INTERACTIVE EFFECTS OF GLOBAL CHANGE ON
SOIL MICROBIAL COMMUNITY COMPOSITION AND FUNCTION

by

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CHAPTER 1

INTRODUCTION

Anthropogenic forces have extensively modified global biogeochemical cycles and have reduced biodiversity on a global scale (Vitousek et al., 1997). Fossil fuel burning has increased the concentration of CO₂ and O₃ in the atmosphere (Neftel et al., 1985; Finlayson-Pitts and Pitts, 1997), and the rates of atmospheric N deposition (Galloway et al., 1995). Furthermore, intensive land use and habitat conversion have accelerated the loss of plant species in many portions of the Earth (Pimm et al., 1995). These human-induced changes alter the input of growth-limiting resources that shape soil microbial communities (Rogers et al., 1994; Wolters et al., 2000; Matson et al., 2002; Andersen, 2003), and this could trigger changes in heterotrophic soil microorganisms that rely on plants for their source of energy. Because heterotrophic soil microorganisms control the process of organic matter decomposition and N mineralization, it is essential to determine the response of microbial communities to these global change factors to predict the function of terrestrial ecosystems under elevated CO₂, O₃, N deposition, and lower plant diversity. The potential for interactions among these factors exists; however, they have received little attention and investigating the combined effects of multiple environmental changes on ecosystem function should be a research priority (Gregg et al., 2003; Zavaleta et al., 2003). The objective of my dissertation was to determine how concurrent global change components, namely atmospheric CO₂ and O₃ enrichment, atmospheric N
deposition, and declining plant species richness, interact to alter microbial community function and composition, and subsequently ecosystem processes (Fig 1.1).

Elevated CO$_2$ and O$_3$ can affect plant production in opposing ways; elevated CO$_2$ increases plant productivity and stimulates root growth, whereas high O$_3$ levels can lower root biomass and accelerate leaf senescence (Rogers et al., 1994; Anderson, 2003). Studies on forest ecosystems exposed to concurrent CO$_2$ and O$_3$ enrichment show that increased plant production under elevated CO$_2$ can be counteracted by elevated O$_3$ (Isebrands et al., 2001; King et al., 2001a). Therefore, greater foliage and root production under elevated CO$_2$ can provide more organic substrates for microbial metabolism, which could increase the rates of C compound decomposition and N mineralization; elevated O$_3$ can dampen this response (Larson et al., 2002; Holmes et al., 2003). Moreover, the combined effects of CO$_2$ and O$_3$ enrichment on plant litter biochemistry also can alter C and N cycling rates. For example, elevated CO$_2$ can increase non-structural carbohydrate and tannin concentration, and decrease N concentration in plants (Mansfield et al., 1999; King et al., 2001b). However, plants exposed to both elevated CO$_2$ and O$_3$ had greater starch and tannin concentration, and C:N ratio than plants grown under only elevated CO$_2$ (Lindroth et al., 2001; Kopper and Lindroth, 2003). These studies indicate that high levels of tropospheric O$_3$ are an important modifier of plant and microbial function under elevated CO$_2$; thus, they have the potential to alter the composition and metabolism of soil microbial communities that control C and N cycling (Fig 1.1).
Figure 1.1. A conceptual model depicting the links between plants and soil microbial communities, and how these may be altered by concurrent environmental changes. Interactive effects of elevated CO$_2$ and O$_3$ on microbial community composition and function were studied in Chapter 2. In Chapter 3, the degradative potential and community composition of soil microorganisms under elevated CO$_2$, atmospheric N deposition, and declining plant species richness were determined. Interactive effects of N deposition and decreasing plant species richness on microbial metabolism and community composition were investigated in Chapter 4.
In Chapter 2, Compositional and Functional Shifts in Fungal Communities under Elevated CO₂ and O₃, I analyzed microbial community composition and function in the Aspen FACE (Free-Air Carbon dioxide Enrichment) experiment in Wisconsin, U.S.A., in which northern hardwood species (*Populus tremuloides*, *Betula papyrifera*, and *Acer saccharum*) were exposed to elevated CO₂ and O₃. Prior studies have shown that fungal metabolism of cellulose and chitin was enhanced under elevated CO₂ and repressed under elevated O₃ (Larson et al., 2002; Phillips et al., 2002). I tested the hypothesis that the activity of fungal enzymes that degrade plant litter will increase under CO₂ enrichment, and that O₃ enrichment will counteract this. My main hypothesis was that there will be a concurrent shift in fungal community composition with changes in fungal metabolism. I employed extracellular enzyme analysis to measure degradative potential, and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) to determine fungal community composition.

Knowledge of the relationship between plant species richness and soil microbial communities is limited, and how this relationship may be modified by elevated CO₂ and N deposition remains uncertain. Studies that have examined the relationship between plant litter diversity and decomposition have found no consistent results; positive, negative, and non-additive effects of plant litter diversity on decomposition rates have been reported (Blair et al., 1990; Wardle and Nicholson, 1997; Bardgett and Shine, 1999; Hector et al., 2000; Knops et al., 2001). However, plant litterbag studies may not accurately determine how microbial communities respond to declining plant species richness, because temporal and spatial variation in resource availability to heterotrophic
microorganisms by living plants cannot be simulated. Moreover, potential interactions among plant species richness, CO₂ enrichment, and N deposition on microbial communities can be best understood by studying microorganisms that are associated with plant communities subjected to decreasing plant species richness, elevated CO₂, and N deposition.

Because each plant species has a distinct phenology, physiology, morphology, and biochemical composition (Craine et al., 2002; Murphy et al., 2002; Berg and McClaugherty, 2003), plant species richness could have a significant influence on plant production and the range of litter constituents in an ecosystem (Hector et al., 2000; Tilman et al., 2001). Greater plant species richness can increase plant production through complementary resource uptake (Tilman et al., 1996). Increased aboveground and root litter input to soil can enhance microbial metabolism through greater substrate availability, and this can increase rates of C cycling. However, higher organic substrate input can elevate microbial demand for N, and decreased net N mineralization rates could potentially reduce N availability for plants. Therefore, microbial responses to declining plant species richness can feedback to alter plant production.

Furthermore, plant species richness can determine the response of plants to increased CO₂ and N, because species-rich plant communities have a broader range of plant traits to acquire C and N than species-poor communities (Navas et al., 1999; He et al., 2002; Craine et al., 2003). In a grassland study where interactive effects of elevated CO₂, atmospheric N deposition, and declining plant species richness on ecosystem functions were studied, plant production increased with greater plant species richness,
and this response was amplified by elevated CO₂ and added N (Reich et al., 2001). Interactive effects of declining plant species richness, elevated CO₂, and N deposition on plant production and litter biochemistry can propagate to alter microbial community composition, and this can affect soil C storage and N availability for plants (Fig 1.1).

Atmospheric N deposition can alter microbial community composition and function by increasing plant production and plant N concentration, but it can also directly affect microbial-mediated processes by biological and chemical mechanisms. Higher N input to soil can enhance the decomposition of labile litter, but repress lignin-degrading enzymes produced by white-rot basidiomycetes (Fog, 1988; Carreiro et al., 2000; Saiya-Cork et al., 2002). Moreover, increased N availability may retard the rate of C cycling by forming recalcitrant complexes with by-products of lignin decomposition (Berg and McClougherty, 2003). Therefore, atmospheric N deposition by itself has the potential to alter C cycling in terrestrial ecosystems, but how this will interact with decreases in plant species richness and elevated CO₂ to alter soil microbial community composition and function remains to be determined.

In Chapter 3, Soil Microbial Community Composition and Function under Declining Plant Species Richness, Elevated CO₂, and Atmospheric N Deposition, I studied how these global change factors interact and alter microbial function and composition. I examined soil microbial communities in the BioCON (Biodiversity, CO₂, and Nitrogen) experiment located at the Cedar Creek Natural History Area in Minnesota, U.S.A. In this experiment, 1, 4, 9, and 16 grassland species are exposed to factorial CO₂ and N deposition treatments. Plant production and species richness showed a positive
relationship, and the increase in plant production in response to elevated CO₂ and N deposition was greater in species-rich plant communities (Reich et al., 2001). Therefore, I hypothesized that greater substrate input under higher plant species richness will increase microbial biomass, alter community composition, and enhance metabolic rates. I also tested the hypothesis that microbial activity under higher plant species richness will show larger enhancement in response to elevated CO₂ and added N. Here, I measured extracellular enzyme activities, and employed phospholipid fatty acid (PLFA) analysis to gain insight into microbial composition.

In Chapter 4, Microbial Assimilation of New Photosynthate under N Deposition and Decreasing Plant Species Richness, I determined how the flow of new photosynthate into microbial communities is affected by atmospheric N deposition and declining plant species richness. In the BioCON experiment, plants under elevated CO₂ have significantly lower $^{13}$C/$^{12}$C ratio than those under ambient CO₂ due to the $^{13}$C-depleted CO₂ fumigation gas that is used. This difference in C isotopic ratio allowed me to trace the incorporation of the new photosynthate produced under CO₂ enrichment into microbial groups. I reasoned that increased plant litter input under N deposition and greater plant species richness could enhance microbial metabolism, because the growth of heterotrophic microbial communities is limited by organic substrate availability. By coupling PLFA analysis with stable isotope ratio mass spectrometry (IRMS), I tested the hypothesis that microbial groups under N deposition will incorporate more new photosynthate compared to those under ambient N deposition, and this will be accompanied by greater microbial biomass and higher extracellular enzyme activity. I
also hypothesized that increased plant production under higher plant species richness will enhance the rates of organic matter degradation and microbial incorporation of new photosynthate. In Chapter 5, I summarized the major findings of my research described in Chapters 2, 3, and 4.
LITERATURE CITED


CHAPTER 2

COMPOSITIONAL AND FUNCTIONAL Shifts IN FUNGAL COMMUNITIES UNDER ELEVATED CO₂ AND O₃

ABSTRACT

Atmospheric CO₂ and O₃ concentrations are increasing due to human activity and both trace gases have the potential to alter C cycling in forest ecosystems. Because soil microorganisms depend on plant litter as a source of energy for metabolism, changes in the amount or the biochemistry of plant litter produced under elevated CO₂ and O₃ could alter microbial community function and composition. In this experiment, elevated CO₂ increased the microbial metabolism of cellulose and chitin, whereas elevated O₃ dampened this response. Based on this previous study, I hypothesized that this change in metabolism under CO₂ and O₃ enrichment would be accompanied by a concomitant change in fungal community composition. I tested my hypothesis at the Aspen Free-Air CO₂ and O₃ Enrichment (FACE) experiment at Rhinelander, Wisconsin, in which *Populus tremuloides*, *Betula papyrifera*, and *Acer saccharum* were grown under factorial CO₂ and O₃ treatments. I employed extracellular enzyme analysis to assay microbial metabolism, phospholipid fatty acid (PLFA) analysis to determine change in microbial community composition, and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) to analyze fungal community composition. The activities of 1,4-β-glucosidase (+37%) and 1,4-β-N-acetylglucosaminidase (+84%) were
significantly increased under elevated CO₂, whereas 1,4-β-glucosidase activity (-25%) was significantly suppressed by elevated O₃. There was no significant main effect of elevated CO₂ or O₃ on fungal relative abundance, as measured by PLFA. I identified 39 fungal taxonomic units from soil using DGGE, and found that O₃ enrichment significantly altered fungal community composition. I conclude that fungal metabolism is altered under elevated CO₂ and O₃, and that there was a concomitant change in fungal community composition under elevated O₃. Thus, changes in plant inputs to soil under elevated CO₂ and O₃ can alter microbial community composition and function, and in turn, the cycling of C in soil.

**INTRODUCTION**

Fossil fuel burning and land-use conversion have increased atmospheric CO₂, which has the potential to alter rates of C cycling in forest ecosystems (Zak et al., 1993; DeLucia et al., 1999). The major impacts of CO₂ enrichment on plants include stimulated photosynthesis, accumulation of nonstructural carbohydrates, and reduced tissue N concentration (Mooney et al., 1991; Körner, 2000). In plants, a substantial portion of photosynthate is allocated to root growth and maintenance, and elevated CO₂ can further stimulate belowground plant growth (Rogers, 1994). For example, many studies observed an increase in root biomass and possibly higher rhizodeposition in response to elevated CO₂ (Allen et al., 2000; Matamala and Schlesinger, 2000; Mikan et al., 2000; Pregitzer et al., 2000). Much of this additional belowground photosynthate eventually becomes available to soil microorganisms; hence, atmospheric CO₂ enrichment may have major impacts on energy flow through microbial food webs in soil. Higher plant litter
production and a change in litter biochemistry of CO$_2$-enriched plants could alter soil microbial community function and composition, and this in turn may alter C and N cycling in soil. However, understanding of how the aforementioned responses will be modified by other climate change factors, like elevated O$_3$, which could counteract the effect of elevated CO$_2$, is incomplete.

Ozone is an atmospheric pollutant that also has increased globally over the past century due to fossil fuel burning (Finlayson-Pitts and Pitts, 1997). In contrast to CO$_2$, elevated O$_3$ has detrimental effects on plant growth, because it can decrease leaf photosynthesis, lower root and stem biomass, and accelerate leaf senescence (Findlay and Jones, 1990; Taylor et al., 1994; Karnosky et al., 1996). Moreover, plants typically allocate less to roots when exposed to sufficiently high concentrations of O$_3$ (Coleman et al., 1996; Andersen et al., 1997; Andersen, 2003). Reduced allocation of photosynthate to roots under O$_3$ enrichment has the potential to suppress microbial metabolism, an effect that could counteract that of elevated CO$_2$. How will changes in plant growth under elevated CO$_2$ and O$_3$ alter microbial community function and composition?

