambient N-deposition at those two sites compared to the two northern sites.

Shannon diversity index at our sites for a single tree species was in the same range (H =1.94-1.64, Table 1) as values for a grassland system for two plant species (H = 1.71, Vandenkoornhuyse et al., 2002), lower than in a tropical forest for 2 plant species (H = 2.33, Husband et al., 2002) but much higher than an arable site (H= 0.39, Helgason et al., 1998). However, lower taxa diversity does not necessarily reflect the functional diversity of the AMF species present. For example, Munkvold et al. (2004) found a substantial functional heterogeneity with low AMF species diversity by looking at species isolate characteristics and their benefits to the plant. This intraspecific functional variation raises another challenging aspect of interpreting AMF community composition and diversity and their related function in ecosystem processes (Sanders, 2004; van der Heijden and Scheublin, 2007). By analyzing the effects of N-amendment on community composition and diversity of our obtained AMF sequences at different taxonomic levels (100-95% sequence similarity) we tried to account for some of the effects caused by response at different levels of taxonomic organization.

The AMF community composition that we did find was strongly affected by N-amendment and site at all taxonomic levels, and effects of N-amendment also varied among AMF taxa. Other studies have found similar results, showing variable responses of the dominant AMF taxa under N-amendment (Porras-Alfaro et al., 2007; Jumpponen et al., 2005). Of our most abundant OTU's, Glo5 showed a similar positive response to N-amendment as a closely related environmental isolate found by Jumpponen et al. (2005). We also observed a positive response to N-addition for OTU Acau1, which suggested that our *Acaulospora* species was more tolerant to high nitrogen levels than

some of the *Glomus* taxa found at our sites, or better at competing with species from the *Glomus* genus at higher levels of nitrogen. Negative effects of N-amendment were found for Glo8. A similar negative effect of N addition was observed for close relatives of Glo8 found by Porras-Alfaro et al. (2007), but was contrasted by increased abundance with N-amendment for a closely related isolate found by Jumpponen et al. (2005).

Responses of some AMF taxa to N-amendment within our study were confirmed by similar responses found for those AMF taxa in other studies. Finding this repeated pattern is a prerequisite for determining universal patterns and rules for responses of AMF taxa to environmental change (van der Heijden and Scheublin, 2007). Although we are still far removed from identifying all AMF species within a variety of ecosystems and how they respond to human-accelerated environmental change, this study has contributed to insights of responses of some AMF taxa in sugar maple dominated forests, which form extensive stands in northern temperate biomes. Further analysis of the AMF community data will focus on the effects of additional environmental variables on the AMF community composition, such as litter C and nutrient content, foliar nutrient content, temperature, soil respiration and NPP. These analyses could reveal some indirect effects of increased N-amendment on AMF community composition, and could be useful for the creation of models predicting changes in fungal communities and plant-fungal relationships.

The changes in AMF community composition found at our sites together with an overall decrease in intraradical AMF abundance with N-amendment (van Diepen et al., 2007) could have implications for the functioning of this type of ecosystem. Johnson (1993) found that N-amendment may select less mutualistic AMF taxa, which is reflected through decreased nutrient uptake efficiency or an increased carbon cost for the host plant. AMF taxa that are abundant at high N-levels have been found to be less beneficial or even detrimental to the host plant (Corkidi et al., 2002). Our observed change in AMF community composition thus has the potential to substantially change both nutrient and carbon cycling within northern hardwood forests.

Acknowledgments

We thank the NSF for their continued financial support of this project. We also thank Carrie Andrew for her helpful advice and training with some of the molecular techniques used within this study.

References

Anderson, M.J. 2005. PERMANOVA: a FORTRAN computer program for permutational multivariate analysis of variance. Department of Statistics, University of Auckland, New Zealand.

Anderson, M.J. 2004. CAP: a FORTRAN computer program for canonical analysis of principal coordinates. Department of Statistics, University of Auckland, New Zealand.

Burton, A.J., Pregitzer, K.S., Reed, D.D., 1991. Leaf area and foliar biomass relationships in northern hardwood forests located along an 800 km acid deposition gradient. Forest Science 37: 1041-1059.

Colwell, R. K. 2005. EstimateS: Statistical estimation of species richness and shared species from samples. Version 7.5. User's Guide and application published at: http://purl.oclc.org/estimates.

Colwell, R. K., Coddington, J. A., 1994. Estimating terrestrial biodiversity through extrapolation. Philosophical Transactions of the Royal Society (Series B) 345: 101-118.

Colwell, R. K., Mao, C. X., Chang, J., 2004. Interpolating, extrapolating, and comparing incidence-based species accumulation curves. Ecology 85, 2717-2727.

Corkidi, L., Rowland, D.L., Johnson, N.C., Allen, E.B., 2002. Nitrogen fertilization alters the functioning of arbuscular mycorrhizas at two semiarid grasslands. Plant and Soil 240: 299–310.

Coughlan, A.P., Dalpé, Y., Lapointe, L., Piché, Y., 2000. Soil pH-induced changes in root colonization, diversity, and reproduction of symbiotic arbuscular mycorrhizal fungi from healthy and declining maple forests. Can. J. For. Res. 30: 1543-1554.

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

Eom, A-H., Hartnett, D.C., Wilson, G.W.T., Figge, D.A.H., 1999. The effect of fire, mowing and fertilizer amendment on arbuscular mycorrhizas in tallgrass prairi. American Midland Naturalist 142: 55-70.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41:95-98.

Helgason, T., Daniell, T.J., Husband, R., Fitter, A.H., Young, J.P.W., 1998. Ploughing up the wood-wide web? Nature 394: 431.

Helgason, T., Fitter, A.H., Young, J.P.W., 1999. Molecular diversity of arbuscular mycorrhizal fungi colonizing *Hyacinthoides non-scripta* (Bluebell) in a seminatural woodland. Molecular Ecology 8: 659-666.

Helgason, T., Merryweather, J.W., Denison, J., Wilson, P., Young, J.P.W., Fitter, A.H., 2002. Selectivity and functional diversity in arbuscular mycorrhizas of co-occurring fungi and plants from a temperate deciduous woodland. Journal of Ecology 90: 371–384.

Husband, R., Herre, E.A., Turner, S.L., Gallery, R., Young, J.P.W., 2002. Molecular diversity of arbuscular mycorrhizal fungi and patterns of host association over time and space in a tropical forest. Molecular Ecology 11: 2669-2678.

Johnson, N.C., 1993. Can fertilization of soil select less mutualistic mycorrhizae? Ecological applications 3: 749-757.

Jumpponen A, Trowbridge J, Mandyam K, Johnson L. 2005. Nitrogen enrichment causes minimal changes in arbuscular mycorrhizal colonization but shifts community composition – evidence from rDNA data. Biology and Fertility of Soils 41: 217–224.

Lansing, J.L., 2003. Comparing arbuscular and ectomycorrhizal fungal communities in seven North American forests and their response to nitrogen fertilization. PhD-thesis. University of California, Davis, CA, USA.

Lerat, S., 2003. Étude des relations source/puits de carbone dans la symbiose endomycorhizienne à arbuscules. Study of the relations between carbon source and sinks of an arbuscular endomycorrhizal symbiosis. Ph.D.-thesis.Université Laval. Québec, Canada.

Lilleskov, E.A., Parrent, J.L., 2007. Can we develop general predictive models of mycorrhizal fungal community–environment relationships? New Phytologist 174: 250-256.

Moutoglis, P., Widden, P., 1995. Vesicular-arbuscular mycorrhizal spore populations in sugar maple (*Acer saccharum* marsh. L.) forests. Mycorrhiza 6: 91-97.

Munkvold, L., Kjøller, R., Vestberg, M., Rosendahl, S., Jakobsen, I., 2004. High functional diversity within species of arbuscular mycorrhizal fungi. New Phytologist 164: 357–364

NADP. 2006. National Atmospheric Deposition Program (NRSP-3). NADP Program Office, Illinois State Water Survey, IL, USA.

Porras-Alfaro, A., Herrera, J., Natvig, D.O., Sinsabaugh, R.L, 2007. Effect of long-term nitrogen fertilization on mycorrhizal fungi associated with a dominant grass in a semiarid grassland. Plant and Soil 296: 65-75.

Redecker, D., J. B. Morton, and T. D. Bruns. 2000. Ancestral lineages of arbuscular mycorrhizal fungi (Glomales). Molecular Phylogenetics and Evolution 14: 276–284.

Sanders, I.R., 2004. Intraspecific genetic variation in arbuscular mycorrhizal fungi and its consequences for molecular biology, ecology, and development of inoculum. Canadian Journal of Botany 82: 1057-1062.

Schüßler, A., Gehrig, H., Schwarzott, D., Chris Walker, C., 2001. Analysis of partial *Glomales* SSU rRNA gene sequences: implications for primer design and phylogeny Mycological Research 105: 5-15.

Simon, L., LaLonde, M., Bruns, T.D., 1992. Specific Amplification of 18S Fungal Ribosomal Genes from Vesicular-Arbuscular Endomycorrhizal Fungi Colonizing Roots. Applied and Environmental Microbiology 58: 291-295.

Smith, S. E., Read, D. J., 1997. Mycorrhizal Symbiosis. 2nd edition, Academic Press, New York.

Swofford, D.L., 1998. PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Research 22, 4673-4680.

Treseder, K.K., 2004. A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric CO2 in field studies. New Phytologist 164: 347-355.

Treseder, K.K., Allen, M.F., 2002. Direct nitrogen and phosphorus limitation of arbuscular mycorrhizal fungi: a model and field test. New Phytologist 155: 507-515.

Vandenkoornhuyse, P., Husband, R., Daniel, T.J., Watson, I.J., Duck, J.M., Fitter, A.H., Young, J.P.W., 2002. Arbuscular mycorrhizal community composition associated with two plant species in a grassland ecosystem. Molecular Ecology 11: 1555-1564.

Van der Heijden, M.G.A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., Sanders, I.R., 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396: 69-72.

Van der Heijden, M.G.A., Scheublin, T.J., 2007. Functional traits in mycorrhizal ecology: their use for predicting the impact of arbuscular mycorrhizal fungal communities on plant growth and ecosystem functioning. New Phytologist 174: 244-250.

Van Diepen, L.T.A., Lilleskov, E.A., Pregitzer, K.S, Miller, R.M., 2007. Decline of arbuscular mycorrhizal fungi in northern hardwood forests exposed to chronic nitrogen additions. New Phytologist 176: 175-183.

Wirsel, S.G.R., 2004. Homogenous stands of a wetland grass harbour diverse consortia of arbuscular mycorrhizal fungi. FEMS Microbiology Ecology 48: 129–138.

Table 1 Comparison of P-values of PERMANOVA of AMF community composition and ANOVA of Shannon diversity index for the 3 different taxonomic levels.

Taxonomic level	# OTU's	Community composition			Shannon diversity			Mean Shannon diversity
		Treatment	Site	Treatment * Site	Treatment	Site	Treatment * Site	
RFLP type	27	0.0315	0.0001	0.1637	0.908	0.172	0.035	1.94
97% similarity	17	0.0150	0.0001	0.1046	0.894	0.144	0.029	1.88
95% similarity	12	0.0132	0.0003	0.0998	0.416	0.441	0.810	1.64

OTU's, Operational Taxonomic Units

Table 2 RFLP types grouped by OTU-ID and name at 95% and 97% sequence similarity. Mean percentages of OTU's at 97% similarity for the four study sites by treatment are displayed.

OTU ID	OTU name	RFLP types > 95% similarity	RFLP types > 97% similarity	Site A		Site B		Site C		Site D	
				C	N	C	N	C	N	C	N
1	Glo1	A,B,F,M	A,B,F,M	6.2	12.3	11.1	9.9	6.2	7.4	6.2	8.6
2	Acau1	C,N	C,N	1.9	9.3	0.0	1.2	5.6	20.4	3.7	4.3
3	Glo2	D,O,I	D	51.2	27.8	21.6	12.3	8.0	4.3	16.0	20.4
4	Glo2a		O	1.2	0.6	0.0	0.0	0.0	0.0	0.0	0.0
5	Glo2b		I	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0
6	Glo3	E,P,Q,R	E,P,Q,R	6.8	3.7	4.3	5.6	6.2	0.0	5.6	4.9
7	Glo4	S,T,U	S, T	0.0	0.0	9.3	5.6	4.3	5.6	4.9	1.9
8	Glo4a		U	0.0	0.0	1.2	0.6	0.0	0.0	0.0	0.0
9	Glo5	G	G	8.0	11.7	8.6	17.3	16.7	19.8	11.7	24.7
10	Glo6	H,K,L,V,X	K,L,V	0.6	13.0	13.0	14.8	13.6	4.9	2.5	1.2
11	Glo6a		H	3.7	8.6	1.9	5.6	5.6	2.5	11.1	6.8
12	Glo6b		X	9.3	6.2	4.9	12.3	22.2	21.6	21.0	18.5
13	Glo7	J	J	0.0	4.3	0.0	0.0	0.0	0.0	0.0	0.0
14	Glo8	AA	AA	11.1	2.5	21.6	14.2	11.7	12.3	16.0	8.6
15	Glo9	W	W	0.0	0.0	0.6	0.0	0.0	1.2	0.0	0.0
16	Glo10	Y	Y	0.0	0.0	1.2	0.0	0.0	0.0	0.6	0.0
17	Glo11	Z	Z	0.0	0.0	0.6	0.6	0.0	0.0	0.0	0.0

OTU's, Operational Taxonomic Units

Table 3 Comparison of P-values of ANOVA of dominant OTU's at 97% sequence similarity for the four study sites.

OTU	Treatment	Site	Treatment * Site
Glo2	0.07	<0.001	0.16
Glo5	0.08	0.34	0.77
Glo6b	0.94	0.04	0.76
Glo8	0.09	0.16	0.73
Glo6	0.62	0.01	0.02
Glo1	0.40	0.71	0.77
Acau1	<0.001	<0.001	0.01

OTU's, Operational Taxonomic Units

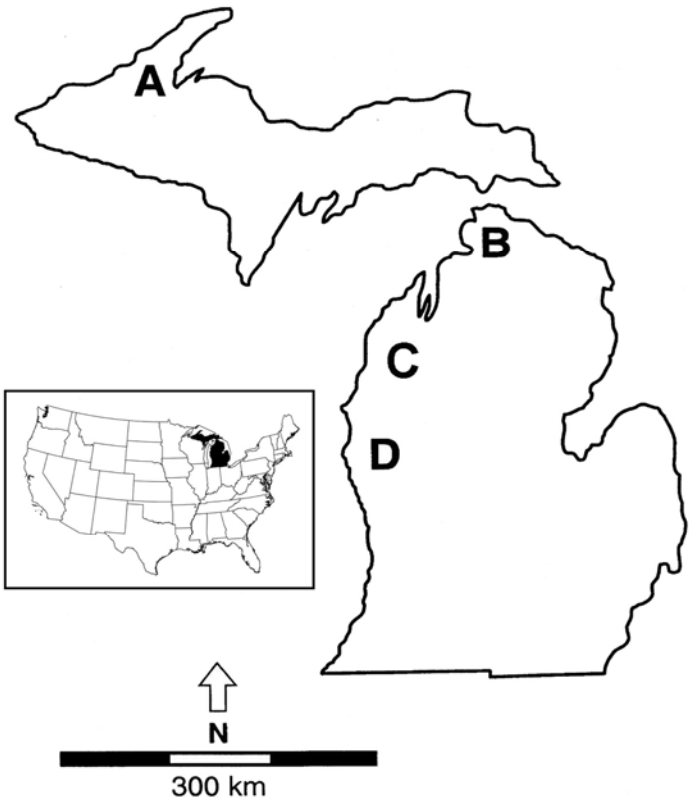

Fig. 1 Locations of the study sites (A-D) in Michigan. The inset shows the location of Michigan (filled area) within the USA.

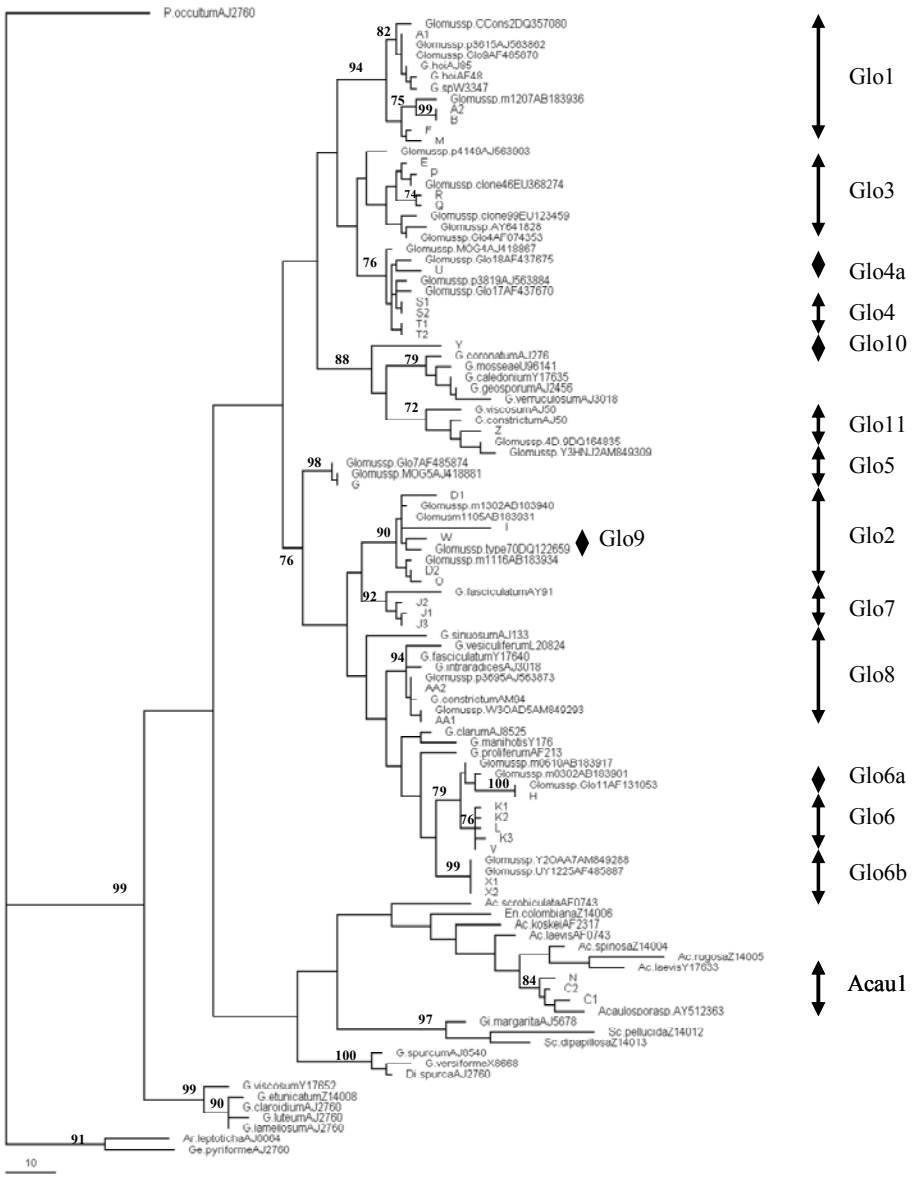

Fig. 2 Maximum parsimony phylogenetic tree of obtained AMF sequences from this study, GenBank sequences of known Glomeromycota, and of other GenBank AMF sequences of the 18S rDNA region highly similar to our sequences. Neighbor-joining phylogenetic tree had the same general topology. Tree is rooted to *Paraglomus occultum* (AJ276082). Bootstrap values of 70% (1000 replicates) and higher are shown, indicating well supported branching. OTU's as defined at >97% sequence similarity (Table 2) are displayed on the right side of the tree.

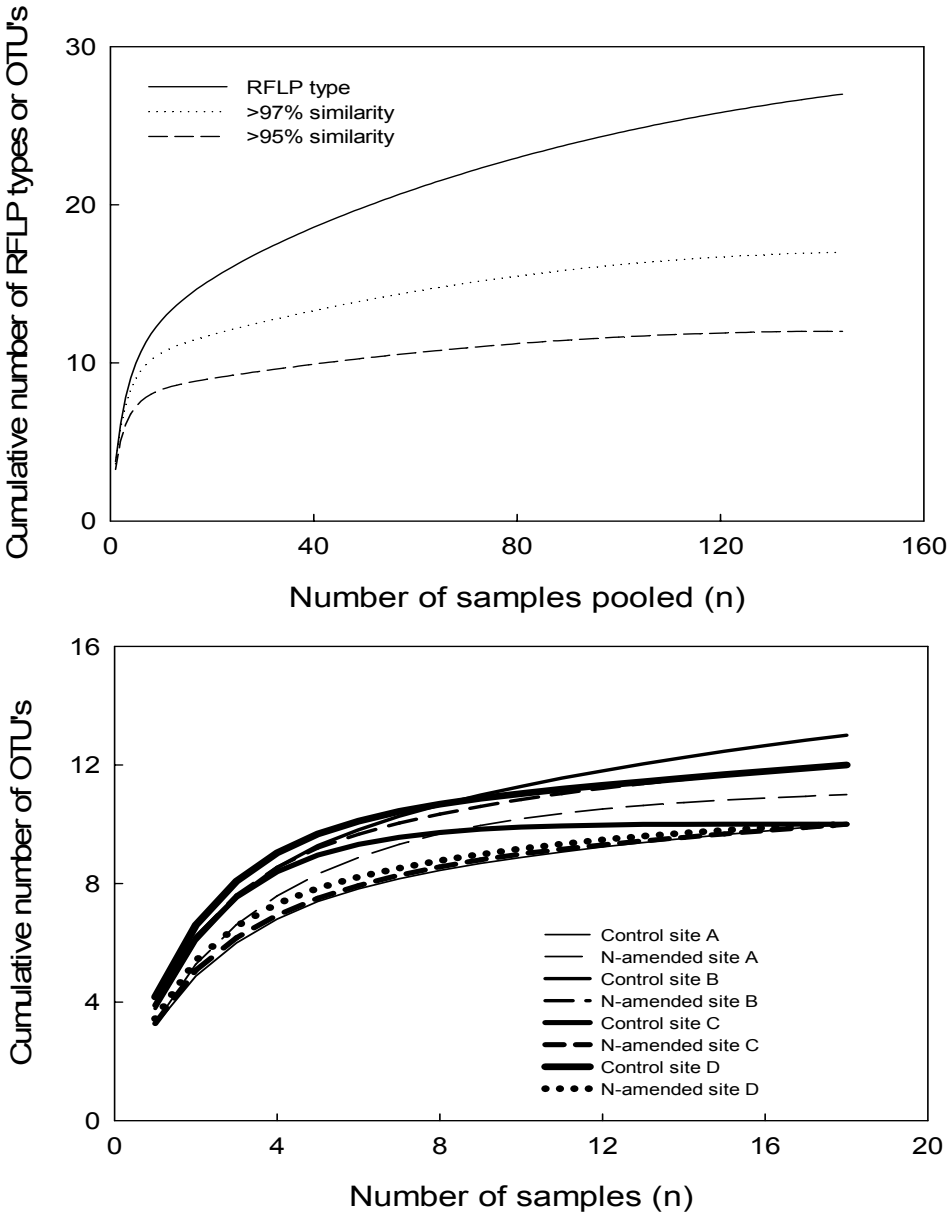

Fig. 3 Rarefaction curves with Mao Tau values estimating species accumulation a) at all taxonomic levels for number of AMF taxa of the pooled samples from all plots, and b) at treatment by site level at the taxonomic level of 97% similarity.

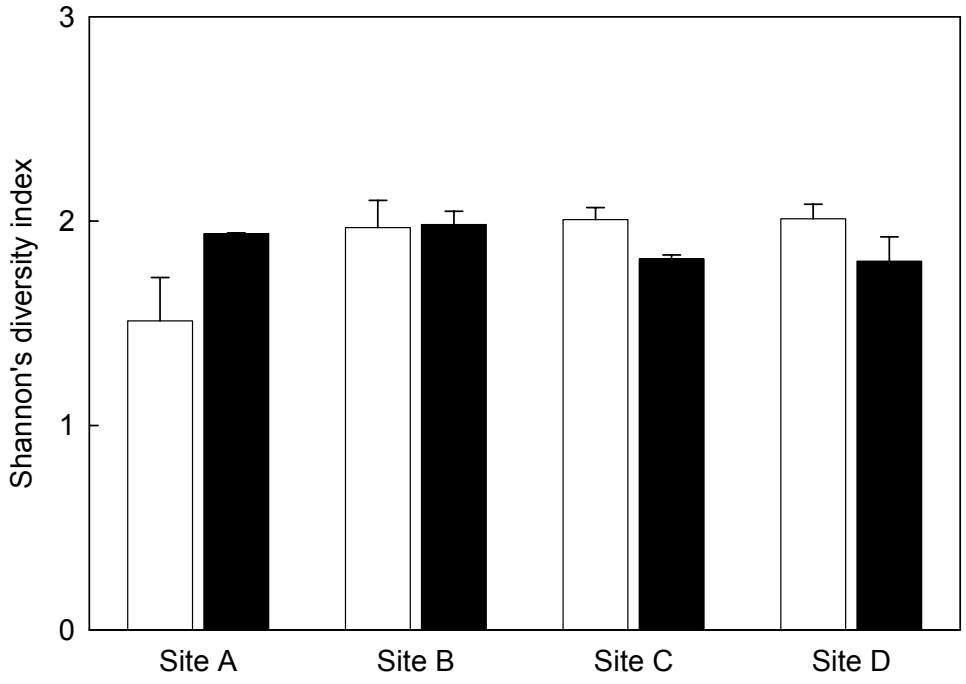

Fig. 4 Mean Shannon diversity index at the taxonomic level of 97% sequence similarity for the four study sites by treatment (open bars, control; closed bars, N-amended). Error bars indicate 1 SE of the mean. Overall treatment and site effect was not significant, but a significant treatment by site interaction was observed.

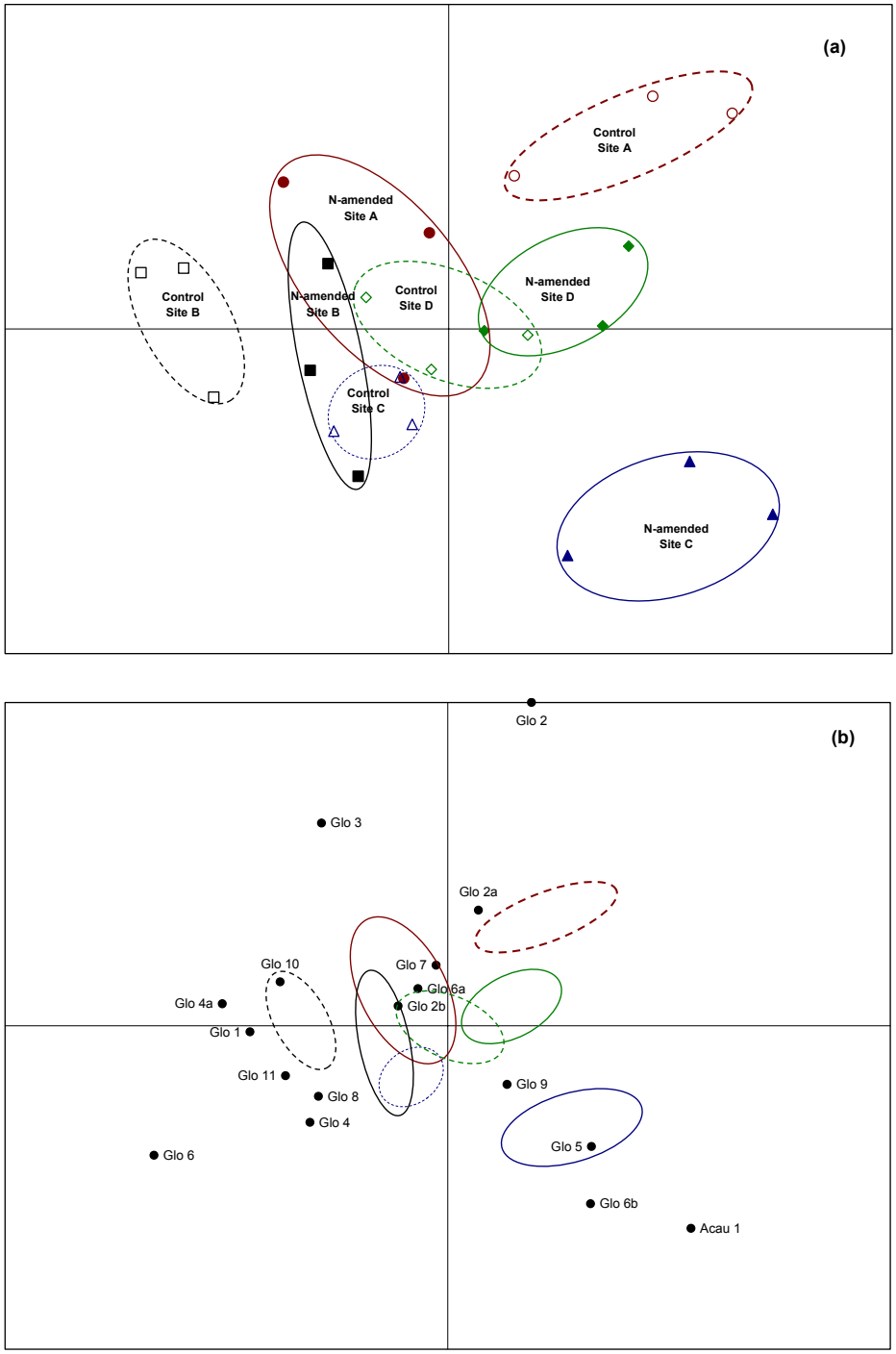

Fig. 5 Canonical Analysis of Principal Coordinates (CAP) biplots based on Bray-Curtis distance measure of (a) AMF taxa composition of plots at treatment by site level with OTU's defined at 97% sequence similarity, and b) AMF taxa together with plots. Biplots of the other taxonomic levels showed similar placement of plots and taxa.

Chapter 3 Chronic nitrogen addition causes a decline of intra- and extraradical abundance of arbuscular mycorrhizal fungi and changes in microbial community composition in northern hardwood forests.

Summary

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 chronic nitrogen (N) addition using phospholipid fatty acid analysis (PLFA). Intra- and extraradical AMF biomass and total microbial biomass were significantly decreased by N addition. N-amendment also significantly affected the microbial community composition, and was dominated by a decrease in fungal to bacterial biomass ratios. Our observed decline in AMF and total microbial biomass together with changes in microbial community composition could have substantial impacts on the nutrient and carbon cycling within northern hardwood forest ecosystems.