I studied microbial community function and composition under elevated CO$_2$ and O$_3$ at the Aspen Free-Air CO$_2$ and O$_3$ Enrichment (FACE) experiment in Rhinelander, Wisconsin. In this experiment, *Populus tremuloides*, *Betula papyrifera*, and *Acer saccharum* have been exposed to factorial elevated CO$_2$ and O$_3$ treatments since 1998 (Dickson et al., 2000). Previous work has demonstrated that fine root biomass increased significantly under elevated CO$_2$, and decreased under elevated O$_3$ (King et al., 2001). Also, the C:N ratio in senescing *Populus* and *Betula* leaves increased significantly under
elevated CO₂, and this change was carried through litter deposition (Lindroth et al., 2001). Along with these changes in litter production and chemistry, they have observed an increase in the fungal metabolism of cellulose and chitin under CO₂ enrichment; O₃ enrichment dampened this response (Larson et al., 2002; Phillips et al., 2002). I hypothesized that this change in fungal metabolism has arisen from a change in fungal community composition, a result of altered substrate availability. To test this hypothesis, I used microbial extracellular enzyme analysis to assay microbial metabolism in our experiment. Because most of the extracellular enzymes that decompose plant litter are synthesized based on the concentration of substrates present in soil (Burns 1982), I reasoned that extracellular enzyme activity would reflect microbial metabolic potential under elevated CO₂ and O₃. I examined the relationship between belowground plant biomass and enzyme activity to confirm if changes in substrate availability in response to elevated CO₂ and O₃ was responsible for altering microbial metabolism. In addition, I used PLFA analysis to determine whether elevated CO₂ and O₃ elicited an overall change in microbial community composition. To specifically analyze fungal community composition, I extracted DNA from soil, and amplified and separated fungal rDNA using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). In this analysis, the generated DNA banding pattern shows the major taxonomic units within a microbial community (Fromin et al. 2002). I used this technique to determine if changes in metabolic activity were accompanied by a shift in fungal community composition.
METHODS

Experimental design and sampling procedures

My study was conducted at the Aspen Free-Air CO₂ and O₃ Enrichment (FACE) experiment in Rhinelander, Wisconsin, U.S.A. In this experiment, factorial CO₂ and O₃ treatments are applied in a randomized complete block (n = 3) design. There are a total of twelve 30-m-diameter-FACE rings, and within each ring, trembling aspen (Populus tremuloides), paper birch (Betula papyrifera), and sugar maple (Acer saccharum) are planted at a density of 1 stem/m². Each ring was split into three sections; half of the ring was planted with aspen; one quarter of the ring was planted with aspen and birch, and aspen and maple were planted in the remaining quarter. Trees were exposed to CO₂ and O₃ treatments beginning in May 1998. The target concentration of elevated CO₂ treatment was 560 μL/L, which is ~200 μL/L above ambient concentration. In 1999, the atmospheric CO₂ concentrations were 346.5 μL/L in ambient plots and 547.8 μL/L in elevated CO₂ plots. The target level of elevated O₃ treatment is determined at the beginning of each day, based on current meteorological conditions. For hot and sunny days, a maximum O₃ concentration of 90 to 100 nL/L is applied; 50 to 60 nL/L is maintained for cool and cloudy days. In 1999, atmospheric O₃ concentrations were 36.9 nL/L (seasonal sum 0, 61.9 μL/L) in the ambient plots and 51.7 nL/L (seasonal sum 0, 89.0 μL/L) in elevated plots (Dickson et al., 2000). Seven soil cores, 2 cm in diameter and 15 cm in depth, were randomly collected from each ring section. Samples were collected in July 2001 (summer), November 2001 (autumn), and May 2002 (spring).
Cores were composited by ring section and immediately frozen. Soil samples were kept at -80 °C prior to enzymatic and molecular analysis.

**Microbial Community Function**

To determine microbial community metabolism, I measured the activities of enzymes that degrade nonstructural carbohydrate, hemicellulose, cellulose, lignin, chitin, and organic P substrate in soil (Table 2.1). I analyzed the activities 1,4-α-glucosidase, 1,4-β-glucosidase, cellobiohydrolase, 1,4-β-xylosidase, 1,4,-β-N-acetylglucosaminidase, and phosphatase using methylumbelliferone (MUB) linked substrates (after Saiya-Cork et al., 2002). One gram of soil from each composite was thawed, and then placed in 125 mL of sodium acetate buffer (pH 5.0). The solution was transferred to a 96-well microplate that contained eight analytical replicates of each enzyme assay. For each enzyme assay, 200 μL of soil-buffer solution and 50 μL of substrate were combined. Plates were incubated at 21 °C for all enzyme assays. Phosphatase and 1,4,-β-N-acetylglucosaminidase assays were incubated for 0.5 hr and 1,4-α-glucosidase, 1,4-β-glucosidase, cellobiohydrolase, and 1,4-β-xylosidase assays were incubated for 2 hr. Fluorescence was analyzed using a f-Max fluorometer (Molecular Devices Corp., Sunnydale, CA), in which the excitation energy was set at 355 nm and emission was measured at 460 nm. Enzyme activities were expressed as nmol 4-MUB g⁻¹h⁻¹.

The activities of lignin-degrading enzymes, phenol oxidase and peroxidase, were determined by colorimetric assay using 25 mM L-3,4-dihydroxy-phenylalanine (L-DOPA) as the substrate (Saiya-Cork et al., 2002). The procedure for measuring the activity of these enzymes was similar to that described above. There were sixteen
Table 2.1. Extracellular enzymes involved in litter decomposition and their substrates.

<table>
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<tr>
<th>Enzymes</th>
<th>Substrate</th>
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<tr>
<td>1,4-β-N-acetylglucosaminidase</td>
<td>Chitin</td>
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<tr>
<td>Acid phosphatase</td>
<td>Organic P</td>
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<tr>
<td>1,4-α-glucosidase</td>
<td>Nonstructural carbohydrate</td>
</tr>
<tr>
<td>1,4-β-xylosidase</td>
<td>Hemicellulose</td>
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<tr>
<td>1,4-β-glucosidase</td>
<td>Cellulose</td>
</tr>
<tr>
<td>Cellbiohydrolase</td>
<td>Cellulose</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Lignin</td>
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<tr>
<td>Phenol oxidase</td>
<td>Lignin</td>
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analytical replicates for each enzyme assay. Following 24-hr incubation at 21 °C, absorbance was read at 450 nm on EL-800 plate reader (Biotek Instruments, Inc., Winooski, VT). Activity was reported as nmol L-DOPA oxidized g⁻¹ h⁻¹. The results of all enzymatic assays are expressed on a dry soil weight basis.

Belowground plant biomass of 2001 was reported by King et al. (in review). I used these data to explore the relationship between belowground plant biomass and extracellular enzyme activity.

**Microbial Community Composition**

Microbial lipids were extracted from 5 grams of freeze-dried soil collected in each ring section. I used a solvent system that included phosphate buffer to extract total lipids, and silicic acid chromatography to separate the total lipids into neutral, glyco- and polar lipids (White et al. 1979; Guckert et al. 1985). Polar lipids were methylated with 0.2 M methanolic KOH to form fatty acid methyl esters (FAMEs). FAMEs were identified and quantified using a Finnigan Delta plus mass spectrometer with a GC/C III interface (ThermoElectron, Austin, TX) coupled to a HP 5973 GC (Agilent Technologies, Palo Alto, CA). Fatty acids 18:2o6 and 18:1o9c were considered fungal biomarkers (Bardgett et al., 1996; Bååth, 2003).

**Fungal Community Composition**

**DNA extraction:** Total soil DNA was extracted using UltraClean™ soil DNA isolation kit (Mo Bio Laboratories, Inc., Solana Beach, CA). One gram from each composite soil sample was placed in a 2-mL tube with glass beads and a buffer solution. The tubes were agitated horizontally for 10 min, allowing DNA from ruptured cells to attach to the glass
beads. DNA was then precipitated by adding Solution S2 and incubating at 4 °C for 5 min. DNA was purified by diluting ten times with 10 mM Tris-Cl buffer, transferring it to a spin-filter, and centrifuging at 10,000 g for 1 min. A tube without soil was subjected to the DNA extraction procedure, and it served as a negative control.

**PCR, DGGE, and DNA sequence analysis:** The extracted DNA was amplified using fungal specific primers FF390 and FR1 that amplify a 390-base pair region of 18S rDNA (Vainio and Hantula, 2000). The reaction mixture was 50 μL in volume, and it contained template DNA, 5 μL of 10x reaction buffer, 1 μL of 10 mM dNTP mixture, 1.75 units of Expand™ High Fidelity PCR system (Roche Diagnostics, Germany), 0.5 μM of forward primer FF390 and 0.5 μM of reverse primer FR1. In the negative control, 1 μL of sterilized water was used as the DNA template. The DNA was amplified according to the following program using a Robocycler temperature cycler (Stratagene, La Jolla, CA): (1) 8 min at 95 °C, (2) 30 sec at 95 °C, (3) 45 sec at 50 °C, (4) 2 min at 72 °C, (5) 29 more times of step (2)-(4), and (6) 10 min at 72 °C. I subsequently separated the PCR products on 1.6% agarose gel to determine if the PCR was successful.

For DGGE analysis, the PCR products of each soil sample were loaded on 7.5% (w/v) acrylamide/bisacrylamide (37.5:1) parallel gradient gel, which was cast using a Model 475 Gradient Delivery System (Bio-Rad, Hercules, CA). In each gel, I loaded twelve PCR products from one block and EZ Load 100bp Molecular Ruler (Bio-Rad, Hercules, CA) (Fig 2.1). Electrophoresis was conducted on Bio-Rad DCode™ Universal Mutation Detection System (Bio-Rad, Hercules, CA) for 18 hr at 50 V and 58 °C (after Vainio and Hantula, 2000). The gel was stained with ethidium bromide, and the gel
Figure 2.1. A PCR-DGGE gel image. PCR products were amplified from soil samples collected in May 2002 (spring) from block 3. MR: EZ Load 100bp Molecular Ruler (The size of each band is shown in base pairs), A: aspen section, B: aspen-birch section, M: aspen-maple section.
image was documented and analyzed by EPI-Chemi Darkroom System (UVP Lab Products, Upland, CA). The size of the bands was assigned using the software LabWorks (UVP Lab Products, Upland, CA) according to the position of each band in relation to molecular size standards. Because the band size was standardized to molecular size markers, we were able to compare bands across multiple gels.

To elute PCR products for sequencing, DNA bands were cut from the DGGE gel and were kept at -20°C for 12 hr, and then at 4°C for another 12 hr in Tris-EDTA buffer. The eluted DNA was re-amplified through PCR, and the PCR product was subject to sequence analysis at the Sequencing Core Facility at University of Michigan (Ann Arbor, MI). Because DNA fragments that have different base composition may migrate at identical rates in a DGGE gel (Sekiguchi et al., 2001), I eluted two bands that were at the same position in a gel and sequenced them. I also eluted two bands of same base pair size from two different gels and compared the DNA sequence. The DNA sequences were identical for bands that were of equal molecular size.

I performed PCR-DGGE on five analytical replicates to determine whether microbial community composition in one gram of soil was representative of the microbial community in the composite soil sample from each ring section. DNA was extracted from five 1-gram subsamples of one composite soil sample, and each DNA extract was amplified using primers described above. DGGE analysis confirmed that the banding pattern of five replicates was identical (data not shown).

To determine the phylogenetic affinity of the sequenced operational taxonomic units (OTU), related sequences were obtained using BLAST (NCBI, Bethesda, MD)
searches. All sequences were imported into Bioedit Sequence Alignment Editor version 6.0.7 (1997-2004, Tom Hall, Isis Pharmaceuticals, Inc., Carlsbad, CA) and aligned using the Clustal W accessory application. Alignments were checked and adjusted manually where needed. Phylogenetic trees were generated using PAUP* version 4.0 b10 for Windows (Sinauer Associates, Inc., Sunderland, MA). A heuristic search was carried out using maximum parsimony, with gaps treated as missing data, ten replicates, and no more than 100 trees saved in each replicate. After initial analysis, I performed a bootstrap analysis using 1000 replicates to generate probability estimates for the branches.

**Statistical Analyses**

Enzyme activities were analyzed using repeated-measures ANOVA for a split-plot randomized complete block design. Block, CO₂, O₃, and species were fixed effects in this model. Carbon dioxide and O₃ treatment combinations were main effects, and they were split by species. Significance of main effects (CO₂ and O₃), split-plot effects (species), time and their interaction was accepted at α = 0.05. I performed linear regression analysis to determine the relationship between belowground plant biomass and enzyme activity.

For the analysis of fungal community composition, multi-response permutation procedures (MRPP) were conducted using PC-ORD (Mjm Software Design, Gleneden Beach, OR). MRPP is a non-parametric method for testing the hypothesis of no difference between two or more communities, and this method does not require distributional assumptions such as multivariate normality and homogeneity of variance (McCune and Grace 2002). The null hypotheses tested through blocked MRPP were as follows: 1) fungal communities under ambient and elevated CO₂ are not different, and 2)
fungal communities under ambient and elevated O₃ are not different. Euclidian distance was measured and compared for each fungal community. I report the level of significance for each comparison procedure; significance between any two groups was accepted at \( \alpha = 0.05 \).

I performed indicator species analysis with PC-ORD to determine if any taxonomic unit was specific to the elevated CO₂ and O₃ treatments. Through this method, I determined how faithfully a taxonomic unit occurs in a particular treatment. The significance of resulting indicator value was tested through Monte Carlo test and was accepted at \( \alpha = 0.05 \).

**RESULTS**

**Extracellular enzyme activity**

As a main effect, elevated CO₂ significantly increased the activities of 1,4-\( \beta \)-glucosidase and N-acetylglucosaminidase (Table 2.2, Fig 2.2). 1,4-\( \beta \)-glucosidase activity was 37% higher under elevated CO₂ (Fig 2.2A), and N-acetylglucosaminidase activity increased under elevated CO₂ by 84% when compared to ambient CO₂ (Fig 2.2B). Elevated CO₂ also enhanced the activities of cellobiohydrolase, 1,4-\( \beta \)-xylosidase, phosphatase, 1,4-\( \alpha \)-glucosidase, and phenol oxidase, but this effect was not statistically significant (Table 2.2, Fig 2.2). Elevated CO₂ had no effect on peroxidase activity (Table 2.2, Fig 2.2G).

Elevated O₃ significantly (main effect) reduced the activity of 1,4-\( \beta \)-glucosidase by 25% relative to the activity of this enzyme at ambient O₃ (Table 2.2, Fig 2.3A).
Table 2.2. The influence of elevated CO$_2$ and O$_3$ on microbial extracellular enzyme activity. P-values for microbial extracellular enzyme activities analyzed by repeated-measures analysis of variance (ANOVA) are shown. P-values equal to or lower than 0.05 are in bold face print.