Introduction

Of the different classes of mycorrhizal fungi, arbuscular mycorrhizal fungi (AMF) are most common in terrestrial ecosystems (Smith and Read, 1997). Most herbaceous plants, agricultural plants and tropical tree species have a symbiotic relationship with AMF. However, in northern high latitude forests ectomycorrhizal (EM) fungi predominate and only a few tree families are associated with AM fungi, e.g. Aceraceae, Rosaceae and Oleaceae (Smith and Read, 1997). Consequently, fewer studies within boreal and higher-latitude temperate forests have focused on the function of AMF in ecosystems.

Mycorrhizal fungi play a pivotal role in plant carbon and nutrient balance, supplying nutrients to the plant in exchange for carbon that they receive from their host plant (Smith and Read, 1997). Mycorrhizal fungi may thus be influenced by changes in nitrogen

availability caused by natural disturbance, fertilization or atmospheric nitrogen deposition. The effects of nitrogen addition on AMF abundance has been studied in several different ecosystems and has generally been found to be negative (van Diepen et al., 2007; Treseder, 2004 and references therein). However most of these N addition studies have focused on the intraradical abundance of AMF, and only a few have been performed in a field setting.

Although intraradical hyphae are responsible for exchange of resources with the host plant, the extraradical AMF mycelium is responsible for the initial uptake of nutrients from the soil. Therefore, when studying nutrient cycling within ecosystems, the function of both the intra- and extraradical AMF are of importance. Extraradical AMF can constitute up to 30% of the total microbial biomass in the soil (Leake et al., 2004), but have not been as extensively studied as intraradical AMF. This lack in understanding of the functioning of extraradical mycelium is mainly caused by the difficulty of estimating their presence within the soil environment.

Fatty acid (phospho and neutral lipids) analyses have been successfully used to estimate AMF intra-radical and extraradical biomass by using the fatty acid 16:1ω5 as AMF biomarker (e.g. van Diepen et al., 2007; Balser et al., 2005; Olsson, 1999). Phospholipids are part of the cell membrane, present in relatively stable ratios with AMF biomass, and are degraded quickly after cell death. This makes them suitable markers for AMF live biomass. In contrast, neutral lipids are storage lipids, the abundance of which can vary greatly over time, and are therefore better indicators of proportional allocation to storage (van Diepen et al., 2007). Correct estimation of AMF biomass in soil is complicated by soil bacteria which also contain small amounts of the phospholipid fatty acid 16:1ω5 (Olsson, 1999). However, these bacteria tend not to use lipids as energy reserves, and therefore have very low NLFA (neutral lipid fatty acid) 16:1ω5 storage lipid content. In soils with an NLFA:PLFA 16:1ω5 ratio greater than one, PLFA 16:1ω5 can be assumed to be mainly derived from AMF (Olsson and Wilhelmsson, 2000; Olsson, 1999; Olsson et al., 1997).

The performance of PLFA analyses on soil for estimating AMF extraradical abundance has the extra advantage of simultaneously providing insight into the total

microbial biomass and composition of the rest of the microbial community. Phospholipid fatty acid analysis has been proven to be successful for identification and biomass estimation of groups of bacteria and fungi within the soil environment (Frostegård and Bååth, 1996; Tunlid and White, 1992) and has been applied to understand changes in soil microbial communities caused by various disturbances (e.g. Allison et al., 2005; Waldrop et al., 2004; Zak et al., 1996). Field studies have observed a decrease in total microbial biomass and/or changes in microbial community composition with increased N deposition. For example both Compton et al. (2004) and DeForest et al. (2004) found a decrease in total microbial biomass with N-amendment for 11 and 7 years, respectively. However, in these studies only Compton et al (2004) found changes in microbial community composition. The study by DeForest et al.(2004) was carried out in the same study system investigated in the present study, providing the opportunity to compare medium and longer-term impacts of N inputs on microbial communities.

In this study we examined the effects of increased chronic nitrogen addition on the intra- and extraradical abundance of AMF in relation to the rest of the microbial community using PLFA analyses. We hypothesized that both intra- and extraradical AMF and microbial biomass would decrease and also changes in soil microbial community composition would be observed after 12 years of N-amendment.

Materials and methods

Study sites

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

Soil and root sampling,

For soil and root sampling, all the plots were divided into six equally sized subplots (10 x 15m). Two paired soil cores, 2 cm diameter and 10 cm deep, were taken randomly in each subplot, at the beginning of October 2006. Each soil core of the first set was washed through a 2mm and 1mm screen until most soil particles were washed out. Maple (*Acer spp.*) roots were handpicked from the screens and cleaned more thoroughly with DI water and by sonication for 5 min. The maple roots were divided up into two diameter classes, fine roots (<0.5mm) and larger roots (>0.5mm), and weights of both 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 paper), and the other subsample was composited at the plot level, frozen, freeze dried and ground (SPEX Certiprep Mill, 8000M), and used for PLFA analysis to estimate AMF intra-radical abundance.

The second set of soil cores were composited at the plot level, stored on ice for transport and frozen at -20 ˚C within 12 hours after sampling. The composite soil samples were freeze-dried (Labconco, Freezone 4.5), sieved through a 2mm sieve, and the <2mm fraction was mixed thoroughly. Subsequently, a subsample of ~10g mixed soil sample (<2mm) was cleaned by manually removing roots and litter particles. The cleaned freeze-dried soil (< 2mm) was used for PLFA analysis to estimate abundance of AMF extraradical hyphae and other members of the microbial community.

Lipid extraction and analysis

Lipids were extracted from approximately 2.5 g of cleaned freeze-dried soil (< 2mm) or 15mg of freeze-dried ground roots, vortexed in a one-phase mixture of methanol, chloroform and phosphate buffer (pH 7.4) with a volume ratio of 2:1:0.8, using the Frostegård et al. (1991) modification of the Bligh and Dyer (1959) method. The extracted lipids were separated into neutral-, glyco-, and phospholipid fractions by silicic acid column chromatography by eluting with chloroform, acetone and methanol, respectively. The neutral- and phospholipids were methylated to free fatty acid methyl esters

(FAME's) using a mild alkaline solution. The FAME's were then dissolved in hexane and analyzed by gas chromatography using a 6890N GC (gas chromatograph; Agilent Technologies, Palo Alto, CA) with an Ultra 2 column (30m, 0.2mm ID, 0.33µm film), an FID detector and hydrogen as carrier gas. Conditions (temperature and time) for the GC analyses are set by the Microbial Identification System (Sherlock version 4.5, MIDI Inc., Newark, DE). The FAME 19:0 (Matreya Inc., State College, PA) was used as internal quantitative standard and the FAME's were identified by a Microbial Identification System (Sherlock version 4.5, MIDI Inc., Newark, DE) using a eukaryotic mix (MIDI Inc., Newark, DE) as qualitative standards. To confirm MIDI software identification several samples were also analyzed using a GC-MS (gas chromatograph-mass spectrometer) at Argonne National laboratory (R.M. Miller laboratory) in parallel to the analysis using the GC with MIDI software.

The fatty acid 16:1ω5c was used as an indicator for AM fungi (Olsson, 1999; Olsson & Johansen, 2000; Allison et al., 2005). Phospholipid fatty acids (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 et al., 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 et al., 2005).

Calculations of AMF intra- and extraradical biomass

To understand the effects of chronic N addition on the 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, and 3) proportional allocation to AMF as nmol of fatty acid 16:1ω5c per maple aboveground biomass. For calculation details, see van Diepen et al. (2007). For the calculations of the proportional allocation metric only maple aboveground biomass was used. Some of the extraradical AMF hyphal biomass could also come from the tree species black cherry (*Prunus serotina*) and white ash (*Fraxinus Americana*) at our sites which are also associated with AMF. However on average these two species represented less than 5 % of the total

standing biomass at our study sites in 2006, and therefore their contribution to AMF extraradical hyphae would be very minor.

Statistical analysis

Differences in dependent variables (all PLFA values) between treatments were determined using a two-way ANOVA with N treatment (n = 2) and site (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 were analyzed using permutational multivariate analysis of variance (PERMANOVA, Anderson, 2005) based on the Bray–Curtis distance measure. PERMANOVA does not provide graphical data display, so the community data were visualized using biplots of CAP (Canonical Analysis of Principal Coordinates, Anderson, 2004) output. CAP was performed with each treatment at each site as a separate group (total of eight groups).

Results

AMF intra- and extra radical biomass

AMF indicator PLFA 16:1ω5c was found to be significantly reduced by N-amendment in maple (*Acer spp.*) fine roots (intraradical) and in soil (extraradical) and total biomass (intra- and extraradical) by using all three metrics (Table 1, Fig. 2). The mean percentage of decrease differed among metrics for both intra- and extraradical AMF biomass, and ranged from 36 to 51% (Table 1). Similar results were found for NLFA 16:1ω5c, with the exception of stand level maple AMF extraradical abundance which had only a marginal decrease with N-amendment (Table 1). N-amendment on average reduced NLFA 16:1ω5c by 28 % for both root and soil colonization intensity and by 38% for proportional allocation to total AMF biomass (Table 1). Significant site effects were only observed for the neutral lipid measurements of AMF extraradical biomass, and total AMF biomass (Table 1, Fig. 2d and 2f, respectively). Some significant treatment by site interactions were found for PLFA and NLFA 16:1ω5c values for all

three metrics (Table 1, Fig. 2), which indicated different treatment responses among sites. For example, proportional allocation to total AMF biomass measured as NLFA 16:1ω5c was more affected by N-amendment at site A and C compared to site B and D (Fig. 2f).

NLFA to PLFA ratios of 16:1ω5c in soil and roots were greater than 1 on all plots (mean of 2.7 and 20, respectively), and the ratio did not differ among treatment ($p = 0.56$ and $p = 0.37$, respectively, Fig. 3). Therefore PLFA 16:1ω5c could be used as a biomass estimator for AMF live biomass.

The percentage of intraradical AMF compared to total AMF biomass 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 nitrogen effects were found on the proportion of intraradical AMF compared to total AMF biomass (Table 1), thus both intra- and extraradical AMF biomass estimates were equally negatively affected by N-amendment.

Microbial biomass and community

Total microbial biomass, represented by total PLFA, decreased significantly with N-amendment by 24% (Table 2). No significant differences among sites were found, and only a marginal treatment by site interaction (Table 2). Among the different microbial groups, as defined by PLFA analysis, all were significantly negatively affected by N-amendment, except for gram positive bacterial phospholipids, and fungal (not including AMF) neutral lipids which showed no significant effect (Table 2). Total bacterial biomass represented on average 74% of total PLFA and only 18% of total NLFA, confirming the fact that bacteria have low levels of NLFA.

The composition of the microbial community was also significantly affected by treatment ($p = 0.0007$), and differed among sites ($p = 0.0001$) (Fig. 4a). The relative abundance of fungi and bacteria was largely responsible for the variation among treatment and sites (Fig. 4b). The different patterns for bacteria and fungi among treatment and sites is also indicated by the significant treatment and site effect found for total fungal to bacterial ratio (Table 2). Total fungal to bacteria biomass ratio decreased with N-amendment by phospholipid measurement (Fig. 5a), and similar results were

found for AMF to bacteria biomass ratio (Table 2, Fig. 5b) indicating that bacteria increase in abundance relative to fungi in general and AMF in particular under chronic N addition. Chronic N addition only marginally affected the saprotrophic fungi to bacteria ratio (Table 2, Fig 5c). Furthermore, the saprotrophic to AM fungal ratio as measured by PLFA increased significantly with N-amendment (Table 2) indicating that AMF decreased in abundance relative to saprotrophic fungi with N-amendment.

Discussion

AMF intra- and extra radical biomass

In the present study, N-amendment suppressed both intra- and extraradical AMF biomass as measured by PLFA 16:1ω5c. We found similar results for intraradical AMF biomass at these sites in 2005 (van Diepen et al., 2007). In contrast, in 2001 extraradical AMF biomass measured as soil PLFA 16:1ω5c was not found to be significantly reduced by N-amendment ($p = 0.121$), but there was a trend in this direction (DeForest et al., 2004). This disparity in extraradical AMF biomass response to N-amendment between 2001 and 2006 could be caused by a lag-period in the effect of N-amendment or cumulative dose effect, as was also observed for soil respiration at our sites (Burton et al., 2004). Plotting the soil PLFA 16:1ω5c values of 2006 against 2001 showed a significant linear relationship ($p = 0.01$), but a low R^2 of 0.24, confirming the disparity in AMF biomass response between 2001 and 2006. However, the low R^2 could also indicate noise in the sampling, e.g. because of low number of cores per plot or time of year effects.

In contrast with the observed long-term effect of N amendment on AMF in forests, short-term field studies that analyzed both intra- and extraradical AMF biomass showed different results (Table 3). Garcia et al. (2008) and Treseder et al. (2007) found an increase in intraradical AMF colonization and no effect on extraradical AMF mycelium as measured by hyphal length in a temperate forest after one year of N-amendment of 100 kg ha^{-1} yr^{-1}, and in a boreal forest after 2 years of 100 kg ha^{-1} yr^{-1} N-amendment, respectively. The lack of effect of N-amendment or increase in intraradical AMF in both studies could have been caused by the short period of N-amendment combined with lags

in, or cumulative dose effects on, AMF response, as found in the present study (Table 3). In our study, dose was higher (~360 kg N) and fertilization period longer (12 yr) than in the other two studies.

The effect of long term N-amendment might also be related to the type of ecosystem that was studied (Table 3). For example, in a tallgrass prairie Eom et al. (1999) still found increased biomass production of intra- and extraradical AM fungal mycelium after 9 years of 100 kg ha^{-1} yr^{-1} N addition (dose of 900 kg N, Table 3). In contrast, in a desert and agricultural ecosystem Johnson et al. (2003) found significantly decreased AMF hyphal lengths in the soil after three to ten years of N-amendment (100-170 kg ha^{-1} yr^{-1} N, dose of 300-1700 kg N) (Table 3). And in a similar ecosystem compared to our study sites (sugar maple dominated forest) Phillip and Fahey (2007) showed a reduction in intraradical AMF abundance with two years of 167 kg ha^{-1} yr^{-1} N amendment (Table 3). Their cumulative dose of N (334 kg) was comparable to the total amount of N addition in our study (360 kg N). The effects of N-amendment thus vary with length of N-amendment and cumulative dose of N in combination with the type of ecosystem (Table 3).

Other reasons 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. For example, the increased AMF biomass that Eom et al. (1999) still observed after 9 years of N-amendment could be explained by lower soil inorganic phosphorus (P) concentration in the tall grass-prairie. The plants in that study therefore had to compensate for higher N levels by increasing P-uptake using AMF. 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 compensation was necessary.

Another reason for the observed decrease in total AMF (intra- and extraradical) biomass by N-amendment in our study was possibly caused by reduced carbon (C) allocation to AMF by the host plant. The host plant was able to assimilate enough nitrogen at the N-amended plots, with less help from AMF. Furthermore, decreased soil

respiration (Burton et al., 2004) together with increased tree growth (Pregitzer et al., 2008) with N-amendment found at our study sites confirmed the lower carbon investment in belowground biomass.

Most of the mentioned studies on effects of N amendment on AMF biomass used percentage root colonization (using a traditional staining technique) as an estimate for intraradical AMF biomass and hyphal length measurements in soil as an estimate for extraradical AMF biomass. However, with these techniques, colonization intensity within roots and changes in root biomass are not taken into account, and no distinction can be made between dead and live extraradical AMF mycelium. PLFA analyses (phospholipids) using the biomarker fatty acid 16:1ω5c have been shown to be a good estimator of live intraradical AMF biomass (van Diepen et al., 2007) and Balser et al. (2005) found that soil PLFA 16:1ω5 was a good indicator of extraradical hyphal biomass by comparing hyphal length measurements with PLFA 16:1ω5 in soil samples. Another advantage of using similar biomass estimators for both intra- and extraradical AMF biomass is the comparability of values when scaled up to a stand level.