<table>
<thead>
<tr>
<th></th>
<th>1,4-α-glucosidase</th>
<th>1,4-β-glucosidase</th>
<th>Cellobiohydrolase</th>
<th>1,4-β-xylosidase</th>
<th>1,4-β-N-acetylglucoseaminidase</th>
<th>Phosphatase</th>
<th>Peroxidase</th>
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<td></td>
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<td>CO$_2$</td>
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<td>0.14</td>
<td>0.48</td>
<td><strong>0.01</strong></td>
<td>0.16</td>
<td>0.63</td>
<td>0.08</td>
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<tr>
<td>O$_3$</td>
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<td>0.10</td>
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<td>0.10</td>
<td>0.11</td>
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<td>0.26</td>
<td>0.99</td>
<td>0.06</td>
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<tr>
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<tr>
<td>Time x CO$_2$ x O$_3$ x Species</td>
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<td>0.40</td>
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<td>0.79</td>
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</table>
Figure 2.2. Main effect of CO$_2$ on extracellular enzyme activity. Enzyme activity was averaged across three sampling seasons. Error bars indicate standard error of the mean.
Figure 2.3. Main effect of O₃ on extracellular enzyme activity. Enzyme activity was averaged across three sampling seasons. Error bars indicate standard error of the mean.
N-acetylglicosaminidase, cellobiohydrolase, 1,4-β-xylosidase, phosphatase, 1,4-α-glucosidase, peroxidase and phenol oxidase activities were suppressed under elevated O₃, but these reductions were not statistically significant (Table 2.2, Fig 2.3). Although there was no significant interaction between elevated CO₂ and O₃ (Table 2.2), the activity of cellulose-degrading enzymes under elevated CO₂ was dampened by elevated O₃. For example, 1,4-β-glucosidase and cellobiohydrolase activities under both elevated CO₂ and O₃ were lower than the activities under elevated CO₂ and ambient O₃, and were not different from those under ambient CO₂ and O₃ (Fig 2.4A, B). However, N-acetylglicosaminidase activity under elevated CO₂ and O₃ was not different from that under elevated CO₂ alone (Fig 2.4C). 1,4-β-glucosidase, cellobiohydrolase and 1,4-β-N-acetylglicosaminidase activity in July 2001 showed significant positive correlation with total root biomass [1,4-β-glucosidase activity (nmol g⁻¹hr⁻¹) = 0.4(root biomass (gm⁻²)) + 45.6, n = 36, r² = 0.20, P = 0.01]; [Cellobiohydrolase activity (nmol g⁻¹hr⁻¹) = 0.21(root biomass (gm⁻²)) – 41.6, n = 33, r² = 0.15, P = 0.02]; [N-acetylglicosaminidase activity (nmol g⁻¹hr⁻¹) = 0.15(root biomass(gm⁻²)) + 24.4, n = 36, r² = 0.20, P = 0.01].

1,4-β-glucosidase and 1,4-β-N-acetylglicosaminidase displayed a strong seasonal pattern (Table 2.2), wherein activities were highest in July compared to other sampling dates; peroxidase activity was highest in November (data not shown). 1,4-α-glucosidase, cellobiohydrolase, 1,4-β-xylosidase, phosphatase and phenol oxidase did not show a significant temporal pattern (Table 2.2). There was a significant main effect of tree species on peroxidase activity, and it was highest under aspen. Tree species composition and elevated CO₂ had a significant interaction effect on 1,4-β-glucosidase activity (Table
Figure 2.4. Effect of CO$_2$ and O$_3$ on activity of 1,4-β-glucosidase, celllobiohydrolase, and 1,4-β-N-acetylglucosaminidase. Error bars indicate one standard error of the mean.
2.2). 1,4-β-glucosidase activity in aspen-maple section under ambient CO₂ was lower than that under aspen and aspen-birch section, but 1,4-β-glucosidase activity under elevated CO₂ in all three tree compositions were not different (data not shown).

**Microbial community composition**

Biomass of each microbial group was determined by PLFA analysis, and the percentage of fungal biomass comprising total biomass was considered the relative abundance of fungi in the soil microbial community. Fungal relative abundance in July 2001 was 30% higher under elevated CO₂, but this was only marginally significant (P = 0.10). Fungal relative abundance under elevated O₃ was 17% higher than that under ambient O₃, but this result also was not statistically significant. There was no significant interaction between elevated CO₂ and O₃ on fungal abundance. Tree species composition had no significant effect on fungal relative abundance.

**Fungal community composition**

A total of 39 operational taxonomic units (OTU) were identified using DGGE. Sixteen prominent OTUs were sequenced; ten OTUs were in the Basidiomycota, four in the Ascomycota and two in the Zygomycota (Fig 2.5). Of the ten OTUs in the Basidiomycota, one was in the Tremellomycetidae (Unknown 15), a clade containing many mycorrhizas. All others were in the Homobasidiomycetes. One cluster in the Homobasidiomycetes that includes six OTUs (Unknown 2, 7, 8, 9, 10, and 11) resided on a long branch with unknown affinity. Two OTUs in the Basidiomycota (Unknown 5 and 13) may be related to *Inocybe*, an ectomycorrhizal genus in the Cortinariaceae that fruits commonly in these plots, although bootstrap support for this grouping was less than 50%
Figure 2.5. Phylograms showing the placement of the sequenced OTUs amplified from soils at the Rhinelander FACE site for A) Basidiomycota, and B) Ascomycota and Zygomycota. Numbers above the clades indicate bootstrap support (%), shown only for the clades containing the 16 unknowns.
(Fig 2.5A). One OTU (Unknown 3) appears to be related to the genus *Cortinarius*, also in the Cortinariaceae. Two OTUs in the Ascomycota (Unknown 4 and 16) clustered with the Pezizaceae. One OTU (Unknown 12) was affiliated with *Verticillium spp.*, which are soil-borne pathogens. The two OTUs (Unknown 1 and 6) in the Zygomycota clustered with the Mortierellaceae (Fig 2.5B).

Fungal community composition under ambient and elevated CO₂ was not different across all three sampling seasons (P = 0.85). No change in fungal community composition under ambient and elevated CO₂ was detected in spring (P = 0.23), summer (P = 0.74), or fall (P = 0.63). To determine if any taxonomic unit was an indicator of the elevated CO₂ treatment, I performed indicator species analysis. Presence/absence data for each fungal taxonomic unit were analyzed for their occurrence under ambient and elevated CO₂ treatment. In summer, one OTU (Unknown 2 in Fig 2.5A) occurred in 11% of the soil samples under ambient CO₂, whereas it was present in 67% of the soil samples under elevated CO₂. This species was a significant indicator of elevated CO₂ treatment (P < 0.01).

Elevated O₃ significantly altered fungal community composition across all three sampling seasons (P = 0.02). Fungal community composition under ambient and elevated O₃ was different in spring (P = 0.04) and summer (P = 0.02), but no change was detected in fall (P = 0.57). Indicator analysis showed that one OTU (Unknown 1 in Fig 2.5B) was a significant indicator of elevated O₃ treatment in spring (P = 0.02). This OTU was present in 6% of the soil samples under ambient O₃, whereas it occurred in 44% of the soil samples under elevated O₃ in spring.
DISCUSSION

Because soil microbial communities carry out key processes in soil C and N cycling, determining how microbial community composition and function may change under CO₂ and O₃ enrichment is central in predicting how ecosystem function will be altered by these rising trace gases. In my experiment, plant production increased and the N content of the litter was lower under elevated CO₂, whereas plant production was suppressed by O₃ enrichment (Kull et al., 1996; Lindroth et al., 2001). Here, I demonstrate that 1,4-β-glucosidase, cellobiohydrolase and N-acetylglucosaminidase activities are enhanced under CO₂ enrichment, and that the increased activities of 1,4-β-glucosidase and cellobiohydrolase were dampened by elevated O₃ enrichment. These changes in extracellular enzyme activity were accompanied by alteration in fungal community composition under elevated O₃. My results indicate that increases in atmospheric CO₂ and O₃ can induce changes in plant growth that cascade into the soil food web to modify fungal community composition and function.

Activities of cellulose-degrading enzymes 1,4-β-glucosidase and cellobiohydrolase significantly increased beneath plants exposed to elevated CO₂ alone, but this response was dampened by O₃, (i.e., in elevated CO₂ and elevated O₃ treatment) suggesting that elevated O₃ may counteract the effect of elevated CO₂. Cellulose is a major component of the plant tissue, and plant production, especially belowground production will determine cellulose input to soil. King et al. (2001) have found similar pattern in fine root production in this study site; elevated CO₂ increased the production of fine roots, whereas elevated O₃ dampened this response. Total belowground biomass was
significantly correlated with 1,4-β-glucosidase and cellubiohydrolase, indicating that the activities of cellulose-degrading enzymes were induced according to the amount of cellulose entering soil, thereby closely reflecting the cellulose availability under elevated CO₂ and O₃. This observation is consistent with the idea that plant growth responses to these trace gases will drive the response of microbial communities in soil.

Activity of chitin-degrading enzyme N-acetylglucosaminidase was significantly higher under elevated CO₂, and this could indicate that there is a higher input of fungal litter under elevated CO₂. Chitin is the main component of the fungal cell wall, which is built from N-acetylglucosamine subunits (Swift et al., 1979). N-acetylglucosaminidase is produced by a diverse group of fungi, and its activity is positively correlated with fungal biomass (Miller et al., 1998). Elevated CO₂ enhanced fungal incorporation of ¹³C-labelled N-acetylglucosamine in this study (Phillips et al., 2002), and taken together this suggests that there may be higher fungal biomass under CO₂ enrichment.

Fungi are major producers of 1,4-β-glucosidase and N-acetylglucosaminidase in soil (Hayano and Katami, 1977; Miller et al., 1998), and greater activities of these two enzymes under elevated CO₂ indicate that fungal metabolism is stimulated by changes in plant growth due to this trace gas. This is consistent with greater total hyphal lengths, culturable and active fungi under elevated CO₂ (Rillig et al., 1999). Jones et al. (1998) also demonstrated that cellulose-decomposing fungi had higher biomass under elevated CO₂, probably accounting for the increased decomposition rates of cotton strips. Because I have observed higher 1,4-β-glucosidase and N-acetylglucosaminidase activity under elevated CO₂ and dampened 1,4-β-glucosidase activity under elevated O₃, I then
wondered if these physiological responses were accompanied by a change in fungal community composition.

There was no significant difference in fungal relative abundance in the soil microbial community as determined by PLFA analysis. My amplification of fungal rDNA also suggests that fungal communities under ambient and elevated CO₂ did not differ, but there was one indicator OTU (Unknown 2 in Fig 2.5A) that occurred more frequently under elevated CO₂ in summer. Elevated O₃ significantly altered fungal community composition from that under ambient O₃, and one OTU (Unknown 1 in Fig 2.5B) was an indicator of elevated O₃ treatment in spring. This OTU was closely related to the genus Mortiellera, a common group of saprophytic fungi capable of producing chitinolytic, proteolytic, and cellulolytic enzymes (De Boer et al., 1999; Lähn et al., 2002). I cannot infer whether the biomass of this OTU changed under elevated O₃ treatment because PCR-DGGE is not quantitative, but this OTU occurred significantly more frequently in soils under elevated O₃. This fungus may have an advantage over other fungi when there is less belowground production under O₃ enrichment.

I have demonstrated that cellulose- and chitin-decomposing enzyme activities are significantly higher under CO₂ enrichment and that cellulose-degrading enzyme activities are dampened by O₃ enrichment; and there was a significant change in fungal community composition in response to O₃. Although not statistically significant, activities of other enzymes responded in a similar way under CO₂ and O₃ enrichment, further supporting my contention that microbial metabolism is enhanced under elevated CO₂ and suppressed under elevated O₃. I conclude that the change in substrate availability under CO₂ and O₃
enrichment altered microbial community function, and this was accompanied by change in fungal community composition, at least in response to elevated O₃. This indicates that a change in plant production and litter biochemistry under elevated CO₂ and O₃ may alter the function and composition of fungal communities. Taken together, my results imply that concurrent changes in fungal community function and composition under elevated CO₂ and O₃ are driven by changing substrate quantity and quality, and this may in turn alter soil C cycling as CO₂ and O₃ accumulate in the atmosphere.
LITERATURE CITED


CHAPTER 3
SOIL MICROBIAL COMMUNITY COMPOSITION AND FUNCTION UNDER DECLINING PLANT SPECIES RICHNESS, ELEVATED CO₂, AND ATMOSPHERIC N DEPOSITION

ABSTRACT

Human activities are concurrently increasing the atmospheric CO₂ concentration, rates of atmospheric N deposition, and the loss of plant species. All of these factors have the potential to influence soil microbial communities by altering plant production and litter biochemistry. However, we have little knowledge of how reduced plant species richness, elevated CO₂, and N deposition may interact and alter microbial activity and nutrient cycling in terrestrial ecosystems. I determined how microbial community composition and function change under the aforementioned global change factors in a field experiment in which plant communities of increasing species richness were exposed to elevated CO₂ and N deposition. In a Free-Air CO₂ Enrichment (FACE) experiment, 1, 4, 9, or 16 grassland species were grown under factorial CO₂ and N deposition treatments. Because more diverse plant assemblages had greater plant biomass in response to elevated CO₂ and N deposition, I reasoned that heterotrophic microbial communities would have higher biomass and greater metabolic activity, thus potentially altering soil C and N cycling. Therefore, I hypothesized that microbial community response to elevated CO₂ and N deposition will depend on plant species richness. Microbial community composition was quantified using PLFA analysis, and extracellular enzyme analysis was used to assess
microbial community function. Higher plant species richness fostered greater microbial biomass (65.5 ± 4.4 nmol PLFA/g in 1-species vs. 89.6 ± 6.3 nmol PLFA/g in 16-species treatment), higher cellulolytic capacity, and greater fungal relative abundance and arbuscular mycorrhizal (AM) abundance. Nitrogen addition significantly increased 1,4-β-glucosidase activity (27.3 ± 1.7 nmol g⁻¹h⁻¹ under ambient N vs. 32.3 ± 1.8 nmol g⁻¹h⁻¹ under added N) and phosphatase activity (13.0 ± 0.8 nmol g⁻¹h⁻¹ under ambient N vs. 16.4 ± 1.0 nmol g⁻¹h⁻¹ under added N), and decreased AM abundance. My results demonstrate that plant species richness independently altered microbial community composition and function through combined effects of increased plant production, greater cellulose concentration in litter, and complementary resource uptake. N addition stimulated cellulolytic potential and increased microbial demand for P. My study also demonstrates that microbial activity is less affected by interactions among plant species richness, elevated CO₂, and N addition than by individual environmental changes. The main effects of plant species richness were more important to microbial community composition and function than the interactions among plant species richness, elevated CO₂, and N deposition, and this suggests that soil microbial metabolism and composition could be significantly altered as plant species numbers decrease in grassland ecosystems.

**INTRODUCTION**

Human activity has altered global C and N cycling by increasing atmospheric CO₂, increasing rates of atmospheric N deposition, and decreasing plant diversity (Vitousek, 1994; Chapin et al., 2000). Due to fossil fuel combustion, atmospheric CO₂ has risen 100 µL/L since the mid 1800s, and atmospheric N deposition has increased tenfold in eastern
North America (Neftel et al., 1985; Galloway et al., 1995). In addition, land-use change increased the rate of plant extinction by 100 to 1000 times when compared to pre-human extinction rates (Pimm et al., 1995). Although individual effects of elevated CO₂, increased N deposition, and declining plant diversity on ecosystem function have received substantial attention (Norby, 1998; Körner, 2000; Naeem, 2002), we have limited knowledge of the combined impacts of these global change factors on ecosystem function (Reich et al., 2001a). Moreover, the main focus of many studies has been on plant processes (Navas et al., 1999; He et al., 2002; Shaw et al., 2002; Zavaleta et al., 2003) and the response of soil microbial communities to concurrent environmental changes remains a gap in our knowledge (Niklaus et al., 2001a).