At the stand level most of the live AMF biomass (92.5% ± 2.1 of phospholipid 16:1ω5) was extraradical, while stored energy (neutral lipid 16:1ω5) was more equally divided over intra- and extraradical AMF biomass (35 and 65% ± 5.3, respectively). Leake et al. (2004) assessed from several studies that most of the photosynthetically derived carbon of the host plant is allocated to the extraradical hyphae of associated mycorrhizae. Arbuscular mycorrhizal roots contain vesicles that can store large amounts of neutral lipids. The concentration of neutral lipids in roots has been found to increase compared to phospholipids at the end of the growing season (van Diepen et al., 2007), which is when our samples were taken. Therefore the proportion of intraradical to extraradical NLFA 16:1ω5 could be different when sampled in the middle of the growing season. However, most of the spore production also takes place at the end of the growing season, which would cause higher concentrations of NLFA 16:1ω5 in the soil compared to earlier in the growing season.

Microbial community

The decrease in total microbial biomass and lower fungal to bacteria ratio that we observed with N-amendment has been found in several other studies. Three different long term N addition experiments in forest ecosystems compared by Wallenstein et al. (2006) showed a significant decrease in total soil microbial biomass and fungal to bacterial activity ratio. In contrast, Allison et al. (2008) did not find an effect of short-term N fertilization on microbial biomass, nor the fungi to bacteria ratio in boreal forests, which were sampled in the same study sites that also did not find an effect of N-amendment on extraradical AMF biomass (Treseder et al., 2007). This again might indicate that long term N-amendment is needed to observe some of the effects of increased N addition. This was also confirmed at our study sites, where after 7 years of N-amendment an overall decrease in total microbial biomass was observed, but no detectable changes in microbial community composition (DeForest et al., 2004).

A possible cause of lower fungal to bacteria ratios with N-amendment is changes in the quality of organic matter. Fungi in general have a higher C:N ratio compared to bacteria (Paul and Clark, 1996) and to maintain this they therefore likely need to consume organic matter of high C:N ratio (Wallenstein et al., 2006). This hypothesis is supported in the present study by the positive correlation of fungal:bacterial biomass ratio and C:N ratio of sugar maple litter (A.J. Burton, unpublished data) at our study sites ($p = 0.003$, $R^2 = 0.33$). Furthermore, the reduction in saprotrophic fungal biomass is consistent with the observed decline in lignolytic enzyme activity used to degrade lignin (DeForest et al., 2004), which is primarily associated with saprotrophic fungi.

Under chronic N addition saprotrophic fungi were less affected by N-amendment than AMF. As a result, chronic N addition only marginally affected the saprotrophic fungi to bacteria ratio (Table 2, Fig 5c), while AMF to bacterial biomass ratio significantly decreased (Table 2, Fig. 5b) and the saprotrophic to AM fungal ratio increased with N-amendment (Table 2).

Implications for forest ecosystem

Soil microbes play a critical role in nutrient cycling, 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 cycling with these forest ecosystems. Our observed decline in microbial biomass is consistent with the increase in organic matter with no changes in organic matter input, indicating slower organic matter decomposition (Zak et al., 2008).

AMF can make up a large part of the microbial biomass, especially the extraradical mycelium (Leake et al., 2004). AMF also produce a glycoprotein, glomalin, which has a very long residence time in soil, and can represent between 3-8% of SOC (soil organic carbon) (Rillig et al., 2001). A decrease in AMF biomass could therefore have large consequences for soil organic matter content. However, in the present study soil C actually appears to be increasing (Pregitzer et al., 2008), suggesting that other factors, such as a decrease in the activity of saprotrophic fungi (DeForest et al., 2004) might be coming into play.

In conclusion, after 12 years of N-amendment both the intra- and extraradical AMF biomass and total microbial biomass were significantly decreased in sugar maple dominated hardwood forests. The microbial community composition was also different under N-amendment and was dominated by a decrease in fungal to bacterial biomass ratios. The difference in results found for microbial community composition after 7 years and 12 years of N-amendment suggests that a lag or cumulative dose effect exists. Our observed declines in AMF biomass and fungal to bacterial ratio could have significant implications for both the nutrient and carbon cycling within sugar maple dominated forest ecosystems.

Acknowledgments

We thank the NSF for their continued financial support of this project. Further, we are thankful to Cheryl Krol and Mike Miller for analyzing some of the PLFA samples within their lab (Argonne National Laboratory).

References

Allison, S.D., Czimczik, C.I., Treseder, K.K., 2008. Microbial activity and soil respiration under nitrogen addition in Alaskan boreal forest. Global Change Biology 14: 1156–1168.

Allison, V.J., Miller, R.M., Jastrow, J.D., Matamala, R., Zak, D.R., 2005. Changes in soil microbial community structure in a tallgrass prairie chronosequence. Soil Science Society of America Journal 69:1412–1421.

Anderson, M.J., 2005. PERMANOVA: a FORTRAN computer program for permutational multivariate analysis of variance. Department of Statistics, University of Auckland, New Zealand.

Anderson, M.J., 2004. CAP: a FORTRAN computer program for canonical analysis of principal coordinates. Department of Statistics, University of Auckland, New Zealand.

Balser, T.C., Treseder, K.K., Ekenler, M., 2005. Using lipid analysis and hyphal length to quantify AM and saprotrophic fungal abundance along a soil chronosequence. Soil Biology and Biochemistry 37: 601-604.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37: 911-917.

Burton, A.J., Pregitzer, K.S., Crawford, J.N., Zogg, G.P., Zak, D.R., 2004. Simulated chronic NO_3^- addition reduces soil respiration in northern hardwood forests. Global Change Biology 10:1080-1091.

Burton, A.J., Pregitzer, K.S., Reed, D.D., 1991. Leaf area and foliar biomass relationships in northern hardwood forests located along an 800 km acid deposition gradient. Forest Science 37: 1041-1059.

Compton, J.E., Watrud, L.S., Porteous, L.A., DeGrood, S. Response of soil microbial biomass and community composition to chronic nitrogen additions at Harvard forest. Forest Ecology and Management 196: 143–158.

Deforest, J.L., D.R. Zak, K.S. Pregitzer, and A.J. Burton, 2004. Atmospheric nitrate deposition, microbial community composition, and enzyme activity in northern hardwood forests. Soil Science Society of America Journal 68:132-138.

Eom, A-H., Hartnett, D.C., Wilson, G.W.T., Figge, D.A.H., 1999. The effect of fire, mowing and fertilizer amendment on arbuscular mycorrhizas in tallgrass prairie. American Midland Naturalist 142: 55-70.

Frostegård, A., Bååth, E., 1996. The use of phospholipids fatty acid analysis to estimate bacterial and fungal biomass in soil. Biology and Fertility of Soils 22: 59–65.

Frostegård, A., Tunlid, A., Bååth, E., 1991. Microbial biomass measured as total lipid phosphate in soils of different organic content. Journal of Microbiological Methods 14: 151-163.

Garcia, M.O., Ovasapyan, T., Greas, M., Treseder, K.K., 2008. Mycorrhizal dynamics under elevated CO2 and nitrogen fertilization in a warm temperate forest Plant Soil 303: 301–310.

Johnson, N.C., Rowland, D.L., Corkidi, L., Egerton-Warburton, L.M., Allen E.B., 2003. Nitrogen enrichment alters mycorrhizal allocation at five mesic to semiarid grasslands. Ecology 84: 1895–1908.

Leake, J., Johnson, D., Donnelly, D., Muckle, G. Boddy, Read, D., 2004. Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. Canadian Journal of Botany 82: 1016-1045.

NADP., 2006. National Atmospheric Deposition Program (NRSP-3). NADP Program Office, Illinois State Water Survey, IL, USA.

Olsson, P.A., 1999. Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. FEMS Microbiology Ecology 29:303-310.

Olsson, P.A., Bååth, E., Jakobsen, I., 1997. Phosphorus effects on the mycelium and storage structures of an arbuscular mycorrhizal fungus as studied in the soil and roots by analysis of fatty acid signatures. Applied and Environmental Microbiology 63: 3531-3538.

Olsson, P.A., Johansen, A., 2000. Lipid and fatty acid composition of hyphae and spores of arbuscular mycorrhizal fungi at different growth stages. Mycological Research 104: 429-434.

Olsson, P.A., Wilhelmsson, P., 2000. The growth of external AM fungal mycelium in sand dunes and in experimental systems. Plant and Soil 226: 161–169.

Paul, E.A., Clark, F.E., 1996. Ammonification and nitrification. In: Paul EA, Clark FE, eds. Soil microbiology and biochemistry. San Diego, CA, USA. Academic Press: 131–146.

Pregitzer, K.S., Burton, A.J., Zak, D.R., Talhelm, A.F., 2008. Simulated chronic nitrogen deposition increases carbon storage in northern temperate forests. Global Change Biology 14: 142-153.

Phillips, R.P., Fahey, T.J., 2007. Fertilization effects on fine root biomass, rhizosphere microbes and respiratory fluxes in hardwood forest soils. New Phytologist 176: 655–664.

Rillig, M.C., 2004. Arbuscular mycorrhizae and terrestrial ecosystem processes. Ecology Letters 7: 740-754.

Rillig, M.C., Wright, S.F., Nichols, K.A., Schmidt, W.F., Torn, M.S., 2001. Large contribution of arbuscular mycorrhizal fungi to soil carbon pools in tropical forest soils. Plant and Soil 233: 167–177.

Smith, S. E., Read, D. J., 1997. Mycorrhizal Symbiosis. 2nd edition, Academic Press, New York.

Treseder, K.K., 2004. A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric CO2 in field studies. New Phytologist 164: 347-355.

Treseder, K.K., Turner, K.M., Mack, M.C., 2007. Mycorrhizal responses to nitrogen fertilization in boreal ecosystems: potential consequences for soil carbon storage. Global Change Biology 13: 78-88.

Tunlid, A., White, D.C., 1992. Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of microbial communities in soil. Soil Biochemistry 7: 229–262.

Van Diepen, L.T.A., E.A. Lilleskov, K.S. Pregitzer, and R.M. Miller, 2007. Decline of arbuscular mycorrhizal fungi in northern hardwood forests exposed to chronic nitrogen additions. New Phytologist 176: 175-183.

Waldrop, M.P., Zak, D.R., Sinsabaugh, R.L., 2004. Microbial community response to nitrogen deposition in northern forest ecosystems. Soil Biology and Biochemistry 36: 1443-1451.

Wallenstein, M.D., McNulty, S., Fernandez, I.J., Boggs, J., Schlesinger, W.H., 2006. Nitrogen fertilization decreases forest soil fungal and bacterial biomass in three long-term experiments. Forest ecology and management 222: 459-468.

Zak, D.R., Holmes, W.E., Burton, A.J., Pregitzer, K.S., Talhelm, A.F., 2008. Atmospheric NO_3^- deposition increases soil organic matter by slowing decomposition in a northern hardwood ecosystem. Ecological Applications, in press.

Zak, D.R., Ringelberg, D.B., Pregitzer, K.S., Randlett, D.L., White, D.C., Curtis, P.S., 1996. Soil microbial communities beneath Populus grandidentata grown under elevated atmospheric CO2. Ecological Applications 6: 257-262.

Table 1 Comparison of p-values and mean N-effect for all three metrics of intra- and extraradical AMF abundance measured as phospho- and neutral lipid fatty acids (PLFA and NLFA) across a nitrogen deposition gradient in Michigan, USA.

		Treatment		Site		Treatment x site		Mean N-effect (%)	
Metric	**Measurement**	**PLFA**	**NLFA**	**PLFA**	**NLFA**	**PLFA**	**NLFA**	**PLFA**	**NLFA**
AMF colonization intensity	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	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 extra radical	<0.001	0.003	0.15	0.02	0.04	0.03	-42	-34
Proportional allocation to AMF	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 extra radical	<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

Mean N-effect: negative numbers mean a decrease with N-amendment and positive number an increase

Table 2 Comparison of p-values and mean N-effect for the abundance of different soil microbial groups measured as phospho- and neutral lipid fatty acids (PLFA and NLFA) across a nitrogen deposition gradient in Michigan, USA.

Microbial group	Fatty acid	Treatment	Site	Treatment x site	Mean N-effect (%)
Total microbial biomass	Phospholipid	0.02	0.43	0.05	-24
	Neutral lipid	0.02	0.33	0.06	-15
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	-25
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

TOT_F:B, total fungal to bacteria biomass; AMF:B, arbuscular mycorrhizal fungal to bacteria biomass; SapF:B, Saprotrophic fungal to bacterial biomass

Mean N-effect: negative numbers mean a decrease with N-amendment and positive number an increase

Table 3 Comparison of different studies on nitrogen addition effects on AMF intra- and/or extraradical biomass.

Study	Ecosystem	Application rate (kg N ha^{-1} yr^{-1})	Length of study (yrs)	Cumulative dose (kg N ha^{-1})	N-amendment effect on AMF biomass	
					Intraradical	Extraradical
Eom et al., 1999	Tallgrass prairie	100	9	900	↑	↑
Johnson et al., 2003	Agricultural	120	9	1080	Not measured	↓
	Agricultural	170	10	1700	Not measured	↓
	Desert	100	3	300	Not measured	↓
Treseder et al., 2007	Boreal forest	100 (200 in 1^{st} yr)	2	300	↑	↔
Garcia et al., 2008	Temperate forest	100	1	100	↑	↔
Phillips and Fahey, 2007	Temperate forest	167	2	334	↓	Not measured
DeForest et al., 2004	Temperate forest	30	7	210	Not measured	↔
Van Diepen et al., 2007	Temperate forest	30	11	330	↓	Not measured
This study	Temperate forest	30	12	360	↓	↓

↓, significant decrease; ↔, no significant change; ↑, significant increase

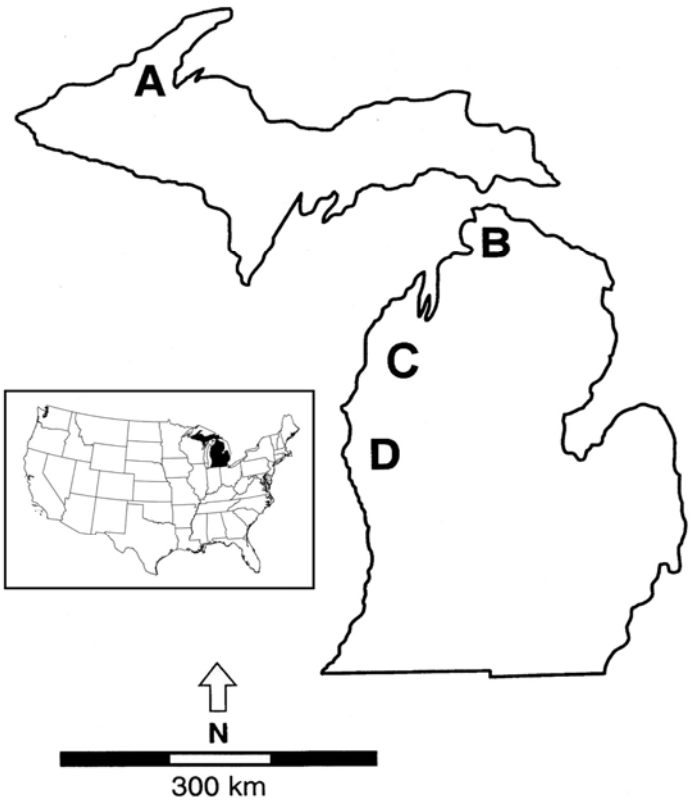

Fig. 1 Locations of the study sites (A-D) in Michigan. The inset shows the location of Michigan (filled area) within the USA.