There are reasons to expect that plant growth responses to elevated CO₂, N deposition, and declining plant species richness will interact to modify soil microbial community composition and function. Because heterotrophic soil microorganisms derive energy from plant litter, the amount and quality of resources provided by plants determine the composition and metabolism of microbial communities. Evidence suggests that plant responses to greater CO₂ and N availability depend on plant species richness (Niklaus et al., 2001b; He et al., 2002), and this effect may be propagated via litter production to heterotrophic soil microorganisms. For example, Niklaus et al. (2001b) found that plant production increased as plant diversity increased, and elevated CO₂ enhanced this response. A mesocosm study also demonstrated that under CO₂ enrichment and high nutrient (N, P, and K) availability, plant species richness increased plant production (He et al., 2002). These studies indicate that the relationship between plant
production and plant species richness can be modified by CO₂ and soil N availability, and this may alter C cycling mediated by soil microorganisms by increasing the amount and modifying the organic compounds contained in litter.

The extinction rate of all species is estimated to have increased 100 to 1000 times by human activity, and this loss of species diversity may negatively affect ecosystem function (Cox, 1993; Pimm et al., 1995; Matson et al., 1997; Chapin et al., 2000). Grassland studies in North America and Europe have demonstrated that plant productivity increased as plant species richness increased, implying that plant species richness may be positively related to ecosystem function (Tilman et al., 1996; Hector et al., 1999; Tilman et al., 2001). Higher diversity also can enhance the stability of ecosystems by conferring greater resistance and resilience to disturbances (Naeem and Li, 1997; McCann, 2000). Niche complementarity, the concept that greater diversity incorporates a wider range of species traits and that these interspecific differences allow more complete use of resources, is one mechanism by which biodiversity can influence ecosystem function (Tilman et al., 1997; Tilman et al., 2001). Alternatively, the “sampling effect” suggests that plant communities of higher diversity have a greater chance of including a few species with a dominant trait that drives ecosystem function (Huston, 1997). Although we are beginning to understand the relationship between plant diversity and ecosystem function, most attention has focused on defining how primary producers respond to changing diversity, and little is known about the relationship between plant species richness and soil microbial communities that mediate nutrient cycling (Loreau et al., 2001a; Catovsky et al., 2002).
Fossil fuel burning has increased atmospheric CO₂ and N deposition, and this also may alter ecosystem function (Mellilo et al., 1993; Vitousek, 1994). Plants grown under elevated CO₂ have higher productivity, greater root biomass, and lower N concentration (BassiriRad et al., 2001; Norby et al., 2001; Nowak et al., 2004). On the other hand, plants exposed to high levels of atmospheric N deposition increase production and have greater N content (Falkengren-Grerup, 1998; Fenn et al., 1998). Increased plant production and changes in litter biochemical constituents by CO₂ enrichment and N deposition can directly alter the rates of heterotrophic microbial metabolism in soil, and consequently the nutrient flow through the soil food webs. Moreover, elevated CO₂ and increased N deposition could interact with plant species richness to modify substrate availability for microbial metabolism in soil.

I determined how microbial community composition and function change with decreasing plant species richness, elevated CO₂, and increased N deposition at the BioCON (Biodiversity, CO₂, and N) experiment at the Cedar Creek Natural History Area in east-central Minnesota, U.S.A. In this experiment, plant production increased with plant species richness, an effect that was amplified by CO₂ enrichment and N deposition (Reich et al., 2001a). In addition, soil CO₂ flux increased under elevated CO₂, high N deposition, and greater plant species richness (Craine et al., 2001), and these results suggest that increased plant productivity may change microbial metabolism in soil. I hypothesized that plant species richness will determine microbial responses to elevated CO₂ and N deposition. More diverse plant assemblages will accrue higher plant biomass under CO₂ enrichment and N deposition, and I reasoned that this would provide soil
microorganisms with greater energy for metabolism. I also hypothesized that microbial biomass and extracellular enzyme activity, a measure of metabolic capability, will increase with plant species richness due to greater substrate availability induced by higher rates of plant litter production. My predictions are different from those of Loreau (2001b)'s theoretical model, which suggests that higher diversity of plant organic compounds will reduce nutrient cycling efficiency, and will have negative, or no effect on ecosystem processes mediated by soil microorganisms. To test my hypotheses, I employed PLFA analysis to characterize microbial community composition, and I determined the rates of microbial function by assaying the activity of extracellular enzymes that mediate key functions in plant litter decomposition.

METHODS

Experimental design and sampling procedures

My study was conducted at the BioCON (Biodiversity, CO₂, and Nitrogen) experiment established at the Long Term Ecological Research site in the Cedar Creek Natural History Area in Minnesota, U.S.A (Reich et al., 2001a, 2001b). A split-plot design was employed with CO₂ treatment as the whole-plot, and plant species richness and N treatments as the split-plots. The experiment was established in secondary successional grassland on sandy soil. To destroy the seedbank, plots were tilled and methyl bromide was applied. The soils were then inoculated with unfumigated soil from outside the plots. The experiment consists of six 20-m diameter Free-Air CO₂ Enrichment (FACE) rings. In each FACE ring, there are sixty-one 2-m x 2-m plots. Elevated CO₂ was applied to three rings at the concentration of 560 μL/L, which is ~200 μL/L above ambient CO₂
concentration. Plants were exposed to elevated CO₂ treatment during daylight hours from May to September, and 1-minute averages were within 10% of the target concentration 94% of the time in 1998, and 95% of the time in 1999 (Reich et al., 2001b). In 1997, each 2 m x 2 m plot was planted with 1, 4, 9, or 16 perennial grassland species randomly chosen from 16 locally occurring plant species. In 1999, the average number of plant species in the treatments was 1, 4, 8, and 14 (Reich et al., 2001a). The 16 species used in this experiment were native or have naturalized in the Cedar Creek Natural History Area; they include four C₄ grasses (Andropogon gerardii, Boutelia gracilis, Schizachyrium scoparium, Sorghastrum nutans), four C₃ grasses (Agropyron repens, Bromus inermis, Koeleria cristata, Poa pratensis), four legumes (Amorpha canescens, Lespedeza capitata, Lupinus perennis, Petalostemon villosum), and four forbs (Achillea millefolium, Anemone cylindrica, Asclepias tuberosa, Solidago rigida). Plots were regularly weeded to remove unwanted species. Half of the randomly selected plots were treated with 4 g N m⁻² yr⁻¹ in the form of NH₄NO₃. All plots were burned in the spring of 2000, 2002, and 2003. There was little variation in the mean concentration of elevated CO₂ among plant species richness treatments (560 µL/L under 1-species, 561µL/L under 4-species, 561µL/L under 9-species, and 559 µL/L under 16-species treatments; mean standard deviation was 10 µL/L) or between ambient N and N amended plots (560 µL/L under ambient N and 561µL/L for added N plots; mean standard deviation was 10 µL/L) (Reich et al., 2001a, Supplementary Information).

In July 2003, I collected soil samples from 184 of the 366 plots. There were 64 replicates for 1-species treatment and 40 replicates each of the 4-, 9-, and 16-species
treatments. For the 1-species treatment, there were 16 replicates of each CO₂ by N treatment combination. For 4-, 9-, and 16-species treatments, there were 10 replicates for each CO₂ by N treatment combination. Six soil cores, 2 cm in diameter and 15 cm in depth, were randomly collected from each 2-m x 2-m plot. Cores were composited by plot, immediately frozen, and stored at -80 °C. Soil subsamples were thawed, ground, and analyzed for C and N content using a Flash EA 1112 (ThermoQuest, Austin, TX). The results of soil C and N analysis are summarized in Table 3.1.

**Microbial Community Composition**

**Phospholipid Fatty Acid Analysis:** Lipids were extracted from 5 g of freeze-dried soil with a solvent system that included methanol, chloroform, and a phosphate buffer (Guckert et al., 1985). Total extracted lipids collected in the organic phase were fractionated into neutral, glyco-, and polar lipids with chloroform, acetone, and methanol using silicic acid chromatography (Gehron and White, 1983). Polar lipids were methylated to form fatty acid methyl esters (FAME) by subjecting them to 0.2 M methanolic KOH (White et al., 1979). The resulting FAMEs were analyzed by a Finnigan Delta plus mass spectrometer with a GC/C III interface (ThermoElectron, Austin, TX) connected to a HP 5973 GC (Agilent Technologies, Palo Alto, CA). The recovery rate of FAMEs was calculated based on the amount of an internal standard 21:0 added prior to the analysis and present at the end of the analysis. FAMEs were identified and quantified based on the retention time and peak area of FAME standards.

Bacterial specific PLFAs were i15:0, a15:0, i16:0, 16:1ω7c, 16:1ω9c, 10Me16:0, i17:0, a17:0, cy17:0, 17:0, 18:1ω7c, 18:1ω7t, and cy19:0a (Frostegard et al., 1993;
Table 3.1. Soil C, N, and C:N ratio in BioCON experiment measured in year 2003. Standard errors are shown in parentheses. Within a treatment, means in a column followed by the same letter are not significantly different at $\alpha = 0.05$, as determined by Tukey's HSD test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Soil C (mg C/g)</th>
<th>Soil N (mg N/g)</th>
<th>Soil C:N</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plant species richness</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.19\textsuperscript{a} (0.15)</td>
<td>0.51\textsuperscript{a} (0.01)</td>
<td>12.3\textsuperscript{a} (0.14)</td>
</tr>
<tr>
<td>4</td>
<td>6.31\textsuperscript{a} (0.26)</td>
<td>0.52\textsuperscript{ab} (0.02)</td>
<td>12.3\textsuperscript{a} (0.40)</td>
</tr>
<tr>
<td>9</td>
<td>6.24\textsuperscript{a} (0.21)</td>
<td>0.52\textsuperscript{ab} (0.02)</td>
<td>12.1\textsuperscript{a} (0.23)</td>
</tr>
<tr>
<td>16</td>
<td>6.88\textsuperscript{a} (0.28)</td>
<td>0.57\textsuperscript{b} (0.02)</td>
<td>12.3\textsuperscript{a} (0.40)</td>
</tr>
<tr>
<td><strong>CO\textsubscript{2} treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient</td>
<td>6.48\textsuperscript{a} (0.16)</td>
<td>0.53\textsuperscript{a} (0.01)</td>
<td>12.2\textsuperscript{a} (0.20)</td>
</tr>
<tr>
<td>Elevated</td>
<td>6.28\textsuperscript{a} (0.14)</td>
<td>0.52\textsuperscript{a} (0.01)</td>
<td>12.3\textsuperscript{a} (0.20)</td>
</tr>
<tr>
<td><strong>N treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ambient</td>
<td>6.25\textsuperscript{a} (0.15)</td>
<td>0.51\textsuperscript{a} (0.01)</td>
<td>12.4\textsuperscript{a} (0.20)</td>
</tr>
<tr>
<td>N added</td>
<td>6.48\textsuperscript{a} (0.15)</td>
<td>0.54\textsuperscript{a} (0.01)</td>
<td>12.1\textsuperscript{a} (0.19)</td>
</tr>
</tbody>
</table>
Pennanen et al., 1998; Grayston et al., 2001). Bacterial biomass and relative abundance were calculated using the sum of all the bacterial PLFAs. Relative abundance of bacteria in the microbial community was calculated as the percentage of bacterial biomass comprising total microbial biomass. Biomarkers for saprophytic fungi were PLFAs 18:1ω9c and 18:2ω6 (Bardgett et al., 1996; Stahl and Klug, 1996). The sum of two fungal PLFAs was used to estimate fungal biomass and relative abundance. The PLFA 16:1ω5c can be a biomarker for arbuscular mycorrhizal (AM) fungi (Olsson, 1999; Madan et al., 2002; Olsson et al., 2003), but this interpretation requires some caution because this PLFA can also exist in some bacteria (Olsson, 1999). However, the bacterial concentration of 16:1ω5c is considerably lower than AM fungi, particularly if the soil organic matter content is low (Olsson, 1999). Because soil C content in this study was less than 1%, I used this PLFA to infer the biomass and relative abundance of AM fungi. PLFAs i14:0, 14:0, 15:0, 16:1ω5c, 16:0, 20H-16:0, 18:3ω3, 18:1ω5c, 18:0, 22:0, and 24:0 are common to both bacteria and fungi. Total PLFA was used as an index of living microbial biomass.

**Microbial Community Function**

To gain insight into microbial community function, I performed fluorometric assays using methylumbelliferone (MUB) linked substrates to determine the activities of 1,4-β-glucosidase, cellulbiohydrolase, 1,4-β-N-acetylg glucosaminidase, and phosphatase, which are enzymes that mediate key functions during the microbial degradation of litter (Saiya-Cork et al., 2002). 1,4-β-glucosidase and cellobiohydrolase are cellulose-degrading enzymes. 1,4-β-N-acetylg glucosaminidase decomposes chitin, and acid phosphatase
degrades organic P. Peroxidase and phenol oxidase are lignin-degrading enzymes. Two grams of each composite soil sample were combined with 125 mL of sodium acetate buffer (pH 5.0). The soil slurry was loaded on a 96-well microplate and there were eight replicates of each enzyme assay. For each enzyme assay, I combined 200 μL of soil slurry and 50 μL of substrate specific for each enzyme. All enzyme assays were incubated at 21 °C. The incubation time was 0.5 hr for the phosphatase and 1,4-β-N-acetylglucosaminidase assays, and 2 hr for the 1,4-β-glucosidase and cellobiohydrolase assays. I determined fluorescence using an f-Max fluorometer (Molecular Devices Corp., Sunnydale, CA); excitation energy was 355 nm and emission was measured at 460 nm. Enzyme activity is expressed as nmol 4-MUB g⁻¹ h⁻¹.

The activity of phenol oxidase and peroxidase was measured using 25 mM L-3,4-dihydroxy-phenylalanine (L-DOPA) as the substrate (Saiya-Cork et al., 2002). Procedures for the colorimetric assay were similar to those of the fluorometric assay described above. Phenol oxidase and peroxidase assays each had sixteen replicates for each soil sample. The 96-well microplates were incubated for 24 hr at 21 °C, and absorbance was measured at 450 nm on EL-800 plate reader (Biotek Instruments, Inc., Winooski, VT). Activity was expressed in nmol L-DOPA converted g⁻¹ h⁻¹.

**Statistical analyses**

I analyzed PLFA and enzyme activities using ANCOVA for a split-plot design. The fixed effects in this model were block, plant species richness, CO₂, and N. Carbon dioxide treatment was the whole-plot factor, and the sub-plot factors were plant species richness and N treatment. In a neighboring plant diversity experiment at this site, plant diversity
effects could be partly explained by legume presence (Tilman et al., 2001). In the BioCON experiment, the presence of two dominant legume species Lespedeza capitata and Lupinus perennis significantly increased aboveground biomass (Craine et al., 2003). Moreover, analysis of plant monocultures showed that these two legume species had significantly greater total plant biomass and total plant N under elevated CO₂, and tissue N concentration in legume was roughly 2 times higher than other plant functional groups (Reich et al., 2001b). Therefore, I used legume proportion of the plant aboveground biomass as a covariate to account for the unique role and litter biochemistry of this functional group. I also used the relative abundance of C4 plants as a covariate to adjust for their distinct characteristics. C4 plants have significantly higher C:N ratio and lignin concentration than C3 plants (Wedin and Tilman, 1990; Wedin et al., 1995; Murphy et al., 2002), and two species from this group, Schizachyrium scoparium and Sorghastrum nutans partly accounted for the plant diversity effect in another plant diversity experiment at Cedar Creek Natural History Area (Tilman et al., 2001). Total plant biomass was used as a covariate to adjust for the effects of plant production on microbial community composition and function. Significant effects of plant species richness, CO₂, and N treatments and their interaction were accepted at α = 0.05. Tukey’s honestly significant difference (HSD) test was performed to assess which group means differ from other means within the group (P < 0.05).
RESULTS

Plant species richness, CO$_2$, and N interacted to influence living microbial biomass, fungal relative abundance, and bacterial relative abundance (Table 3.2, Fig 3.1). Total microbial biomass and fungal relative abundance had the tendency to increase with higher plant species richness under the combinations of elevated CO$_2$ and ambient N deposition, and ambient CO$_2$ and experimental N deposition. However, this trend diminished under both ambient CO$_2$ and ambient N, and both elevated CO$_2$ and added N. There was no clearly interpretable trend in bacterial relative abundance related to the three-way interaction (Fig 3.1). Carbon dioxide and N had significant interactive effects on living microbial biomass ($P = 0.05$). For example, microbial biomass displayed no response to N addition under ambient CO$_2$ (77.9 ± 5.2 nmol PLFA/g under ambient N vs. 78.9 ± 6.6 nmol PLFA/g under added N), whereas N amendment decreased microbial biomass by 18% under elevated CO$_2$ (83.7 ± 7.2 nmol PLFA/g under ambient N vs. 69.0 ± 5.0 nmol PLFA/g under added N). Microbial community composition was not significantly influenced by any two-way interaction between treatments (Table 3.2).

Carbon dioxide enrichment did not alter microbial community composition as a main effect. For example, elevated CO$_2$ had no significant effect on fungal relative abundance, bacterial relative abundance, or arbuscular mycorrhizal (AM) relative abundance (Table 3.2). Nitrogen amendment reduced AM relative abundance by 9% ($P = 0.05$; 7.5 ± 0.3% under ambient N vs. 6.8 ± 0.2% under added N), but had no main effect on microbial biomass, fungal relative abundance, or bacterial relative abundance (Table 3.2).
Table 3.2. Influence of elevated CO₂, N addition, and plant species richness on microbial biomass and community composition. P-values for total microbial biomass and community composition analyzed by analysis of covariance (ANCOVA) using legume relative abundance as covariate are shown. P-values equal to or lower than 0.05 are in bold face print.

<table>
<thead>
<tr>
<th></th>
<th>Total microbial biomass</th>
<th>Fungal relative abundance</th>
<th>Bacterial relative abundance</th>
<th>AM relative abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂</td>
<td>0.85</td>
<td>0.25</td>
<td>0.07</td>
<td>0.44</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>0.23</td>
<td>0.78</td>
<td>0.18</td>
<td>0.05</td>
</tr>
<tr>
<td>Species richness</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>0.50</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>CO₂ x Species richness</td>
<td>0.68</td>
<td>0.90</td>
<td>0.85</td>
<td>0.90</td>
</tr>
<tr>
<td>Nitrogen x Species richness</td>
<td>0.99</td>
<td>0.70</td>
<td>0.92</td>
<td>0.93</td>
</tr>
<tr>
<td>CO₂ x Nitrogen</td>
<td>0.05</td>
<td>0.55</td>
<td>0.68</td>
<td>0.11</td>
</tr>
<tr>
<td>CO₂ x Nitrogen x Species richness</td>
<td>0.05</td>
<td>&lt; 0.01</td>
<td>0.05</td>
<td>0.52</td>
</tr>
<tr>
<td>Legume relative abundance</td>
<td>0.04</td>
<td>0.01</td>
<td>0.05</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>
Figure 3.1. Interactive effects of elevated CO₂, experimental N deposition, and plant species richness on microbial biomass and community composition. Error bars indicate one standard error of the mean.
As a main effect, plant species richness significantly increased total microbial biomass, fungal relative abundance, and AM relative abundance (Table 3.2). Living microbial biomass in the 16-species treatment was significantly greater than in the 1-species treatment (Fig 3.2). Fungal (P < 0.01) and AM (P < 0.01) relative abundance increased significantly with plant species richness, and the pattern was similar to the increase in living microbial biomass (Fig 3.2). The relative abundance of both fungal biomarkers 18:1ω9c (P < 0.01) and 18:2ω6 (P < 0.01) significantly increased under greater plant species richness (data not shown). For example, relative abundance of 18:1ω9c was 5.5 ± 0.1% under the 1-species treatment, whereas it was 6.0 ± 0.1% under the 16-species treatment. Relative abundance of 18:2ω6 was 2.1 ± 0.1% under the 1-species treatment, and it was 2.5 ± 0.2% under the 16-species treatment.

The relative abundance of the bacterial group, as a whole, was not different among plant species richness treatments (Table 3.2, Fig 3.2). However, at the individual PLFA level, the relative abundance of bacterial biomarkers 16:1ω7c (P < 0.01), cy17:0 (P = 0.02), and 18:1ω7c (P < 0.01) increased, whereas the relative abundance of i15:0 (P < 0.01), a15:0 (P = 0.04), i16:0 (P < 0.01), and 10Me16:0 (P < 0.01) decreased with increasing plant species richness (data not shown). Legume relative abundance, but not total plant biomass (P = 0.15–0.98), was a significant covariate for total microbial biomass and microbial community composition. The relative abundance of C4 plants was a significant covariate for fungal (P = 0.04), bacterial (P = 0.01), and AM (P < 0.01) relative abundance.
Figure 3.2. The influence of plant species richness on microbial biomass and community composition. PLFA values were averaged across CO₂ and N treatments. Error bars indicate one standard error of the mean. Means with the same letter are not significantly different at α = 0.05, as determined by Tukey’s HSD test.
There was no three-way interaction among the plant species richness, CO$_2$, and N treatments on the activity of any extracellular enzymes. Extracellular enzyme activity also was not significantly affected by any two-way interaction between treatments. Elevated CO$_2$ had no main effect on the activity of any enzymes (Table 3.3). As a main effect, N treatment significantly increased the activity of 1,4-β-glucosidase by 18% and that of phosphatase by 26% (Fig 3.3). Plant species richness significantly increased 1,4-β-glucosidase, cellobiohydrolase, and 1,4-N-acetylglucosaminidase activity. In the 16-species treatment, 1,4-β-glucosidase (23%), cellobiohydrolase (75%), and 1,4-N-acetylglucosaminidase (20%) activity was significantly higher than in the 1-species treatment. Activities of phosphatase, peroxidase, and phenol oxidase were not significantly influenced by plant species richness (Table 3.3, Fig. 3.4). Legume relative abundance was a significant covariate for 1,4-N-acetylglucosaminidase. Total plant biomass was a significant covariate for phosphatase (P = 0.01), but not for other extracellular enzymes (P = 0.16–1.00). The relative abundance of C4 plants was a significant covariate for peroxidase activity (P = 0.05).

**DISCUSSION**

Concurrent changes in plant diversity and atmospheric chemistry driven by human activities have the potential to interact and alter nutrient cycling in terrestrial ecosystems (Niklaus et al., 2001a; Shaw et al., 2002). In my study, plant species richness, elevated CO$_2$, and experimental N deposition significantly interacted to alter microbial biomass and community composition, but these changes did not propagate to alter microbial
Table 3.3. Influence of elevated CO₂, N addition, and plant species richness on microbial enzyme activity. P-values for extracellular enzyme activities analyzed by analysis of covariance (ANCOVA) using legume relative abundance as covariate are shown. P-values equal to or lower than 0.05 are in bold face print.

<table>
<thead>
<tr>
<th></th>
<th>1,4-β-glucosidase</th>
<th>Cellobio-hydrolase</th>
<th>1,4-β-N-acetyl-glucosaminidase</th>
<th>Phosphatase</th>
<th>Peroxidase</th>
<th>Phenol oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂</td>
<td>0.53</td>
<td>0.53</td>
<td>0.53</td>
<td>0.14</td>
<td>0.63</td>
<td>0.47</td>
</tr>
<tr>
<td>Nitrogen</td>
<td><strong>0.01</strong></td>
<td>0.66</td>
<td>0.62</td>
<td><strong>0.01</strong></td>
<td>0.23</td>
<td>0.43</td>
</tr>
<tr>
<td>Species richness</td>
<td><strong>0.03</strong></td>
<td>&lt; <strong>0.01</strong></td>
<td><strong>0.01</strong></td>
<td>0.18</td>
<td>0.19</td>
<td>0.96</td>
</tr>
<tr>
<td>CO₂ x Species richness</td>
<td>0.97</td>
<td>0.36</td>
<td>0.74</td>
<td>0.96</td>
<td>0.68</td>
<td>0.32</td>
</tr>
<tr>
<td>Nitrogen x Species richness</td>
<td>0.15</td>
<td>0.37</td>
<td>0.60</td>
<td>0.56</td>
<td>0.12</td>
<td>0.68</td>
</tr>
<tr>
<td>CO₂ x Nitrogen</td>
<td>0.80</td>
<td>0.46</td>
<td>0.30</td>
<td>0.86</td>
<td>0.76</td>
<td>0.92</td>
</tr>
<tr>
<td>CO₂ x Nitrogen x Species richness</td>
<td>0.37</td>
<td>0.45</td>
<td>0.29</td>
<td>0.64</td>
<td>0.79</td>
<td>0.12</td>
</tr>
<tr>
<td>Legume relative abundance</td>
<td>0.55</td>
<td>0.72</td>
<td><strong>0.05</strong></td>
<td>0.12</td>
<td>0.21</td>
<td>0.79</td>
</tr>
</tbody>
</table>
Figure 3.3. The influence of N addition on 1,4-β-glucosidase and phosphatase activity. Enzyme activities were averaged across plant species richness and CO₂ treatments. Error bars indicate one standard error of the mean.
Figure 3.4. The influence of plant species richness on extracellular enzyme activity. Enzyme activities were averaged across CO$_2$ and N treatment. Error bars indicate one standard error of the mean. Means with the same letter are not significantly different at $\alpha = 0.05$, as determined by Tukey's HSD test.
degradative potential. On the other hand, plant species richness and N addition exerted important but non-interacting effects on both microbial community composition and function. These results imply that soil microbial function may not be largely affected by interactive effects of global changes that directly modify plant production. Higher plant species richness increased microbial biomass, fungal relative abundance, AM relative abundance, and cellulolytic capacity in microbial communities, and this suggests that plant species richness could have significant influence on microbial community composition and the rate of cellulose decomposition. Enhanced β-glucosidase and phosphatase activity in N amended soil further indicate that added N can stimulate cellulose degradation and increase microbial demand for P.

My results show that organic matter decomposition by soil microorganisms may not be strongly affected by interactions among elevated CO₂, atmospheric N deposition, and plant species richness. Alterations in microbial biomass and community composition by simultaneous changes in the level of atmospheric CO₂, N deposition rate, and plant species richness were not accompanied by changes in microbial degradative potential. The pattern of total microbial biomass and fungal relative abundance did not reflect that of plant production under concurrent changes in CO₂, N, and plant species richness, and analysis of plant litter biochemistry under elevated CO₂, atmospheric N deposition, and decreasing plant species richness may explain alterations in microbial community composition observed in this study. For example, greater plant species richness increased plant production under factorial CO₂ and N treatment (Reich et al., 2001a), but the positive effect of higher plant species richness on total microbial biomass and fungal
relative abundance was eliminated under the combinations of ambient CO$_2$ and ambient N, and elevated CO$_2$ and added N. Current evidence suggests that interactive effects of elevated CO$_2$, N deposition, and decreasing plant species richness may not significantly affect microbial degradative potential, and a long-term plant litter decomposition study is needed to determine if interactive effects will be manifested in long term due to a lag effect.

Decreased total microbial biomass in N amended soil under elevated CO$_2$ is likely to be due to a direct inhibitory effect of N addition on microbial communities, rather than an effect mediated through increased plant production. Carbon dioxide enrichment and N addition had no significant interactive effects on plant production, but the two treatments independently increased plant biomass (Reich et al., 2001b). Therefore, increase in plant biomass in response to N addition under elevated CO$_2$ cannot explain reduced microbial biomass. Although not statistically significant, biomass of both fungi and bacteria were lower in N amended soil under CO$_2$ enrichment (data not shown), which indicates that effect of N addition was not confined to a specific microbial group. Plants grown under elevated CO$_2$ can have higher lignin or phenolic content (Gebauer et al., 1998; Henry et al., 2005), and plants under CO$_2$ enrichment in this experiment had lower N concentration (Reich et al., 2001b; Lee et al., 2003). If plant litter under elevated CO$_2$ had higher lignin:N ratio than that under ambient CO$_2$, N amendment can reduce the biomass of white-rot basidiomycetes that degrade more recalcitrant litter (Fog, 1988). However, N addition alone increased plant N content (Reich et al., 2001a), and none of the extracellular enzymes showed similar pattern with microbial biomass (data not shown),
which makes this type of interpretation equivocal. Therefore, interactive effects of CO₂ and N on microbial biomass warrants further study in conjunction with analysis of plant litter biochemistry.

Total microbial biomass increased with greater plant species richness, and this may have been due to higher substrate availability from greater plant production (Fig 3.5). Although not statistically significant, soil C content in this experiment increased as plant species richness increased, and this is most likely due to higher plant production with increasing species richness (Reich et al., 2001a). Greater soil organic matter content in higher plant species richness treatments may have provided more substrate for microbial metabolism, leading to the observed increase in microbial biomass.