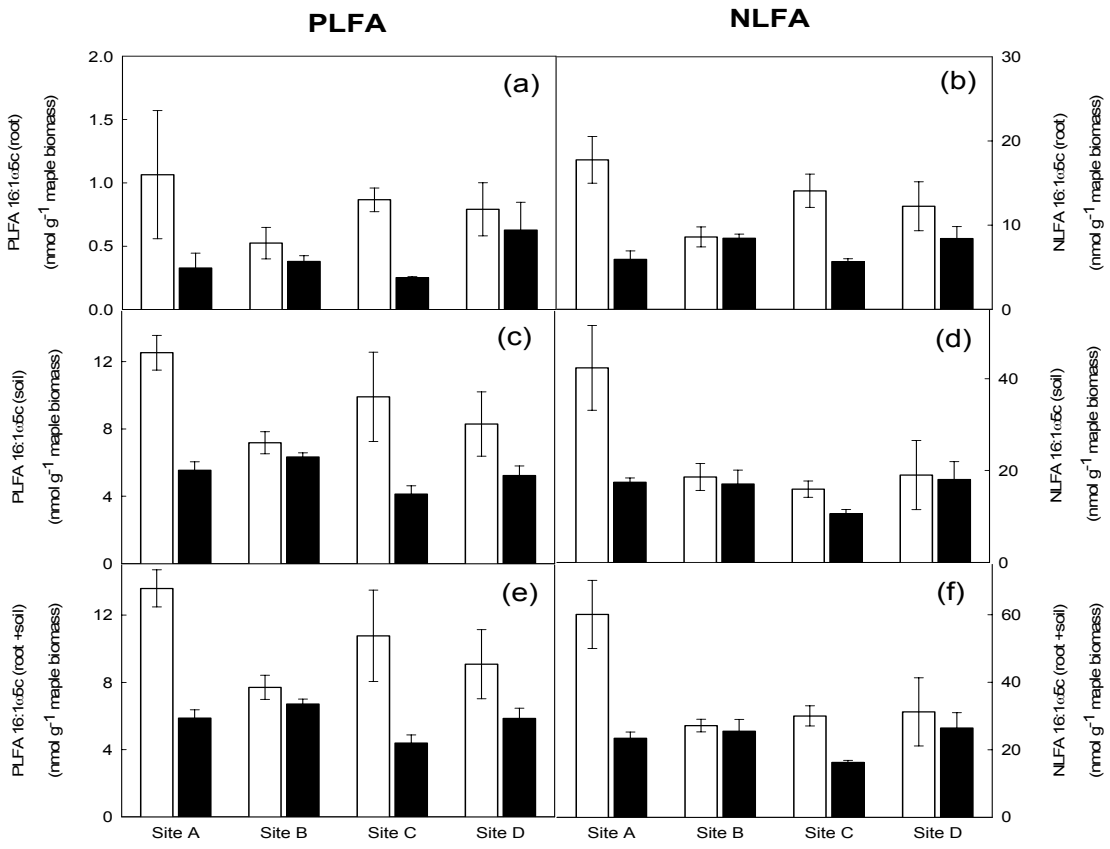

Fig. 2 Mean phospholipid fatty acid (PLFA) and neutral lipid fatty acid (NLFA) 16:1ω5c proportional allocation to maple (*Acer spp.*) fine roots (a,b), soil (c,d), and roots plus soil (e,f) for the four study sites by treatment (open bars, control; closed bars, N-amended). Error bars indicate 1 SE of the mean. Overall N treatment effect was significant in all cases (see Table 1 for details).

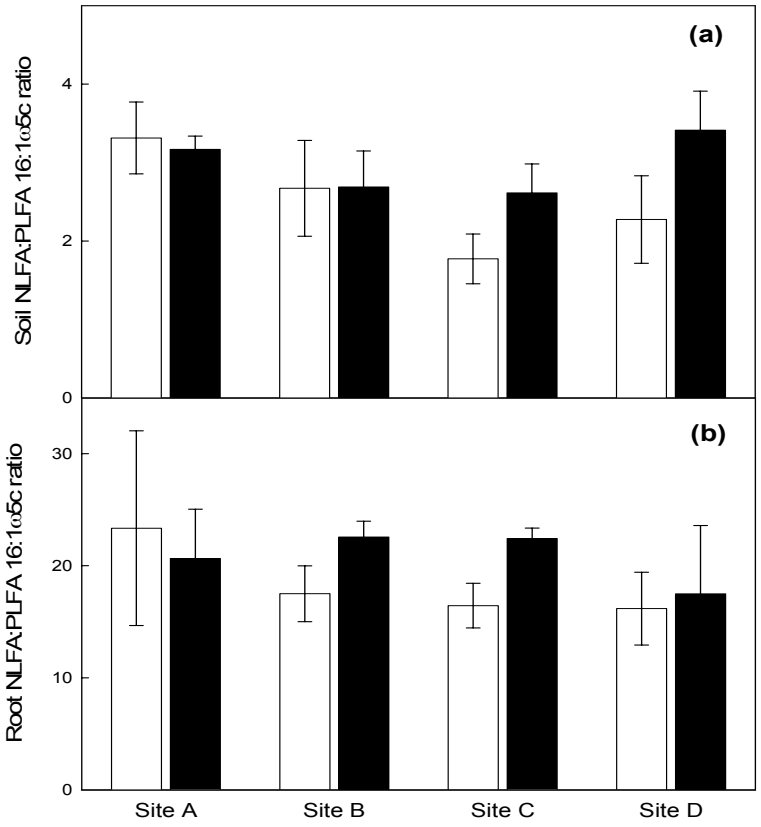

Fig. 3 Mean NLFA to PLFA 16:1ω5c ratios within a) soil and b) maple (*Acer spp.*) fine roots for the four study sites by treatment (open bars, control; closed bars, N-amended). Error bars indicate 1 SE of the mean.

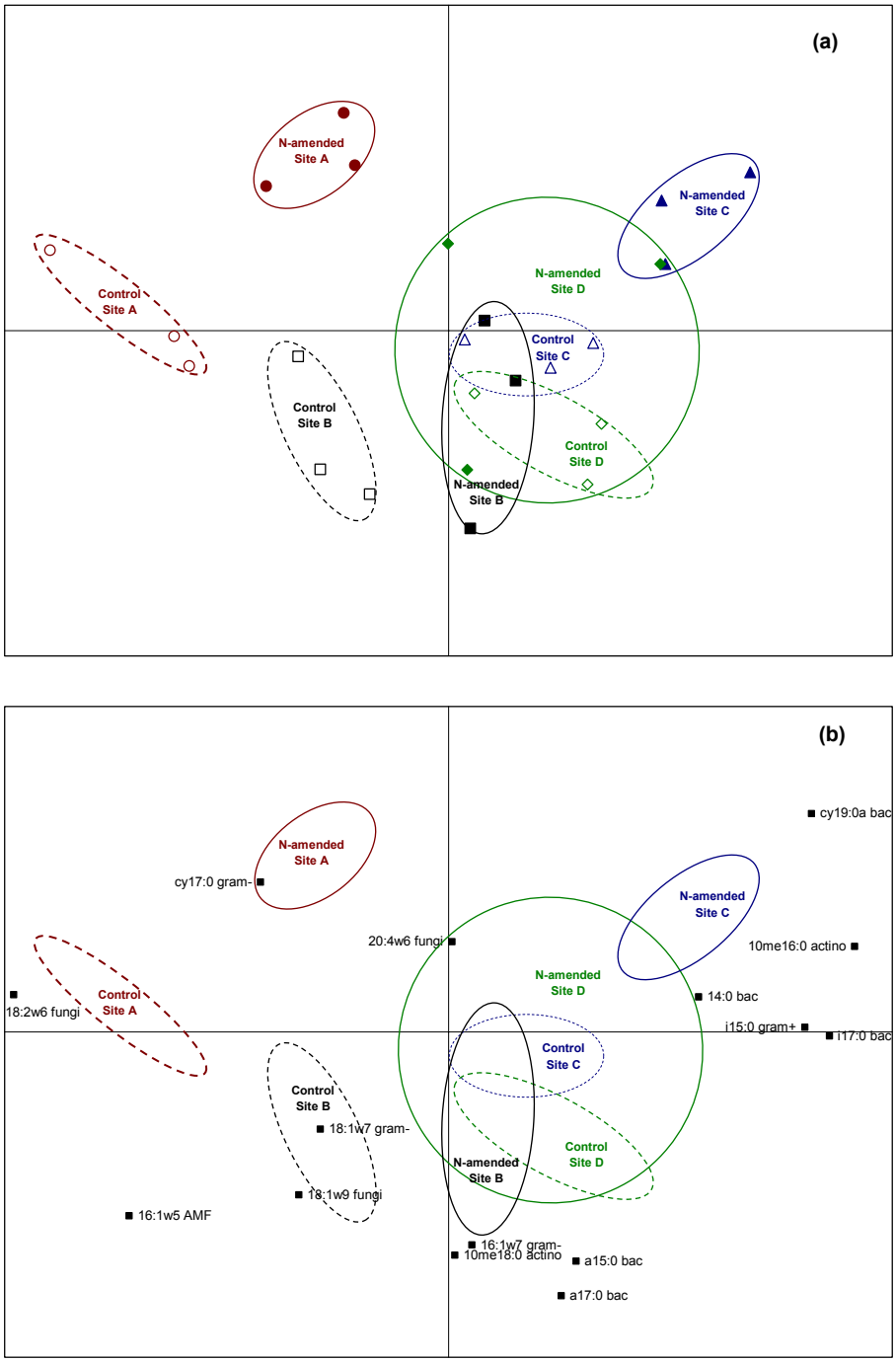

Fig. 4 Canonical Analysis of Principal Coordinates (CAP) biplots based on Bray-Curtis distance measure of (a) microbial community composition of plots at treatment by site level based on phospholipid fatty acids (PLFAs), and b) PLFAs with description of microbial group together with plots. First two axes explain 74% of total variation among plots.

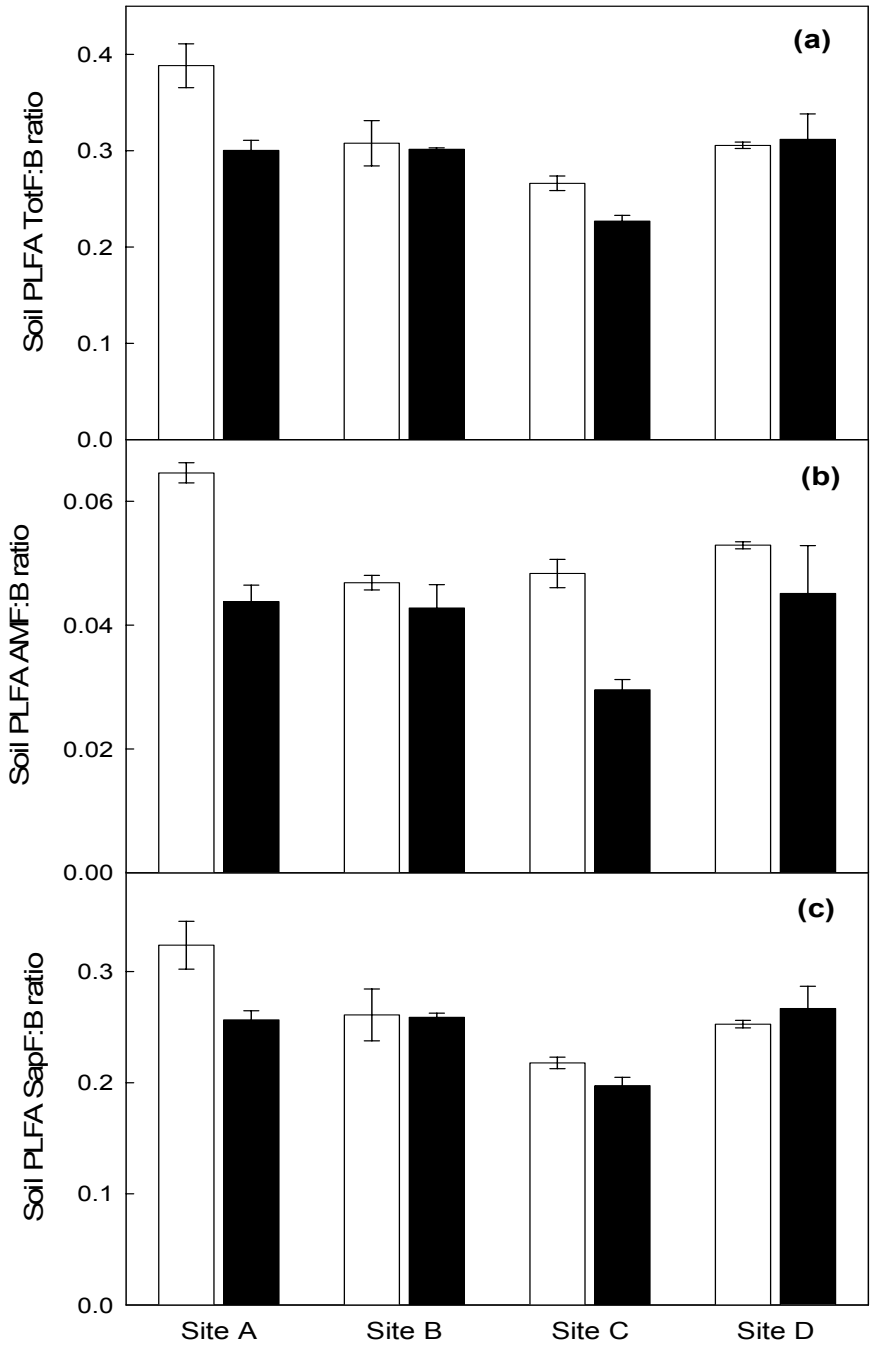

Fig. 5 Mean a) total fungal to bacterial biomass ratio, b) arbuscular mycorrhizal fungal (AMF) to bacterial biomass ratio, and c) saprotrophic to bacterial biomass ratio within soil for the four study sites by treatment (open bars, control; closed bars, N-amended). Error bars indicate 1 SE of the mean.

Chapter 4 Effects of chronic nitrogen deposition on respiration of extraradical mycelium of arbuscular mycorrhizal fungi in northern hardwood forests.

Summary

Soil respiration is a major pathway of carbon efflux, but the contributions of the different components to soil respiration are still not completely clear. Using mycorrhizal hyphal in-growth bags we attempted to estimate the contribution of arbuscular mycorrhizal (AM) mycelium to soil respiration in northern hardwood forests. We also estimated the AM fungal (AMF) mycelium production using the in-growth bags and studied how AMF mycelium production and respiration were affected by chronic N addition. Annual net AMF mycelium production was significantly negatively affected by N addition, but only a trend in decreased AMF hyphal CO_2 flux was found. AMF hyphal respiration was found to be significantly positively related to AMF hyphal biomass within the in-growth bags. It is suggested that the decrease in soil respiration with N-amendment observed at our study sites could partially be explained by the decline in AMF hyphal biomass with N addition.