Plant species richness had a significant influence on microbial biomass even after accounting for variation in total aboveground biomass and soil organic matter (data not shown), and this suggests that plant species richness per se can influence microbial biomass. Spehn et al. (2000) also found that microbial biomass increased as plant species richness increased, even when adjusting for variation attributable to plant production. Studies have shown that mixing litter of different plant species either increased or decreased microbial biomass (Wardle et al., 1997; Bardgett and Shine, 1999). Soil microorganisms also derive their energy from root exudates and fine roots which are more labile and have greater turnover than other parts of plants (Berg and McClaugherty, 2003), so it could be the case that plant species richness plays a significant role in determining microbial biomass when active parts of plants as well as plant litter are considered.
Figure 3.5. Main effect of plant species richness on C and N cycling in the BioCON experiment. The figure has been modified from Craine et al., 2003a. The percent difference in each ecological trait between 1-species and 16-species treatments are shown in parentheses.

* P < 0.05
\(^a\) Reich et al., 2001a
\(^b\) Dijkstra et al., 2004
\(^c\) Craine et al., 2001
Plant species in this experiment each have unique phenology, physiology, and morphology (Craine et al., 2003a; Craine et al., 2003b), and this diversity in plant traits under higher species richness treatments may have provided microbial communities with more resources. Plant communities of higher diversity can utilize space more completely due to interspecific differences than plant communities of lower diversity (Tilman, 1999). For instance, there was up to a 40-fold difference in fine root biomass among plant species in this study, and plants differed significantly in their distribution of fine roots throughout the soil profile (Craine et al., 2003a). Due to the complementary soil occupation of roots by diverse plant species, energy for soil microorganisms can be supplied more homogeneously in the soil volume. Also, differences in phenology of plant species led to lower variation in plant production in the species-rich experimental communities (Craine et al., 2003b). This implies that microorganisms may derive energy from plants at a more consistent level with smaller temporal fluctuation.

Increases in total microbial biomass could explain lower net N mineralization rate under species-rich plant communities (Reich et al., 2001a). Net N mineralization was 68% lower in the 16-species treatment than under the 1-species treatment (Reich et al., 2001a), and this could have been due to higher microbial demand for N in response to greater C availability in species-rich plant communities. Although the proportion of legume increased with plant species richness (Reich et al., unpublished data), there was a 26% decrease in plant N concentration in the 16-species treatment when compared to that under the 1-species treatment (Reich et al., 2001a; Reich et al., 2004). Higher rates of N
immobilization by soil microorganisms may have reduced N availability for plants, and this could negatively affect plant production in long-term (Fig 3.5).

Greater plant species richness increased fungal relative abundance, and these results are consistent with other studies (Smith et al., 2003; Zak et al., 2003). In a neighboring grassland diversity study, greater fungal relative abundance with higher plant species richness could not be explained by changes in soil pH or water potential, which indicates that plant species richness per se can enhance fungal relative abundance (Zak et al., 2003). Smith et al. (2003) have found that cessation of NPK fertilizer use and seed addition increased plant species richness in a grassland ecosystem, which was primarily due to increase in legumes and stress-tolerant plants. Moreover, fungal relative abundance increased with greater plant species richness (Smith et al., 2003). Other studies in this ecosystem also found that shift from high fertilizer input to unfertilized management increases plant diversity, and this change in vegetation was accompanied by higher fungal relative abundance (Bardgett et al., 1996; Bardgett et al., 1999). Because increase in plant diversity in these studies was in response to changes in management practices that could affect soil environment (e.g. soil pH and soil water availability), it is not clear whether plant diversity alone increased fungal abundance. Moreover, the effect of plant species or functional group on microbial community composition was not explicitly tested. Because soil pH or water potential was not measured in the BioCON experiment, it remains to be determined whether changes in soil environment associated with increasing plant species richness fosters fungal abundance.
The significant influence of legumes on microbial communities could be due to the ability of legumes to fix N2 and the labile nature of legume litter (Marschner et al., 2004). Legume proportion increased with plant species richness in the BioCON experiment (Reich et al., unpublished data) and legume relative abundance was a significant covariate for fungal relative abundance, but the effect of plant species richness still remained significant. Legumes can increase N availability for neighboring plants through leaching and decomposition of plant litter high in N, and increased plant production in higher plant diversity communities could be partially explained by the presence of legumes (Tilman et al., 2001; Mulder et al., 2002; Spehn et al., 2002). *Lupinus* was one of the four most productive plant species in the BioCON experiment (Reich et al., 2001a), and the presence of two dominant legumes *Lupinus* and *Lespedeza* had significant influence on plant response to CO2 enrichment and N addition (Reich et al., 2001b; Craine et al., 2003; Lee et al., 2003). Therefore, legumes could have affected soil microbial communities through their influence on plant production, or legume litter could have hastened microbial litter degradation. However, legume abundance was a significant covariate for only N-acetylglucosaminidase activity; this interpretation should be tempered by caution.

Soil microbial communities could be significantly affected by C4 plants due to the unique biochemical composition of this plant functional group (Wedin and Tilman, 1990; Knops and Tilman, 2000). Although *Schizachyrium* and *Sorghastrum* were among the most abundant plant species in a neighboring plant diversity experiment (Tilman et al., 2001), none of the C4 plants were dominant species in the BioCON experiment (Reich et al.,
2001a). Therefore, high lignin concentration and C:N ratio of C4 plant litter, rather than production of this plant functional group, is likely to influence microbial community composition and function. Bacterial relative abundance was negatively correlated with C4 abundance (data not shown), and this indicates that high recalcitrance of C4 plant litter may decrease bacterial abundance because bacteria are more adapted to decomposing labile plant litter than fungi (Paul and Clark, 1996). In Cedar Creek, C4 grasses increased soil C:N (Knops and Tilman, 2000), and this further supports my argument that C4 plants may negatively affect bacterial population through the input of low quality substrate to soil. Significant influence of C4 grasses on peroxidase activity is likely due to high lignin content in this plant functional group.

Greater fungal abundance could be due to a higher cellulose concentration in plant litter of species-rich communities, because fungi are major degraders of cellulose in soil (Lynd et al., 2002). In a neighboring plant diversity study, cellulose content in plant litter increased with greater plant species richness (Waldrop et al., unpublished data). If this is also the case in the BioCON experiment, this change in plant litter biochemistry could favor fungal abundance. In the 16-species treatment, fungal (45%), bacterial (38%), and AM (62%) biomass were higher than in the 1-species treatment, which indicates that all microbial groups contribute to increase in total microbial biomass. However, higher plant species richness preferentially increased the abundance of fungi and AM abundance over bacteria. Activity of N-acetylglucosaminidase increased with higher plant species richness, and this may be due to increase in fungal and arbuscular mycorrhizal abundance. Miller et al. (1998) have found significant positive correlation between N-
acetylglucosaminidase activity and fungal biomass, and the activity of this chitin-degrading enzyme could have increased due to greater fungal abundance in species-rich plant communities.

Arbuscular mycorrhizal fungi are the dominant plant symbionts in this experiment (Wolf et al., 2003), and increase in AM abundance with greater plant species richness could be due to higher root biomass in species-rich plant communities. Although the results were not significant, Wolf et al. (2003) also found higher AM spore abundance and spore biovolume under 16-species treatment than under 1-species treatment. Zak et al. (2003) also found increase in the relative abundance of 16:1ω5c along with greater total N content in plant biomass as plant species richness increased, which indicate that greater AM abundance could have facilitated N acquisition in species-rich plant communities, leading to greater plant production. Therefore, greater AM abundance and root biomass in species-rich plant communities could have facilitated the plant N acquisition (Fig 3.5).

An increase in cellulolytic potential with greater plant species richness is likely to be due to higher plant production and cellulose content in plant litter. Cellulose is the most common component of plant litter, and can constitute between 10 and 50% of plant litter mass (Berg and McClaugherty, 2003). Because the production of cellulose-degrading enzymes is induced by the amount of substrate available (Lynd et al., 2002), increased plant production in response to greater species richness may have resulted in higher 1,4-β-glucosidase and cellobiohydrolase activity. Plants in this study allocate more than 70% of total biomass below the soil surface (Craine et al., 2003a), and root biomass was significantly greater in the 16-species treatment (Reich et al., 2001a). Higher
substrate input to soil through labile parts of plants such as fine roots in the species-rich communities may have increased 1,4-β-glucosidase and cellobiohydrolase activity. Also, because fungi are dominant cellulase producers in soil (Lynd et al., 2002), greater fungal relative abundance under the higher species richness treatments could have led to increased cellulolytic potential. A 21% increase in soil CO₂ flux (Craine et al., 2001) under 16-species treatment than under 1-species treatment could also be due to greater cellulolytic capacity accompanied by higher root biomass (Fig 3.5).

The lack of a CO₂ effect on soil microbial communities may be attributed to a small increase in plant production under elevated CO₂. Other than increasing plant biomass and soil CO₂ flux, elevated CO₂ had no significant main effect on degradative potential, N mineralization, or microbial community composition (Fig 3.6). Photosynthesis of plants in the BioCON experiment was stimulated to a smaller degree than plants of other elevated CO₂ studies (Lee et al., 2001; Reich et al., 2001b), and all of the plant functional groups significantly acclimated to CO₂ enrichment (Lee et al., 2001).

Therefore, a 15% increase in soil CO₂ flux under elevated CO₂ (Craine et al., 2001) could have been mainly due to increase in belowground production and may not be attributed to changes in microbial activity (Fig 3.6).

Addition of N significantly enhanced 1,4-β-glucosidase activity, and this is consistent with a greater soil CO₂ flux in N amended soil (Craine et al., 2001). Nitrogen amendment can enhance the degradation of labile parts of litter, but repress ligninolytic enzyme activity of white-rot basidiomycetes (Fog, 1988; Carreiro et al., 2000). Nitrogen addition repressed phenol oxidase activity by 18%, which is consistent with previous
Figure 3.6. Main effect of elevated CO₂ on C and N cycling in the BioCON experiment. The figure has been modified from Craine et al., 2003a. The percent difference in each ecological trait between ambient and elevated CO₂ are shown in parentheses.

* P < 0.05

² Reich et al., 2001a
³ Dijkstra et al., 2004
⁴ Craine et al., 2001
studies; however, this reduction was not significant (Fig 3.7). Although not statistically significant, both labile and recalcitrant soil C fractions increased in N amended soil (Dijkstra et al., 2004), and this indicates that greater plant production and a decline in ligninolytic potential together could lead to greater soil C storage (Fig 3.7). Greater phosphatase activity is likely due to a higher demand microbial demand for P in N amended soil. Johnson et al. (1998) found that phosphomonoesterase activity and utilization of organic P increased in response to N addition, and a Hawaiian ecosystem study also showed that N amendment increased phosphatase activity in N-limited soils (Olander and Vitousek, 2000). A significant decrease in AM abundance, which could have been due to increased soil N availability, also indicate that N is less limiting in N amended soil. Altogether, N addition is likely to have affected microbial communities directly rather than through increased plant input (Fig 3.7).

I have found that plant species richness is a significant factor that determines microbial community composition and function, and potential rates of litter degradation. Increased plant production and cellulose content in plant litter enhanced microbial biomass, fungal abundance, and AM abundance, and this change in microbial community composition was accompanied by greater cellulytic and chitin-degrading activity. In most cases, plant species richness consistently had a strong positive influence on microbial communities regardless of atmospheric CO₂ or soil N availability. This indicates that the composition and processes mediated by soil microbial communities will be more strongly affected by declining plant species richness than by elevated CO₂ or atmospheric N deposition. The observation that plant species richness fostered a higher
Figure 3.7. Main effect of N addition on C and N cycling in the BioCON experiment. The figure has been modified from Craine et al., 2003a. The percent difference in each ecological trait between ambient and added N are shown in parentheses.

* P < 0.05
a Reich et al., 2001a
b Dijkstra et al., 2004
c Craine et al., 2001
rate of cellulose degradation suggests that rates of soil C cycling may decrease as plant species richness declines in an ecosystem.
LITERATURE CITED


CHAPTER 4
MICROBIAL ASSIMILATION OF NEW PHOTOSYNTHATE UNDER N DEPOSITION AND DECREASING PLANT SPECIES RICHNESS

ABSTRACT

Due to human activity, terrestrial ecosystems are subject to concurrent increases in atmospheric N deposition and the loss of plant species. Soil microbial metabolism and community composition may be altered by these global environmental changes, but studies that have analyzed their interactive effects on soil microbial communities are rare. In the BioCON (Biodiversity, CO₂, and N) experiment, in which plant species richness, atmospheric CO₂ concentration, and N availability are manipulated in concert, I investigated the potential interaction of N deposition and plant species richness on microbial community composition and function. Experimental plant communities composed of 1, 4, 9, and 16 grassland species were subjected to factorial treatments of N deposition (ambient and 4 g N m⁻² yr⁻¹) and atmospheric CO₂ (ambient and 560 μL/L of CO₂). The depleted δ¹³C of fumigation CO₂ enabled me to determine how increased N availability and declines in plant species richness interact to modify the assimilation of ‘new’ photosynthate (C assimilated by plants after the initiation of the experiment) by soil microorganisms. In this experiment, plants accumulated greater biomass in response to N deposition and with increasing plant species richness. Because heterotrophic growth in soil is limited by organic substrate availability, I hypothesized that higher plant litter
production under N deposition and greater plant species richness will increase the metabolism of recently produced photosynthate by soil microbial communities, thereby lowering the δ¹³C of microbial PLFAs.

Under ambient N, plant species richness had the tendency to increase the relative abundance of 18:2o6, which is a biomarker for fungi. However, N deposition eliminated this effect, and 18:2o6 relative abundance was not different across plant species richness treatments. This indicates that N addition and plant species richness can interact to alter fungal abundance. Nitrogen addition as a main effect significantly decreased total microbial biomass (88.1 ± 8.4 nmol PLFA/g under ambient N vs. 69.4 ± 5.4 nmol PLFA/g under added N). Although the result was not significant, N addition decreased phenol oxidase activity (208.4 ± 29.5 nmol g⁻¹h⁻¹ under ambient N vs. 167.2 ± 17.3 nmol g⁻¹h⁻¹ under added N), which indicates that N addition could have inhibited microbial access to soil organic matter, leading to lower microbial biomass. δ¹³C of microbial PLFAs was significantly lower in species-rich plant communities (2.1 to 3.0% lower under 16-species treatment than under 1-species treatment), which suggests that microbial assimilation of new C depleted in ¹³C increased with greater plant species richness. Furthermore, plant species richness increased total microbial biomass (62.6 ± 6.4 nmol PLFA/g under 1-species treatment vs. 97.1 ± 8.4 nmol PLFA/g under 16-species treatment), and cellubiohydrolyase activity (3.6 ± 0.4 nmol g⁻¹h⁻¹ under 1-species treatment vs. 6.5 ± 1.1 nmol g⁻¹h⁻¹ under 16-species treatment). Taken together, these results suggest that higher plant species richness enhanced soil microbial metabolism via greater substrate availability. This study suggests that considering combined effects of N
deposition and plant species richness is important in determining microbial community composition under simultaneous increase in N deposition and declining plant species richness, and that microbial activity in soil could decrease with plant species loss.

INTRODUCTION

Human activities have doubled the amount of N entering terrestrial ecosystems via increased fossil fuel burning, fertilizer, and legume production (Galloway et al., 1995; Matthews, 1994). In eastern North America, atmospheric N deposition has increased tenfold to current levels of 0.5 to 2.0 g N m\(^{-2}\) yr\(^{-1}\) (Galloway et al., 1995). At the same time, plant species are disappearing at an unprecedented rate, mainly due to land-use conversion (Pimm et al., 1995; Purvis and Hector, 2000). Because the productivity of terrestrial ecosystems in temperate regions is generally limited by soil N availability (Vitousek and Howarth, 1991), N deposition can significantly alter ecosystem function; greater N deposition rates can stimulate plant growth, change plant species composition, and alter litter degradation rates (Wedin and Tilman, 1996; Fenn et al., 1998; Norby, 1998; Carreiro et al., 2000). On the other hand, studies have shown that plant diversity is positively related to ecosystem function, as evidenced by increase in plant production, N mineralization rate, ecosystem stability, and resistance (Tilman, 1996; Hector et al., 1999; McCann, 2000; Tilman et al., 2001; Kennedy et al., 2002; Zak et al., 2003). Because ecosystems subjected to N deposition are concurrently experiencing the loss of plant species, it is important to understand the combined impacts of atmospheric N deposition and plant species richness on soil C and N cycling (Vitousek et al., 1997a).
Greater N deposition and plant species loss have the potential to change the composition and activity of soil microbial communities by altering the production and biochemistry of plant litter. Soil microorganisms and plants are dependent on each other for the supply of limiting resources; microorganisms make nutrients available for plants through the balance of immobilization and mineralization, and plants serve as the primary source of energy for heterotrophic microorganisms. Increased plant input to soil and higher N concentration in litter under N deposition can stimulate microbial activity, whereas lower plant production and changes in litter biochemistry under declining plant species richness could alter microbial metabolism. Therefore, atmospheric N deposition and reductions in plant species richness can change the rate of soil C cycling mediated by soil microorganisms via change in organic substrate availability, and this could potentially feedback to alter plant productivity.

Nitrogen addition also can directly affect microbial metabolism by biological and chemical mechanisms. Increased soil N availability can enhance or suppress litter-degrading enzyme activity (Carreiro et al., 2000; Saiya-Cork et al., 2002). Greater N input to soil can hasten the decomposition of labile litter, but inhibit lignin-degrading enzymes produced by white-rot basidiomycetes (Fog, 1988; Carreiro et al., 2000; Saiya-Cork et al., 2002). Nitrogen additions also can decrease the rate of C cycling by forming highly resistant complexes with phenolic compounds and quinones that are by-products of lignin degradation (Berg and McLaugherty, 2003). Although N deposition has the potential to alter C cycling in terrestrial ecosystems (Vitousek et al, 1997b), I am unaware
of studies that have examined the interactive effects of N addition and plant species richness on microbial communities that mediate soil C cycling.

The objective of my study was to determine the combined impacts of N deposition and declining plant species richness on the metabolism of new photosynthate by soil microorganisms. In a grassland experiment in which interactions among elevated CO₂, N deposition, and plant species richness on ecosystem functions were studied, physiological differences in plant species led to complementary C and N acquisition (Reich et al., 2001b; Craine et al., 2003; Reich et al., 2004). I reasoned that interactive effects of N deposition and plant species richness could significantly alter microbial community composition and function in soil through changes in plant production and litter biochemistry. Due to ¹³C-depleted CO₂ fumigation gas used in this experiment, photosynthate under elevated CO₂ has significantly lower δ¹³C (−37.2 ± 0.0‰) than that under ambient CO₂ (−23.0 ± 0.1‰) (Dijkstra et al., 2004). The ¹³C-depleted feature of the new photosynthate is propagated to the soil food web upon release via aboveground litterfall and rhizodeposition, and heterotrophic microbial communities that metabolize plant litter will retain the depleted signature (Niklaus et al., 2001). This feature allowed me to determine the extent to which soil microbial communities metabolized recent photosynthate versus older forms of soil organic matter (Burke et al., 2003). I traced the fate of new photosynthate into microbial groups using the δ¹³C of phospholipid fatty acid analysis. Stable isotope analysis of microbial PLFAs can be a powerful tool to study C utilization in microbial communities, because this method links identity with metabolic activity (Abrahm et al., 1998; Boschker et al., 1998; Treonis et al., 2004).
I hypothesized that microbial communities will assimilate more new photosynthate, will have a greater biomass, and higher extracellular enzyme activity in soil receiving N deposition compared to microbial communities under ambient N deposition. I also hypothesized that increased plant production under higher plant species richness will promote faster rates of microbial metabolism and support greater microbial biomass. To gain insight into microbial metabolism, I measured the δ¹³C of PLFAs and the activity of extracellular enzymes involved in the process of plant litter degradation.

**METHODS**

**Experimental design and sampling procedures**

I implemented my study at the BioCON (Biodiversity, CO₂, and Nitrogen) experiment situated at the Long Term Ecological Research site in the Cedar Creek Natural History Area, Minnesota, U.S.A. The experiment was organized in a split-plot design in which CO₂ treatment was the whole-plot, and plant species richness and N addition were the split-plot factors (for more details see Reich et al., 2001a, 2001b). There were a total of six 20-m diameter FACE rings, and sixty-one 2-m x 2-m plots within each ring. Three experimental rings received elevated CO₂ treatment at the concentration of 560 μL/L, a concentration likely to be reached at the end of this century (Intergovernmental Panel on Climate Change, 2001). The 5 minute average of CO₂ concentration was within 5% of the target concentration for 92-93% of the time during daylight hours from May to September (Reich et al., 2001a). The other three rings were exposed to ambient level of CO₂. The δ¹³C of elevated CO₂ gas mixture was -19.5%, and it was -8.3% for the ambient atmosphere (Ellsworth, unpublished data).
Plots were tilled and treated with methyl bromide to destroy the existing seedbank, and the soils were inoculated with untreated soil from outside the plots. In 1997, each 2-m x 2-m plot was planted with 1, 4, 9, or 16 plant species randomly selected from a pool of 16 perennial grassland species. The average plant species richness was 1, 4, 8, and 14, based on the four aboveground harvests during 1998-1999 (Reich et al, 2001a). These plant species were native or have naturalized to the Cedar Creek Natural History Area. There were four C4 grasses (Andropogon gerardii, Boutelua gracilis, Schizachyrium scoparium, Sorghastrum nutans), four C3 grasses (Agropyron repens, Bromus inermis, Koeleria cristata, Poa pratensis), four legumes (Amorpha canescens, Lespedeza capitata, Lupinus perennis, Petalostemum villosum), and four forbs (Achillea millefolium, Anemone cylindrica, Asclepias tuberosa, Solidago rigida). Plant species richness treatments were maintained by regularly weeding unwanted species. Plots at each plant species richness level were exposed to a factorial CO2 and N treatments. For N treatment, NH4NO3 was applied at the rate of 4 g N m⁻² yr⁻¹.

In July 2003, I collected six soil cores that were 2 cm in diameter and 15 cm in depth from each plot. Cores were composited by plot, immediately frozen, and stored at -80 °C prior to PLFA and extracellular enzyme analysis. In my analysis, I sampled plots only in the elevated CO2 treatment to take advantage of the 13C tracer. I collected soil samples under 1- (n = 32), 4- (n = 20), 9- (n = 20), and 16-species (n = 20) treatments. Half of the plots from each species richness level received N addition. Soil subsamples were ground, and the C and N content was determined using a Flash EA 1112 (ThermoQuest, Austin, TX). Although the δ13C of C3 and C4 plants varies (Wedin et al.,
1995), I included plots with both C3 and C4 plants in my analysis because the proportion of C3 biomass was not significantly different among the plant species richness treatments (70% in 1-species, 69% and 4-species, 68% in 9-species, and 83% in 16-species treatments; P = 0.66). Plant $\delta^{13}$C under ambient CO$_2$ was $-23.0 \pm 0.1\%$ and it was $-37.2 \pm 0.0\%$ under elevated CO$_2$ (Dijkstra et al., 2004). The $\delta^{13}$C of soil C in the bare plots was $-23.6 \pm 0.1\%$ (Dijkstra et al., 2004). Plant and soil characteristics in plots analyzed in my study are summarized in Tables 4.1 and 4.2. Because there was no significant interaction between N addition and plant species richness (P values were > 0.05 for all characteristics analyzed), only main effects of N and plant species richness are shown.

**Phospholipid Fatty Acid (PLFA) Analysis**

I freeze-dried 5 g of soil from each soil composite, and extracted lipids from the soil using a solvent system composed of methanol, chloroform and a phosphate buffer (Guckert et al., 1985). Total extracted lipids were separated into neutral, glyco-, and polar lipids with chloroform, acetone, and methanol. Silicic acid chromatography was used to collect polar lipids. I converted polar lipids to fatty acid methyl esters (FAME) by subjecting them to 0.2 M methanolic KOH. Mild alkaline methanolysis cleaves fatty acids from the glycerol backbone and adds a methyl group to each PLFA (White et al., 1979). The resulting FAMEs were analyzed for their concentration and $\delta^{13}$C employing a Finnigan Delta plus mass spectrometer with a GC/C III interface (ThermoElectron, Austin, TX) coupled to a HP 5973 GC (Agilent Technologies, Palo Alto, CA). I used 21:0 of known concentration as an internal standard to calculate the recovery rate of FAMEs. The retention time and concentration of each FAME were determined using a
Table 4.1. Main effect of N addition on plant and soil characteristics (mean ± 1 SE) under elevated CO₂ in the BioCON experiment. NA: data not available.

<table>
<thead>
<tr>
<th></th>
<th>Ambient N</th>
<th>Added N</th>
<th>% difference&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td><strong>Plant</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total biomass&lt;sup&gt;a&lt;/sup&gt; (g m⁻²)</td>
<td>674 ± 44</td>
<td>867 ± 54</td>
<td>29 %&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Root biomass&lt;sup&gt;a&lt;/sup&gt; (g m⁻²)</td>
<td>471 ± 34</td>
<td>643 ± 49</td>
<td>37 %&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>δ¹³C&lt;sup&gt;b&lt;/sup&gt; (%)</td>
<td>-37.2 ± 0.9</td>
<td>-37.2 ± 0.9</td>
<td>0 %</td>
</tr>
<tr>
<td><strong>Soil</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mass of light soil fraction&lt;sup&gt;b&lt;/sup&gt; (mg/g)</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>10 %</td>
</tr>
<tr>
<td>Mass of heavy soil fraction&lt;sup&gt;b&lt;/sup&gt; (mg/g)</td>
<td>4.2 ± 0.1</td>
<td>4.5 ± 0.1</td>
<td>7 %</td>
</tr>
<tr>
<td>δ¹³C of light soil fraction&lt;sup&gt;b&lt;/sup&gt; (%)</td>
<td>-30.1 ± 0.4</td>
<td>-31.3 ± 0.4</td>
<td>-1 %</td>
</tr>
<tr>
<td>δ¹³C of heavy soil fraction&lt;sup&gt;b&lt;/sup&gt; (%)</td>
<td>-24.9 ± 0.2</td>
<td>-25.2 ± 0.2</td>
<td>-1 %</td>
</tr>
<tr>
<td>N content (mg/g)</td>
<td>0.51 ± 0.01</td>
<td>0.54 ± 0.01</td>
<td>5 %</td>
</tr>
<tr>
<td>C:N</td>
<td>12.4 ± 0.1</td>
<td>12.1 ± 0.1</td>
<td>-2 %</td>
</tr>
</tbody>
</table>

<sup>*</sup> P < 0.05  
<sup>a</sup> Reich et al., unpublished data  
<sup>b</sup> Dijkstra et al., 2004  
<sup>c</sup>% difference between ambient and added N
Table 4.2. Main effect of plant species richness on plant and soil characteristics (mean ± 1 SE) under elevated CO₂ in the BioCON experiment. NA: data not available.

<table>
<thead>
<tr>
<th></th>
<th>1 species</th>
<th>4 species</th>
<th>9 species</th>
<th>16 species</th>
<th>% difference^c</th>
</tr>
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<tbody>
<tr>
<td><strong>Plant</strong></td>
<td></td>
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</tr>
<tr>
<td>Total biomass^a (g m⁻²)</td>
<td>444 ± 40</td>
<td>788 ± 57</td>
<td>969 ± 86</td>
<td>1122 ± 62</td>
<td>153 %*</td>
</tr>
<tr>
<td>Root biomass^a (g m⁻²)</td>
<td>337 ± 36</td>
<td>555 ± 50</td>
<td>681 ± 82</td>
<td>820 ± 62</td>
<td>143 %*</td>
</tr>
<tr>
<td>δ¹³C (‰)</td>
<td>-36.8 ± 0.8</td>
<td>NA</td>
<td>NA</td>
<td>-38.3 ± 0.8</td>
<td>-5 %</td>
</tr>
<tr>
<td><strong>Soil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mass of light soil fraction^b (mg/g)</td>
<td>1.0 ± 0.1</td>
<td>NA</td>
<td>NA</td>
<td>1.5 ± 0.1</td>
<td>50 %*</td>
</tr>
<tr>
<td>Mass of heavy soil fraction^b (mg/g)</td>
<td>4.2 ± 0.1</td>
<td>NA</td>
<td>NA</td>
<td>4.8 ± 0.2</td>
<td>14 %*</td>
</tr>
<tr>
<td>δ¹³C of light soil fraction^b (‰)</td>
<td>-30.5 ± 0.4</td>
<td>NA</td>
<td>NA</td>
<td>-33.1 ± 0.2</td>
<td>-8 %*</td>
</tr>
<tr>
<td>δ¹³C of heavy soil fraction^b (‰)</td>
<td>-24.8 ± 0.2</td>
<td>NA</td>
<td>NA</td>
<td>-26.0 ± 0.2</td>
<td>-5 %*</td>
</tr>
<tr>
<td>N content (mg/g)</td>
<td>0.51 ± 0.01</td>
<td>0.52 ± 0.02</td>
<td>0.52 ± 0.02</td>
<td>0.57 ± 0.02</td>
<td>12 %*</td>
</tr>
<tr>
<td>C:N</td>
<td>12.3 ± 0.1</td>
<td>12.3 ± 0.4</td>
<td>12.1 ± 0.2</td>
<td>12.3 ± 0.4</td>
<td>0 %</td>
</tr>
</tbody>
</table>

* P < 0.05
^a Reich et al., unpublished data
^b Dijkstra et al., 2004
^c % difference between 1 species- and 16 species-treatments
standard FAME mixture that included known concentrations of five PLFAs 12:0, i15:0, 15:0, 10Me16:0, and cy19:0. Stable isotope ratios ($\delta^{13}$C) are expressed as %o difference in $^{13}$C to $^{12}$C ratio of the sample relative to Pee Dee Belemnite (PDB) standard: $\delta^{13}$C (%o) = $\left[\left(\frac{^{13}C}{^{12}C}_{\text{sample}} - \frac{^{13}C}{^{12}C}_{\text{standard}}\right) / \left(\frac{^{13}C}{^{12}C}_{\text{standard}}\right)\right] \times 10^3$.