Introduction

Soil organic carbon is the largest terrestrial carbon storage pool, constituting almost two thirds of the total carbon within terrestrial ecosystems (Schlesinger, 1997). The major pathway of carbon efflux from the soil is through soil respiration, which comprises almost 35% of the net global total (terrestrial and aquatic) C efflux (Schlesinger & Andrews, 2000). Changes in soil respiration can thus influence the global carbon cycle to a great extent. The global carbon cycle has received considerable attention from climate modelers in an attempt to accurately model ecosystem carbon cycling and how it is affected by various disturbances. There is a need to understand the factors influencing

soil respiration and characterize their influence on soil respiration under various climatic conditions.

Increased nitrogen has usually been found to suppress soil respiration (e.g. Bowden et al., 2004; Burton et al., 2004; Lee & Jose, 2003; Söderström et al., 1983). To understand the mechanisms behind decrease in soil respiration with increased nitrogen amendment, it is important to define the different components of soil respiration and their contribution. The two contributors to soil respiration have been defined as autotrophic (plant roots, Ra) and heterotrophic respiration (soil microbes and fauna, Rh) (Subke et al., 2006). Different methods have been designed and applied to measure the contribution of each component to soil respiration separately (Hanson et al., 2000). However, none of the existing methods takes in consideration that mycorrhizal fungi are present in both roots and soil, as intra-radical and extra-radical structures, respectively. Consequently, the contribution of mycorrhizal fungi to soil respiration has been included in estimation of both Ra and Rh.

Mycorrhizal fungi make up a large part of the microbial biomass in soils, with estimates up to 30% for AMF (Olsson and Wilhelmsson, 2000). Estimates as high as 20% of NPP (net primary production) flowing through mycorrhizal fungi have been made (Treseder & Allen, 2000). Most of this photosynthetically derived carbon is allocated to the external hyphae of associated mycorrhizae (Leake et al., 2004). Changes in mycelium biomass could therefore greatly influence soil respiration. Heinemeyer et al. (2007) found that ectomycorrhizal mycelium contributed about 25 % to total soil respiration in a lodgepole pine forest. However, within that study no mycelium biomass measurements were performed, leaving open the question if changes in external hyphal respiration are simply related to changes in external hyphal biomass, or to biomass-specific respiration rates. To fully understand the contribution of mycorrhizal external mycelium to soil respiration and how this is affected by disturbances, we therefore have to measure both the biomass and the respiration of mycorrhizal fungal mycelium.

Estimates of fungal mycelium biomass within soil have been made using microscopic measurements of hyphal length, chemical biomarkers chitin and ergosterol, and more recently using phospholipid fatty acids (PLFAs) and hyphal in-growth bags. All these

methods, except for chitin and ergosterol, are able to separate AM fungi from saprotrophic fungi. PLFA 16:1ω5 has been successfully used as an indicator of AMF abundance within both soil and plant roots (Balser et al. 2005; Olsson & Johansen, 2000; Olsson, 1999). Hyphal in-growth bags filled with sand have been proven to be colonized by mycorrhizal fungi while minimizing colonization by saprotrophic fungi because of the lack of an organic matter source (Wallander et al., 2001). In addition, the hyphal in-growth bags give us the opportunity to study AMF mycelium biomass while it is physically separated from roots, other fungi and soil microbes.

Our objectives were to study the use of hyphal in-growth bags for estimation of AMF mycelium biomass, how increased N addition would affect this AMF mycelium biomass, and the respiration rate of AMF extraradical mycelium biomass.

Materials and methods

Study sites

Four sugar maple dominated forest sites throughout Michigan, USA (Fig. 1) were studied. Each site consisted out of three untreated and three N-amended 30 x 30 m plots. Since 1994, 30 kg N ha^{-1} yr^{-1} has been applied in six equal increments of $NaNO_3$ during the growing season. Ambient wet N deposition at the sites ranged from 3.0 kg N ha^{-1} yr^{-1} near site A to 6.8 kg N ha^{-1} yr^{-1} near site D in 2006 (NADP, 2006). All sites have similar soil development (sandy spodosols), stand age and plant composition. More detailed information about the sites can be found in Burton et al. (1991).

Hyphal in-growth bags

To measure external AMF mycelium biomass production, hyphal in-growth bags were developed after Wallander et al. (2001). The hyphal in-growth bags were made of 50 µm mesh (Sefar Filtration Inc., Depew, NY, USA), and filled with 130 g of Flint silica organic sand (>250µm) (Faulks brother construction Inc., Waupaca, WI, USA). Subsamples of the sand were checked for organic matter content by combustion in a muffle furnace for 6 hours at 600˚C, and confirmed that the sand did not contain

measurable organic matter. The bags had a cylindrical shape, with an average length of 9.11 (± 0.18) cm, diameter of 3.27 (± 0.27) cm, and average volume of 75 cm^3.

The in-growth bags were buried horizontally in the soil at the interface of the organic (O-horizon) and mineral (E horizon) layer at the beginning of the growing season (May 2008) and harvested in September 2008. Five in-growth bags per plot were installed in May, at each corner and the center of a 10 x 10 m square in the center of the 30 x 30 m plot. In September, two days before the harvest, another five in-growth bags were installed within 30cm of the May-installed bags, to serve as blank bags to correct for ambient CO_2 flux of bags removed from the soil.

Previous research by Wallander et al. (2001) has shown that the mycelium of hyphal in-growth bags in a coniferous forest originated from EM fungi and not saprotrophic fungi, using both ^{13}C isotopic measurements and control treatments (trenched systems) to correct for saprotrophic mycelia. Although phospho-lipid fatty acid (PLFA) 16:1w5c has been proven to be a good indicator for the presence and biomass of AM fungi in roots and soil (Olsson, 1999) and within our study sites (van Diepen et al., 2007), we performed an additional experiment to check for potential saprotrophic mycelium growth within our hyphal in-growth bags.

Using an experimental design after Wallander et al. (2001) 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. As an extra measure against plant roots the opening at the bottom of the PVC tube was covered with 50μm mesh. To accomplish this, the PVC tube was briefly removed from the soil after insertion, while keeping the soil inside, followed by attachment of the mesh to the tube with a zip-tie and duct-tape, and reinsertion of the PVC tube into the soil, now with the mesh covering the bottom. By severing the plant roots, a non-mycorrhizal environment inside the tube was created. In November 2004 four paired bags were placed per plot, one hyphal in-growth bag in a 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 analyses (van Diepen et al. 2007) were performed on a subset of mycelia extracted from the in-growth bags outside of the tubes.

Respiration measurements

All respiration measurements were performed with an LI-8100 soil respiration system, using a 10cm survey chamber (LI-COR, Lincoln, NE, USA). A special measurement chamber was designed that would fit the 10 cm survey chamber for placement of the in-growth bag during the measurements. The measurement chamber consisted of a PVC cap (10 cm in diameter, height of 4.6 cm), with a rack constructed of metal wire in the center to support the bag. After removal of the in-growth bags from the soil, bags were cleaned with a brush to remove all soil and roots attached, and immediately placed on the rack in the respiration chamber. After that, the LICOR survey chamber was placed on the respiration chamber and the measurement was started. Measurements were performed similarly to soil respiration measurements, with a total measurement time of 2 min, and a dead band of 30 sec. After the measurement, bags were placed in a zip-lock bag, stored on ice for transport, and frozen at -20 ˚C within 12 hours after harvest. To estimate moisture content, bags were weighed before placement in the soil and directly after harvest. Furthermore, temperature measurements were performed at the time of the harvest at the depth of bag placement.

Hyphal extraction

The frozen in-growth bags were defrosted at room temperature on a clean surface, and the content was emptied into a large beaker. The inside of the mesh bag was further cleaned with a small paint brush to ensure all hyphae were removed from the bag. About 600 ml of water was added to the beaker, stirred, and decanted through a sieve with 50µm mesh. This process was repeated 6 times, after which the hyphae in the sieve were washed into a Petri-dish. The hyphae were further cleaned from any sand particles by multiple washings with DI water and handpicking of sand particles using tweezers by observation through a microscope. The cleaned hyphae were frozen and freeze-dried to

obtain the dry biomass weight. Hyphal biomass was further calculated on a volume basis using the mean volume of 75 cm^3 of the in-growth bags.

Calculation of hyphal respiration

CO_2 flux of the AMF hyphae in the bags were calculated on a per bag basis (μmol CO_2 bag^{-1} s^{-1}) from the CO_2 flux measurements of the LICOR.

Soil has a higher CO_2 concentration compared to the air in which the measurement takes place, leading to artificially high CO_2 fluxes from the bags after removal from the soil. To correct for this, from each sample bag we subtracted a CO_2 flux value estimated from the blank bags. The blank bag flux had to be corrected for the influence of bag soil moisture content on CO_2 flux rate from the bags ($y = 0.8824x + 0.43$, $R^2 = 0.67$, $p < 0.0001$, Fig. 2) where y is the flux rate and x is the bag moisture content. The correction factor for each in-growth bag was composed of the average CO_2 flux of the five blank bags within the same plot. Using the equation for soil moisture effect on CO_2 flux, the CO_2 flux of the blank bags was adjusted to the moisture concentration of the sample in-growth bag. Soil temperature had no measurable influence on the CO_2 flux of the blank bags over the narrow temperature range of the present study ($p = 0.63$), and therefore no corrections were made for this factor. The corrected mean CO_2 flux of the blank bags per plot was subtracted from the measured CO_2 flux of the sample bag to calculate the actual CO_2 flux of a sample bag in nmol CO_2 bag^{-1} s^{-1}.

From this value we could calculate total microbial respiration in the bag. However, it is possible that non-fungal (e.g. bacterial, protist, etc.) respiration is contributing to bag respiration. If all of the respiration is not derived from fungal hyphae, then we would expect to find a positive intercept when fitting the regression of respiration to hyphal biomass. Therefore, to check for and correct for other sources of respiration, we tested for positive intercepts in the hyphal biomass relationship with respiration rate. We found that these linear relationships had significantly positive intercepts ($p < 0.0001$) (Fig. 3). Hence, part of the total CO_2 flux measured from a sample in-growth bag appears to be unrelated to hyphal presence, perhaps as a function of bacterial respiration of dissolved organic carbon. Therefore we made the assumption that the CO_2 flux estimated at zero

mg of AMF hyphal biomass (= intercept of linear regression, Fig. 3) is non-fungal respiration and therefore subtracted this value from the CO_2 flux of the sample bag to derive hyphal respiration.

To calculate the AMF hyphal biomass-specific respiration rate, the blank and intercept-corrected CO_2 flux of a sample bag was simply divided by the dry weight of AMF mycelium extracted from that bag.

Statistical analysis

Differences in dependent variables (hyphal biomass, CO_2 efflux) between treatments were determined using a two-way ANOVA with N treatment (n= 2) and site (n = 4) as factors. Transformations (natural logarithm and sine) were applied as appropriate to ensure a normal distribution and equal variances.

Results

Verification of AMF hyphal growth

The 2005 pilot in-growth bags study showed that hyphal biomass from bags inside the PVC tube (assumed of saprotrophic origin) varied from 5-29% of the biomass extracted from bags outside the tube (assumed of mycorrhizal origin). Phospholipid fatty acid (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 soil was AMF (PLFA 16:1ω5c). We can therefore assume that our mesh bags were strongly dominated by AMF rather than saprotrophic fungi. The average PLFA 16:1ω5c concentration of the AMF hyphae was 1.3 (± 0.3) nmol mg^{-1} dry hyphae.

AMF hyphal biomass

Mean hyphal biomass in the in-growth bags significantly ($p = 0.019$) decreased by 41% with chronic N-addition (Fig. 4). No site effect ($p = 0.612$) or site by treatment interaction ($p = 0.370$) was found.

CO_2 flux

CO_2 flux from the hyphal in-growth bags was significantly positively correlated to the hyphal biomass for both treatments ($p < 0.0001$, $R^2 = 0.36$ (control) and $R^2 = 0.41$ (N-amended), Fig. 3). The linear regression lines of the N-amended plots had a different intercept and slope and a slightly stronger relationship compared to the control plots.

We found no significant difference with N-amendment in total hyphal in-growth bag CO_2 flux after correction for both blank in-growth bags & non-hyphal CO_2 flux ($p = 0.14$ and $p = 0.82$, respectively) but a trend toward decline with an overall mean decrease of 18 and 7%, respectively (Fig. 5a,b). There were also no significant site effects or site by treatment interactions. After the first correction factor for blank in-growth bags CO_2 flux site A, B, and C showed a trend towards a decrease in CO_2 flux (Fig. 5a). And after correction for both blank in-growth bags & non-hyphal CO_2 flux only site B and C showed a trend towards a decrease in CO_2 flux with N-addition (Fig. 5b).

Calculating AMF hyphal biomass-specific respiration rate resulted in a biomass-specific CO_2 flux of 0.32 and 0.51 nmol CO_2 mg hyphae^{-1} s^{-1} for control and N-amended plots, respectively (Fig. 3) and a mean of 0.40 nmol CO_2 mg hyphae^{-1} s^{-1}.

Discussion

The mean 41% reduction in AMF hyphal biomass with N-amendment found in this study using hyphal in-growth bags was very consistent with the mean 41% reduction in extraradical AMF biomass found using soil phospholipid fatty acid (PLFA) 16:1ω5c measurements for the same study sites in a different year (chapter 3).

The decline of AMF hyphal biomass with N-amendment was significantly related to a decline in hyphal in-growth bag CO_2 flux, which suggests that the decline in AMF hyphal CO_2 flux is mainly controlled by the biomass of AMF mycelium present in the system.

We corrected for artificially high CO_2 fluxes from the bags after removal from the soil by subtracting the CO_2 flux of blank in-growth bags. However, the linear relationship between hyphal biomass per sample bag and CO_2 flux per sample bag had an intercept that was significantly different from zero, which suggests that some of the CO_2 flux was

not related to AMF hyphal biomass. More factors thus seemed to be playing a role when measuring CO_2 flux from hyphal in-growth bags that we were not been able to correct for using the blank bags. A possible explanation of an existing CO_2 flux with very low amounts of AMF mycelium is the presence of other unmeasured microorganisms, respiring exudates from live hyphae, dead hyphae, or dissolved organic carbon from outside the bags.

Our AMF hyphal biomass-specific CO_2 flux was quite noisy when the hyphal biomass was lower than 1 mg. The minimum and maximum amounts of hyphal biomass per bag measured were 0.1 mg and 11.9 mg, with 40% of the bags containing 1 mg or less. This suggests that higher hyphal biomass per in-growth bag is needed to be able to make reasonable measurements of biomass-specific CO_2 flux.

A large range has been found in estimates of mycorrhizal biomass specific respiration rates, and our average estimated value of 0.4 nmol CO_2 mg hyphae^{-1} s^{-1} seemed to be near the middle of this range (Table 1). One obvious explanation for the large range in values is the different measurement techniques applied. In addition, the large range that was found from this quick reference review shows that there is a need to organize and verify these findings on fungal biomass specific respiration rate, especially if we want to be able to use the estimated values to calculate fungal respiration on an ecosystem level.

If for example we assume that our average AMF fungal biomass specific respiration of 0.4 nmol CO_2 mg hyphae^{-1} s^{-1} is true and that the estimated hyphal biomass in our in-growth bags is representative of AMF hyphal biomass found in a same volume of the soil, we can calculate the approximate contribution of AMF hyphae to soil respiration by multiplying the hyphal biomass in a bag by the average biomass specific respiration rate to get the respiration rate per bag. We then scaled this value (AMF hyphal respiration per bag (75 cm^3) per second) up to a square meter soil to 10 cm depth to calculate AMF hyphal respiration in μmol CO_2 m^{-2} s^{-1}. We assumed here that most of the hyphal biomass is concentrated in the top 10 cm of the soil, which has been found for fine roots at our study sites. The resulting values ranged from 0.23 to 1.66 μmol CO_2 m^{-2} s^{-1}, and had an average of 0.78 μmol CO_2 m^{-2} s^{-1}. Using soil respiration values taken on the same day (varying from 1.9 to 4.3 μmol CO_2 m^{-2} s^{-1}) for these study sites this would results in an 8

to 63 % (mean of 29%) contribution of AMF mycelium to total soil respiration. This mean value is at least biologically possible although in need of independent verification.

We also performed a similar calculation using the soil PLFA 16:1ω5c values measured in 2006 (Chapter 3) as a representative of AMF extraradical hyphal biomass. Values of PLFA 16:1ω5c are reported in nmol 16:1ω5c per gram of soil. By multiplying this value by bulk density of the soil, and scaling this value up to a square meter soil to 10 cm depth we calculated the AMF hyphal biomass in nmol 16:1ω5c m^{-2} (and 10 cm deep). We then used our estimated value of 1.3 nmol 16:1ω5c mg^{-1} dry hyphae to calculate mg of hyphae m^{-2}. This value was then multiplied by the hyphal biomass specific respiration rate (0.4 nmol CO_2 mg $hyphae^{-1}$ s^{-1}) to get hyphal CO_2 flux values per m^{-2} s^{-1}. This resulted in AMF hyphal CO_2 fluxes varying from 42 to 210 μmol CO_2 m^{-2} s^{-1}, and a mean of 86 μmol CO_2 m^{-2} s^{-1}, which would represent from 1680 % to 7330 % of total soil respiration.

The former is at least a plausible estimate of AMF hyphal respiration rates, whereas the latter indicates a problem with the calculations. Either the PLFA method did not estimate total AMF hyphal biomass correctly or our values of AMF hyphal biomass specific respiration rates are unrealistically high. However, our respiration results are in line with those found for ectomycorrhizal basidiomycete sporocarps (Carrie Andrew, personal communication) which do not suffer from any of the potential methodological artifacts found in the present study. The estimates of AMF biomass using PLFA could be inflated because this PLFA biomarker can also be produced by bacteria. Further work will be needed to determine the AMF contribution to soil respiration.

In conclusion, hyphal in-growth bags used in this study gave a good estimate of the relative differences in AMF hyphal net annual hyphal biomass production between control and N-amended plots. Hyphal in-growth bag CO_2 flux was overall decreased by N-amendment by 18% and 7% depending on the correction factor used, but this decrease was not significant. Hyphal in-growth bag CO_2 flux was also positively related to hyphal biomass, suggesting that our measurement technique is sensitive enough to measure fungal hyphal respiration rates. The observed trend of a decreased hyphal in-growth bag CO_2 flux with N-amendment at some of the sites could possibly explain some of the

decrease found in soil respiration with N-amendment. AMF hyphal biomass-specific respiration rate (nmol CO_2 mg $hyphae^{-1}$ s^{-1}) was found to be in the middle of the range of hyphal biomass-specific respiration rates found in other studies. Depending on the method used, when we tried to estimate the contribution of AMF hyphal respiration to total soil respiration by scaling to a soil volume level, both realistic and unrealistic CO_2 fluxes were observed. More studies are therefore needed to estimate both AMF hyphal biomass-specific respiration rate and AMF mycelial biomass in the soil, and to understand the true contribution of AMF mycelium to soil respiration.

Acknowledgments

We thank the NSF for their continued financial support of this project, and the Ecosystem Science Center for their research grant which supported part of this project.

References

Balser, T.C., Treseder, K.K., Ekenler, M., 2005. Using lipid analysis and hyphal length to quantify AM and saprotrophic fungal abundance along a soil chronosequence. Soil Biology and Biochemistry 37: 601-604.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37: 911-917.

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. Forest Ecology and Management 196: 43-56.

Burton, A.J., Pregitzer, K.S., Crawford, J.N., Zogg, G.P., Zak, D.R., 2004. Simulated chronic NO_3^- addition reduces soil respiration in northern hardwood forests. Global Change Biology 10:1080-1091.

Burton, A.J., Pregitzer, K.S., Reed, D.D., 1991. Leaf area and foliar biomass relationships in northern hardwood forests located along an 800 km acid deposition gradient. Forest Science 37: 1041-1059.

Eltrop, L., Marchner, H., 1996. Growth and mineral nutrition of non-mycorrhizal and mycorrhizal Norway spruce (*Picea abies*) seedlings grown in semi-hydroponic sand culture. New Phytologist 133: 479-486.

Ettema, C.H., Lowrance, R., Coleman, D.C., 1999. Riparian soil response to surface nitrogen input: temporal changes in denitrification, labile and microbial C and N pools, and bacterial and fungal respiration. Soil Biology and Biochemistry 31: 1609–1624.

Frostegård, A., Tunlid, A., Bååth, E., 1991. Microbial biomass measured as total lipid phosphate in soils of different organic content. Journal of Microbiological Methods 14: 151-163.

Hanson, P.J., Edwards, N.T., Garten, C.T., Andrews, J.A., 2000. Separating root and soil microbial contributions to soil respiration: A review of methods and observations. Biogeochemistry 48: 115-146.

Heinemeyer, A., Hartley, I.P., Evans, S.P., Carreira De La Fuente, J.A., Ineson, P., 2007. Forest soil CO_2 flux: uncovering the contribution and environmental responses of ectomycorrhizas. Global Change Biology 13: 1786-1797.

Heinemeyer, A., Ineson, P., Ostle, N., Fitter, A.H., 2006. Respiration of the external mycelium in the arbuscular mycorrhizal symbiosis shows strong dependence on recent photosynthates and acclimation to temperature. New Phytologist 171: 159–170.

Leake, J., Johnson, D., Donnelly, D., Muckle, G. Boddy, Read, D., 2004. Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. Canadian Journal of Botany 82: 1016-1045.

Lee, K.H., Jose, S., 2003. Soil respiration, fine root production, and microbial biomass in cottonwood and loblolly pine plantations along a nitrogen fertilization gradient. Forest Ecology and Management 185: 263-273.

Malcolm, G.M., Lopez-Gutierrezz, J.C., Koide, R.T., Eissenstat, D.M., 2008. Acclimation to temperature and temperature sensitivity of metabolism by ectomycorrhizal fungi. Global Change Biology 14: 1169–1180.

NADP., 2006. National Atmospheric Deposition Program (NRSP-3). NADP Program Office, Illinois State Water Survey, IL, USA.

Olsson, P.A., 1999. Signature fatty acids provide tools for determination of the distribution and interactions of mycorrhizal fungi in soil. FEMS Microbiology Ecology 29:303-310.

Olsson, P.A., Bååth, E., Jakobsen, I., 1997. Phosphorus effects on the mycelium and storage structures of an arbuscular mycorrhizal fungus as studied in the soil and roots by analysis of fatty acid signatures. Applied and Environmental Microbiology 63: 3531-3538.

Olsson, P.A., Johansen, A., 2000. Lipid and fatty acid composition of hyphae and spores of arbuscular mycorrhizal fungi at different growth stages. Mycological Research 104: 429-434.

Olsson, P.A., Wilhelmsson, P., 2000. The growth of external AM fungal mycelium in sand dunes and in experimental systems. Plant and Soil 226: 161–169.

Rygiewicz, P.T., Andersen, C.P., 1994. Mycorrhizae alter quality and quantity of carbon allocated belowground. Nature 369: 58–60.

Schlesinger, W.H., Andrews, J.A., 2000. Soil respiration and global carbon cycle. Biogeochemistry 48: 7-20.

Schlesinger, W.H., 1997. Biogeochemistry: An analysis of global change, 2nd edn. Academic Press, San diego.

Söderström, B., Bååth, E., Lundgren, B., 1983. Decrease in soil microbial activity and biomass owing to nitrogen amendments. Canadian Journal of Microbiology 29: 1500-1506.

Subke, J.A., Inglima, I., Cotrufo, M.F., 2006. Trends and methodological impacts in soil CO2 efflux partitioning: A metaanalytical review. Global Change Biology 12: 921-943.

Treseder, K.K., Allen, M.F., 2000. Mycorrhizal fungi have a potential role in soil carbon storage under elevated CO2 and nitrogen deposition. New Phytologist 147: 189-200.

Van Diepen, L.T.A., E.A. Lilleskov, K.S. Pregitzer, and R.M. Miller, 2007. Decline of arbuscular mycorrhizal fungi in northern hardwood forests exposed to chronic nitrogen additions. New Phytologist 176: 175-183.

Wallander, H., Nilsson, L.O., Hagerberg, D., Bååth, E., 2001. Estimation of the biomass and seasonal growth of external mycelium of ectomycorrhizal fungi in the field. New Phytologist 151:753-760.

Table 1 Comparison of mycorrhizal fungal biomass specific respiration rates found by different studies.

Study reference	Fungal structure	CO_2 flux (nmol CO_2 mg hyphae^{-1} s^{-1})
Heinemeyer et al., 2006	AMF hyphae	$2.9 * 10^{-9}$ to $1.9*10^{-7}$
Ettema et al., 1999	EMF hyphae	$3.9*10^{-5}$
Eltrop & Marschner, 1996	EMF hyphae	0.0805 to 0.223
Malcolm et al., 2008	EMF cultures EMF	$10 * 10^3$ to $15 * 10^4$
	EMF hyphae	$24 * 10^3$
Rygiewicz & Andersen, 1994	EMF sporocarps	$11 * 10^3$ to $16 * 10^3$
C. Andrew (personal communication)	EMC sporocarps	0.05 to 0.25
Our study	AMF hyphae	0.4

AMF, arbuscular mycorrhizal fungi; EMF, ectomycorrhizal fungal

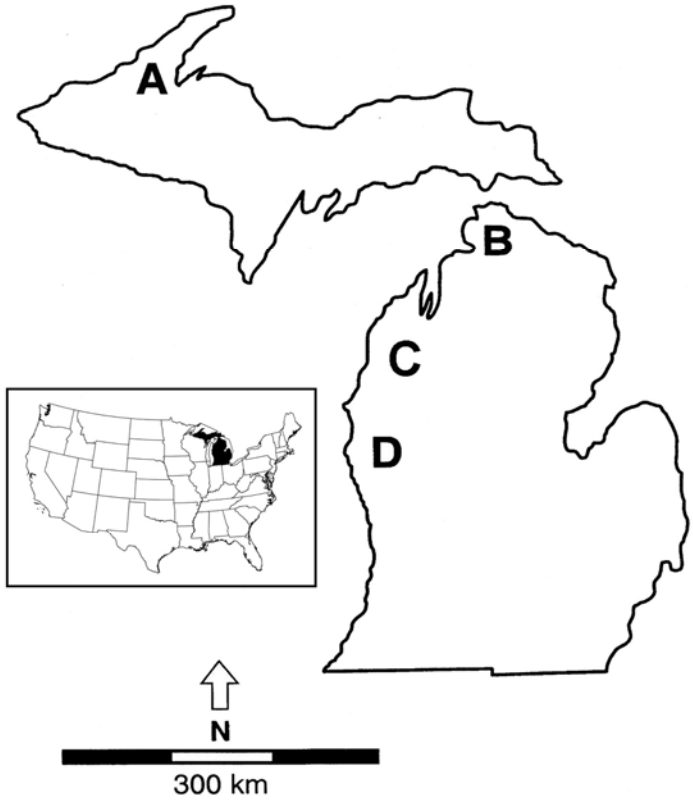

Fig. 1 Locations of the study sites (A-D) in Michigan. The inset shows the location of Michigan (filled area) within the USA.

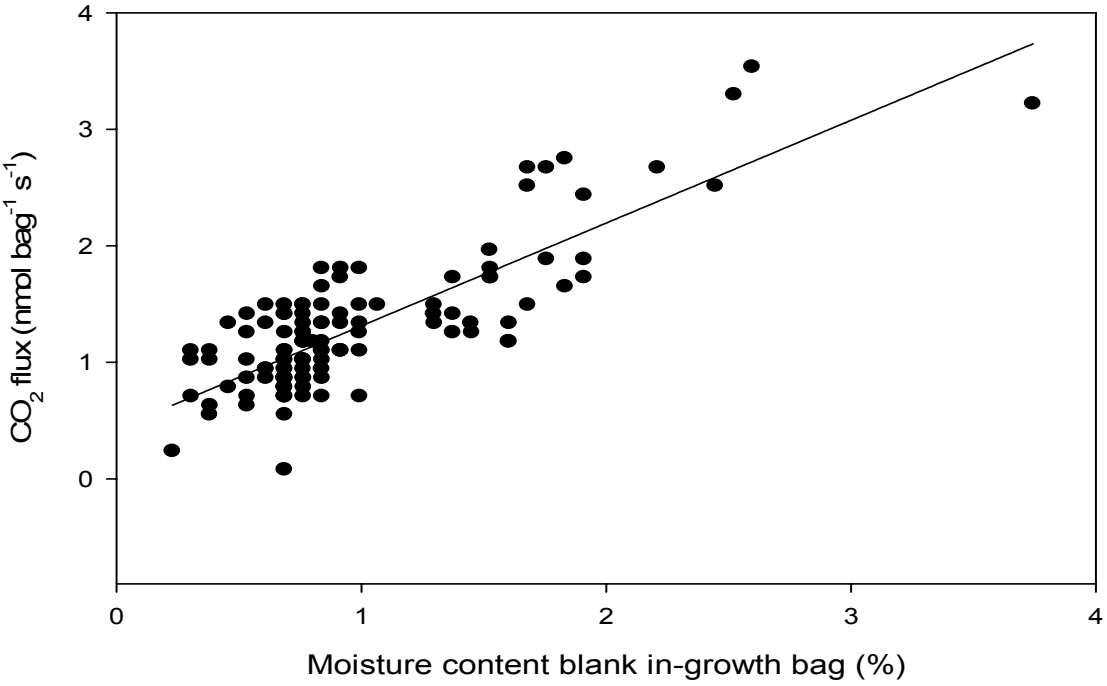

Fig. 2 Linear regression of moisture content and CO_2 efflux of all blank in-growth bags designed to correct for ambient CO_2 efflux of buried hyphal in-growth bags.

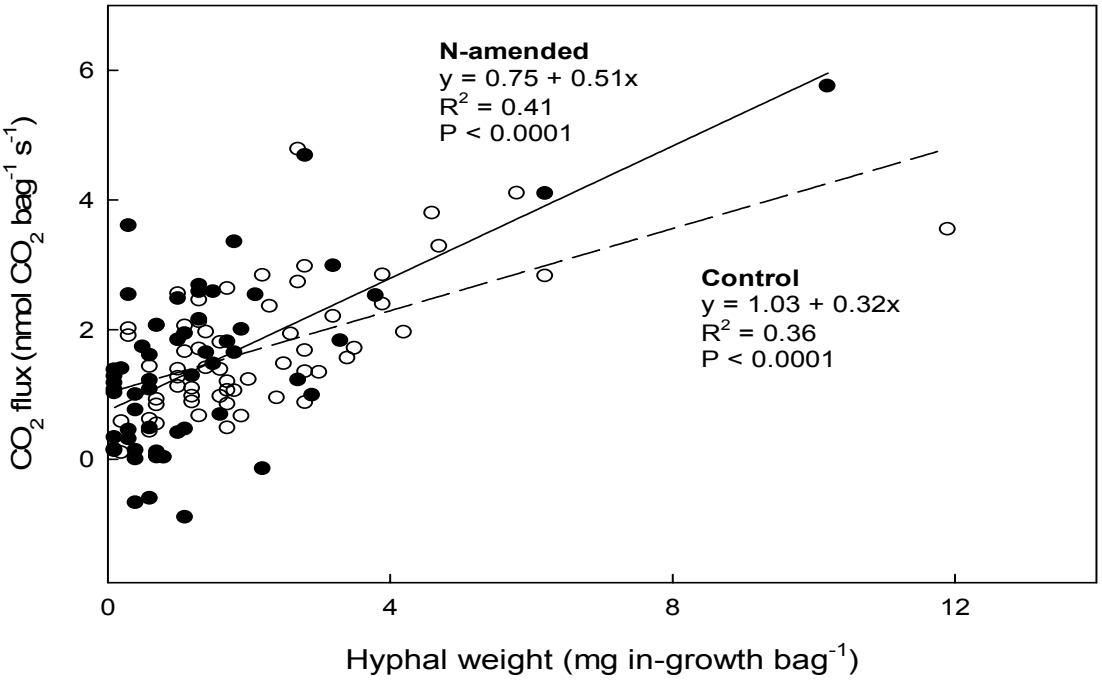

Fig. 3 Linear relationship between hyphal biomass per sample in-growth bag and CO_2 efflux per sample in-growth bag per treatment. Open circles, control plots; closed circles, N-amended plots. Dashed regression line, control plots; solid regression line, N-amended plots.

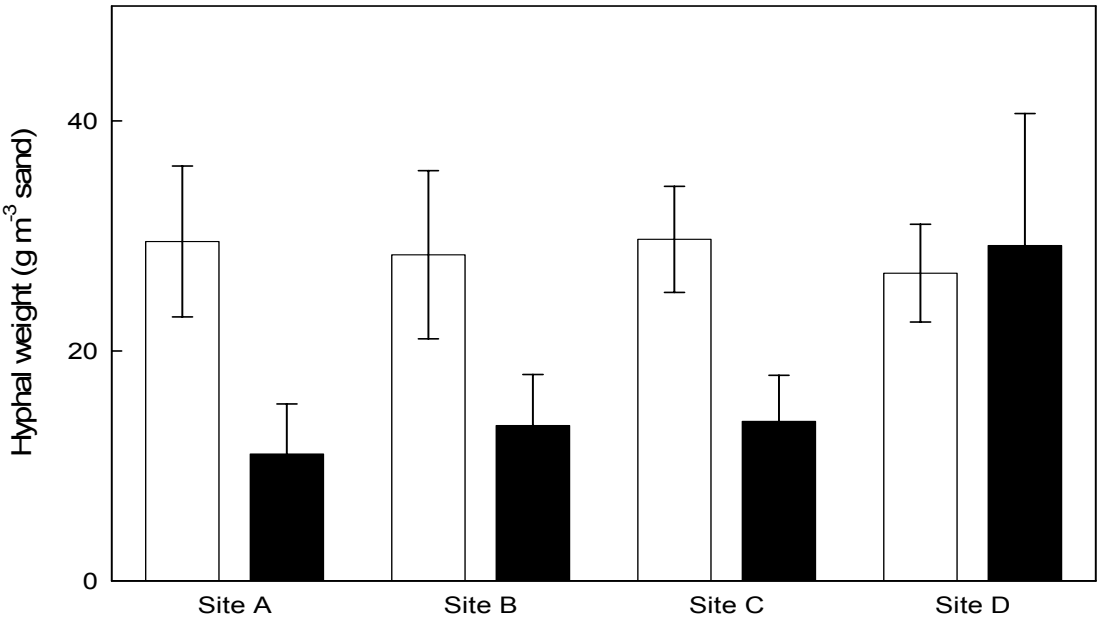

Fig. 4 Mean hyphal biomass extracted from hyphal in-growth bags for the four study sites by treatment (open bars, control; closed bars, N-amended) expressed as g hyphae per m^3 sand. Error bars indicate 1 SE of the mean.

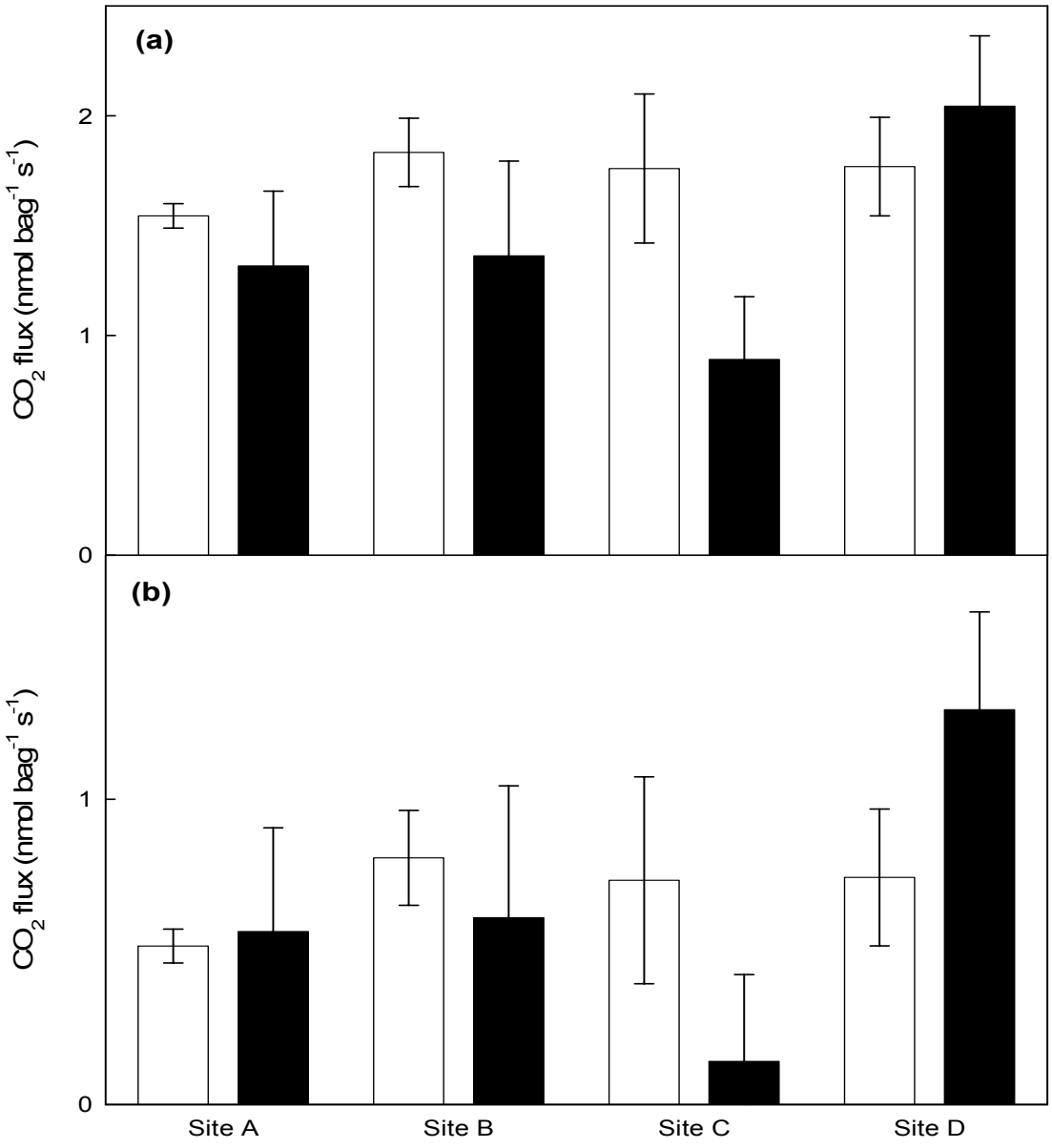

Fig. 5 Mean corrected arbuscular mycorrhizal hyphal CO_2 flux (nmol CO_2 bag^{-1} s^{-1}) after correcting for a) blank bag CO_2 flux and b) blank bag plus non-hyphal CO_2 flux for the four study sites by treatment (open bars, control; closed bars, N-amended). Error bars indicate 1 SE of the mean.

APPENDIX 1

Blackwell Publishing

***New Phytologist*: Exclusive Licence Form**

Author's name: Linda T. A. van Diepen

Author's address: Michigan Tech University, School of Forest Resources & Environmental Science, 1400 Townsend Drive, Houghton, MI 49931

Title of article ("Article"): Decline of arbuscular mycorrhizal fungi in northern hardwood forests exposed to chronic nitrogen additions

Manuscript no. (if known): NPH-MS-2007-0531.L

Names of all authors in the order in which they appear in the Article: Linda T. A. van Diepen, Eric A. Lilleskov, Kurt S. Pregitzer, R. Michael Miller

In order for your Article to be distributed as widely as possible in *New Phytologist* (the Journal) you grant Blackwell Publishing Ltd (Blackwell Publishing) an exclusive licence to publish the above Article on behalf of the New Phytologist Trust, including the abstract in printed and electronic form, in all languages, and to administer subsidiary rights agreements with third parties for the full period of copyright and all renewals, extensions, revisions and revivals. The Article is deemed to include all material submitted for publication with the exception of Letters, and includes the text, figures, tables, author contact details and all supplementary material accompanying the Article.

Please read this form carefully, sign at the bottom (if your employer owns copyright in your work, arrange for your employer to sign where marked), and return the ORIGINAL to the address below as quickly as possible. As author, *you remain the copyright owner of the Article*, unless copyright is owned by your employer. (US Federal Government authors please note: your Article is in the public domain.)

Your Article will not be published unless an Exclusive Licence Form has been signed and received by Blackwell Publishing.

Please note: You retain the following rights to re-use the Article, as long as you do not sell or reproduce the Article or any part of it for commercial purposes (i.e. for monetary gain on your own account or on that of a third party, or for indirect financial gain by a commercial entity). These rights apply without needing to seek permission from Blackwell Publishing.

- **Prior to acceptance:** We ask that as part of the publishing process you acknowledge that the Article has been submitted to the Journal. You will not prejudice acceptance if you use the unpublished Article, in form and content as submitted for publication in the Journal, in the following ways:
  - sharing print or electronic copies of the Article with colleagues;
  - posting an electronic version of the Article on your own personal website, on your employer's website/repository and on free public servers in your subject area.
- **After acceptance:** Provided that you give appropriate acknowledgement to the Journal, the New Phytologist Trust and Blackwell Publishing, and full bibliographic reference for the Article when it is published, you may use the accepted version of the Article as originally submitted for publication in the Journal, and updated to include any amendments made after peer review, in the following ways:
  - you may share print or electronic copies of the Article with colleagues;
  - you may use all or part of the Article and abstract, without revision or modification, in personal compilations or other publications of your own work;
  - you may use the Article within your employer's institution or company for educational or research purposes, including use in course packs;
  - you may post an electronic version of the published Article on your own personal website, on your employer's website/repository and on free public servers in your subject area. Electronic versions of the published Article must include a link to Blackwell *Synergy* together with the following text: 'The definitive version is available at www.blackwell-synergy.com'.

All requests by third parties to re-use the Article in whole or in part will be handled by the New Phytologist Trust. Any permission fees will be retained by the Journal. All requests to adapt substantial parts of the Article in another publication (including publication by Blackwell Publishing) will be subject to your approval (which is deemed to be given if we have not heard from you within 4 weeks of your approval being sought by us writing to you at your last notified address). Please address any queries to journalsrights@oxon.blackwellpublishing.com.

In signing this Agreement:

1. You hereby warrant that this Article is an original work, has not been published before and is not being considered for publication elsewhere in its final form either in printed or electronic form;
2. You hereby warrant that you have obtained permission from the copyright holder to reproduce in the Article (in all media including print and electronic form) material not owned by you, and that you have acknowledged the source;
3. You hereby warrant that this Article contains no violation of any existing copyright or other third party right or any material of an obscene, indecent, libellous or otherwise unlawful nature and that to the best of your knowledge this Article does not infringe the rights of others;
4. You hereby warrant that in the case of a multi-authored Article you have obtained, in writing, authorization to enter into this Agreement on their behalf and that all co-authors have read and agreed the terms of this Agreement;
5. You warrant that any formula or dosage given is accurate and will not if properly followed injure any person;

6. You will indemnify and keep indemnified the Editors, the New Phytologist Trust and Blackwell Publishing against all claims and expenses (including legal costs and expenses) arising from any breach of this warranty and the other warranties on your behalf in this Agreement.

By signing this Agreement you agree that Blackwell Publishing may arrange for the Article to be:

- Published in the above Journal, and sold or distributed, on its own, or with other related material;
- Published in multi-contributor book form or other edited compilations by Blackwell Publishing;
- Reproduced and/or distributed (including the abstract) throughout the world in printed, electronic or any other medium whether now known or hereafter devised, in all languages, and to authorize third parties (including Reproduction Rights Organizations) to do the same;
- You agree to Blackwell Publishing using any images from the Article on the cover of the Journal, and in any marketing material.

You authorize Blackwell Publishing to act on your behalf to defend the copyright in the Article if anyone should infringe it, although there is no obligation on Blackwell Publishing to act in this way.

As the Author, copyright in the Article remains in your name (or your employer's name if your employer owns copyright in your work).

Blackwell Publishing undertakes that every copy of the Article published by Blackwell Publishing will include the full bibliographic reference for your Article, together with the copyright statement.

☐ **BOX A: to be completed if copyright belongs to you**

☐ **BOX B: to be completed if copyright belongs to your employer (e.g. HMSO, CSIRO)**
The copyright holder grants Blackwell Publishing an exclusive licence to publish the Article including the abstract in printed and electronic form, in all languages, and to administer subsidiary rights agreements with third parties for the full period of copyright and all renewals, extensions, revisions and revivals.

Print Name of Copyright holder: .....
This will be printed on the copyright line on each page of the Article. It is your responsibility to provide the correct information of the copyright holder.

☒ **BOX C: to be completed if the Article is in the public domain (e.g. US Federal Government employees)** You certify that the Article is in the public domain. No licence to publish is therefore necessary.

Signature (on behalf of all co-authors (if any)) **Print name:** LINDA VAN DIEPEN

..... **Date:** 4-19-2007

If your employer claims copyright in your work, this form must also be signed below by a person authorized to sign for and on behalf of your employer, as confirmation that your employer accepts the terms of this licence.

Signature (on behalf of the employer of the author (s)) **Print name:**.....

Print name of employer:.....

..... **Date:**.....

The rights conveyed in this licence will only apply upon acceptance of your Article for publication.

Data Protection: The Publisher may store your name and contact details in electronic format in order to correspond with you about the publication of your Article in the Journal. We would like to contact you from time to time with information about new Blackwell publications and services in your subject area. (For European contributors, this may involve transfer of your personal data outside the European Economic Area.) Please check the following boxes if you are happy to be contacted in this way:

☐ (conventional mailing) ☒ (via e-mail)

Please fax a signed copy of this form to: +44 1524 594696

The original signed copy must also be mailed to:

New Phytologist Central Office
Bailrigg House
Lancaster University
Lancaster LA1 4YE
UK

Or

New Phytologist US Office
Oak Ridge Laboratory
Building 1062, PO Box 2008
Oak Ridge TN 37831-6422
USA