**PLFA nomenclature**

Fatty acid notation indicates the number of C atoms and double bonds present in the molecule. The number followed by the symbol \(\omega\) (omega) denotes the position of first double bond relative to aliphatic end of the fatty acid. For instance, 18:2\(\omega6\) has eighteen C atoms and two double bonds, and the first double bond from the aliphatic end occurs at the 6\(^{\text{th}}\) C. Methyl branching relative to the first carbon from the aliphatic end is indicated by the prefix “i” (iso) and from the second carbon by the prefix “a” (anteiso). Methyl branching located elsewhere is expressed by a number from the aliphatic end as a prefix. For example, 10Me16:0 has a methyl branch at the 10\(^{\text{th}}\) C from the aliphatic end. Prefix “c” is used for cyclopropane fatty acids.

**Extracellular enzyme activity**

I determined the degradative potential of microbial communities by measuring the activity of extracellular enzymes that are critical in litter decomposition. The enzymes 1,4-\(\beta\)-glucosidase and cellobiohydrolase degrade cellulose. 1,4-\(\beta\)-N-acetylglicosaminidase decomposes chitin; acid phosphatase breaks down organic P. I also measured the activity of peroxidase and phenol oxidase, enzymes that depolymerize lignin.

Fluorometric assays using methylumbelliferone (MUB) linked substrates were
employed to determine the activities of hydrolytic enzymes 1,4-β-glucosidase, celllobiohydrolase, 1,4-β-N-acetylglucosaminidase, and phosphatase (Saiya-Cork et al., 2002). Two grams of each composite soil sample was mixed with 125 mL of sodium acetate buffer (pH 5.0). On a black 96-well microplate, I loaded 200 μL of soil slurry and 50 μL of substrate specific for each enzyme. There were eight analytical replicates for each enzyme assay. All enzyme assays were incubated at 21 °C. The incubation time was 0.5 hr for phosphatase and 1,4-β-N-acetylglucosaminidase assay, and 2 hr for 1,4-β-glucosidase and celllobiohydrolase assay. I determined the fluorescence from the microplates using f-Max fluorimeter (Molecular Devices Corp., Sunnydale, CA). Excitation energy was set at 355 nm and emission was measured at 460 nm. Enzyme activities are expressed as nmol 4-MUB g⁻¹h⁻¹.

I used 25 mM L-3,4-dihydroxy-phenylalanine (L-DOPA) as the substrate to measure the activity of phenol oxidase and peroxidase (Saiya-Cork et al., 2002). Clear 96-well microplates were used for colorimetric assay, and the procedures are similar to those of fluorometric assay described above. For peroxidase assays, I added 25 μL of 0.12% H₂O₂. There were sixteen analytical replicates each for phenol oxidase and peroxidase assay. The 96-well microplates were incubated for 24 hours at 21 °C, and absorbance was measured at 450 nm on EL-800 plate reader (Biotek Instruments, Inc., Winooski, VT). The unit for peroxidase and phenol oxidase activities is nmol L-DOPA converted g⁻¹h⁻¹.

**Statistical analyses**

The δ¹³C of each PLFA was analyzed using an ANOVA for a split-plot randomized
complete block design. The fixed effects in my model were block, N, and plant species richness. The split-plots were plant species richness and N treatments. I analyzed relative abundance of microbial groups and enzyme activities using ANCOVA for a split-plot randomized complete block design. Because legumes can increase soil N availability through N$_2$ fixation and have significantly higher N concentration in their litter, legume relative abundance was used as a covariate to adjust for the presence of this plant functional group. Root biomass was used as a covariate for enzyme activity analysis to account for the effects of belowground plant production on extracellular enzyme activity. For the analysis of microbial community composition, I used soil C content as the covariate to adjust for the effect of soil organic matter on microbial communities. Treatment effects were considered significant at $\alpha = 0.05$. I performed Tukey’s honestly significant difference (HSD) test to determine which group means differ from other means within the group ($P < 0.05$).

**RESULTS**

Nitrogen addition and plant species richness had a significant interaction effect on the relative abundance of fungal PLFA 18:2ω6 (Table 4.3). At ambient N deposition, the relative abundance of 18:2ω6 had the tendency to increase with greater plant species richness. However, N amendment removed this effect; 18:2ω6 relative abundance was not different across plant species richness treatments (Fig 4.1). Nitrogen addition and plant species richness did not interact to alter $\delta^{13}$C of any microbial PLFAs (Table 4.4).
Table 4.3. The influence of N addition and plant species richness on total microbial biomass and microbial community composition. P-values for total microbial biomass (MB) and relative abundance of each microbial PLFA analyzed by analysis of covariance (ANCOVA) using legume relative abundance as the covariate. P-values equal to or lower than 0.05 are in bold face print.

<table>
<thead>
<tr>
<th></th>
<th>MB</th>
<th>i15:0</th>
<th>i16:0</th>
<th>16:1</th>
<th>16:1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>o9c</td>
<td>o7c</td>
<td>16:0</td>
<td>a17:0</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>0.02</td>
<td>0.93</td>
<td>&lt;0.01</td>
<td>0.99</td>
<td>0.60</td>
</tr>
<tr>
<td>Species richness</td>
<td>0.04</td>
<td>&lt;0.01</td>
<td>0.06</td>
<td>0.14</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Nitrogen x Species</td>
<td>0.16</td>
<td>0.43</td>
<td>0.34</td>
<td>0.42</td>
<td>0.38</td>
</tr>
<tr>
<td>Legume relative</td>
<td>0.22</td>
<td>0.93</td>
<td>0.09</td>
<td>0.19</td>
<td>0.26</td>
</tr>
</tbody>
</table>
Figure 4.1. Interactive effects of N and plant species richness treatments on 18:2ω6 relative abundance. Error bars indicate one standard error of the mean. Means with the same letter are not significantly different at α = 0.05, as determined by Tukey’s HSD test.
Table 4.4. The influence of N addition and plant species richness on microbial PLFA $\delta^{13}$C. P-values for microbial PLFA $\delta^{13}$C were analyzed by analysis of variance (ANOVA). P-values equal to or lower than 0.05 are in bold face print.

<table>
<thead>
<tr>
<th></th>
<th>i15:0</th>
<th>i16:0</th>
<th>16:1 $\omega 9c$</th>
<th>16:1 $\omega 7c$</th>
<th>10Me 16:0</th>
<th>a17:0</th>
<th>cy17:0</th>
<th>18:1 $\omega 7c$</th>
<th>cy 19:0a</th>
<th>10Me 18:0</th>
<th>18:2 $\omega 6$</th>
<th>18:1 $\omega 9c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td>0.47</td>
<td>0.32</td>
<td>0.33</td>
<td>0.48</td>
<td>0.33</td>
<td>0.19</td>
<td>0.42</td>
<td>0.37</td>
<td>0.06</td>
<td>0.17</td>
<td>0.59</td>
<td>0.34</td>
</tr>
<tr>
<td>Species richness</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.01</td>
<td>&lt;0.01</td>
<td>0.33</td>
<td>0.81</td>
<td>0.13</td>
<td>0.91</td>
<td><strong>0.02</strong></td>
<td>0.56</td>
<td><strong>0.05</strong></td>
<td></td>
</tr>
<tr>
<td>Nitrogen x Species richness</td>
<td>0.28</td>
<td>0.47</td>
<td>0.48</td>
<td>0.34</td>
<td>0.27</td>
<td>0.53</td>
<td>0.79</td>
<td>0.23</td>
<td>0.99</td>
<td>0.85</td>
<td>0.99</td>
<td>0.24</td>
</tr>
</tbody>
</table>
Nitrogen as a main effect decreased microbial biomass, as indicated by a 27% decline in total PLFA (Fig 4.2). Nitrogen addition (main effect) significantly increased the relative abundance of the PLFA i16:0 (Table 4.3). Although $\delta^{13}$C of microbial PLFA was 0.4 to 1.3% lower (1.4 to 3.4% more depleted) under added N than under ambient N, the N effect was not statistically significant (Table 4.4, Fig 4.3).

Total microbial biomass increased significantly with plant species richness (main effect) ($62.6 \pm 6.4$ nmol PLFA/g under 1-species treatment vs. $97.1 \pm 8.4$ nmol PLFA/g under 16-species treatment). The relative abundance of PLFAs i15:0 and 10Me16:0 decreased with greater plant species richness, whereas 16:1$\omega$7c, 18:1$\omega$7c, 10Me18:0, and 18:1$\omega$9c relative abundance increased with higher plant species richness. Legume proportion was a significant covariate for the relative abundance of 18:1$\omega$9c (Table 4.3), and soil C content was a significant covariate for the relative abundance of PLFA 18:1$\omega$7c ($P = 0.05$).

The $\delta^{13}$C signature of microbial groups significantly decreased with greater plant species richness. $\delta^{13}$C of bacterial PLFAs i15:0, i16:0, 16:1$\omega$9c, 16:1$\omega$7c, 10Me16:0, and 10Me18:0 was significantly lower in species-rich plant communities than in species-poor plant communities. $\delta^{13}$C of fungal biomarker 18:1$\omega$9c also decreased with higher plant species richness (Table 4.4, Fig 4.4).

There was no significant interaction between N addition and plant species richness on the activity of litter-degrading enzymes (Table 4.5). Nitrogen treatment had no main effect on extracellular enzyme activities (Table 4.5). The activity of 1,4-$\beta$-
Figure 4.2. Main effect of N treatment on total microbial biomass. Total microbial biomass was averaged across plant species richness treatments. Error bars indicate one standard error of the mean.
Figure 4.3. Main effect of N addition on microbial PLFA $\delta^{13}$C. $\delta^{13}$C values were averaged across plant species richness treatment. Error bars indicate one standard error of the mean.
Figure 4.4. Main effect of plant species richness treatment on microbial PLFA $\delta^{13}$C. $\delta^{13}$C values were averaged across N treatment. Error bars indicate one standard error of the mean. Means with the same letter are not significantly different at $\alpha = 0.05$, as determined by Tukey’s HSD test.
Table 4.5. The influence on N addition and plant species richness on microbial enzyme activity. P-values for extracellular enzyme activity analyzed by analysis of covariance (ANCOVA) using legume relative abundance as covariate. P-values equal to or lower than 0.05 are in bold face print. NAGase: 1,4-β-N-acetylglucosaminidase.

<table>
<thead>
<tr>
<th></th>
<th>1,4-β-glucosidase</th>
<th>Celllobio-hydrolase</th>
<th>NAGase</th>
<th>Phosphatase</th>
<th>Peroxidase</th>
<th>Phenol oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td>0.06</td>
<td>0.64</td>
<td>0.53</td>
<td>0.16</td>
<td>0.27</td>
<td>0.46</td>
</tr>
<tr>
<td>Species richness</td>
<td>0.08</td>
<td><strong>0.02</strong></td>
<td>0.16</td>
<td>0.57</td>
<td>0.76</td>
<td>0.40</td>
</tr>
<tr>
<td>Nitrogen x Species richness</td>
<td>0.48</td>
<td>0.90</td>
<td>0.75</td>
<td>0.74</td>
<td>0.25</td>
<td>0.30</td>
</tr>
<tr>
<td>Legume relative abundance</td>
<td>0.49</td>
<td>0.55</td>
<td><strong>0.02</strong></td>
<td>0.15</td>
<td>0.99</td>
<td>0.44</td>
</tr>
</tbody>
</table>
glucosidase (+14%), phosphatase (+21%), and peroxidase (+6%) increased, and the activity of cellobiohydrolase (-6%), 1,4,-β-N-acetylglucosaminidase (-5%), and phenol oxidase (-20%) decreased in N amended soil, but the results were not statistically significant (Table 4.6). As a main effect, plant species richness significantly increased cellobiohydrolase activity (Fig 4.5B). Plant species richness had no significant main effect on 1,4-β-glucosidase, 1,4,-β-N-acetylglucosaminidase, phosphatase, peroxidase, and phenol oxidase (Fig 4.5, Table 4.5). Legume relative abundance was a significant covariate for 1,4,-β-N-acetylglucosaminidase activity (Table 4.5). Root biomass was only marginally significant for phosphatase activity (P = 0.06), and was not a significant covariate for any of the enzymes studied (P = 0.30 - 0.97).

DISCUSSION

Determining how atmospheric N deposition alters the metabolism of soil microorganisms associated with species-poor and species-rich plant communities is important in predicting changes in soil C and N cycling rates in terrestrial ecosystems subjected to both atmospheric N deposition and decreases in plant species richness. Nitrogen amendment diminished the positive effect of plant species richness on fungal relative abundance, which indicates that N deposition and plant species richness can interact to alter fungal abundance. Lower microbial biomass in N amended soil suggests that substrate availability for soil microorganisms is reduced, and this could be due to inhibition of soil organic matter decomposition by white-rot basidiomycetes. Significantly more depleted microbial δ¹³C, larger microbial biomass, and greater
Table 4.6. Extracellular enzyme activity (mean ± 1 SE) under ambient and added N. Enzyme activity is expressed in nmol g⁻¹h⁻¹. Enzyme activities were averaged across plant species richness treatments. NAGase: 1,4-β-N-acetylglucosaminidase.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Ambient N</th>
<th>Added N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4-β-glucosidase</td>
<td>26.3 ± 2.3</td>
<td>30.0 ± 2.3</td>
</tr>
<tr>
<td>Cellulohydrolase</td>
<td>5.7 ± 0.7</td>
<td>5.3 ± 0.5</td>
</tr>
<tr>
<td>NAGase</td>
<td>3.6 ± 0.4</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>Phosphatase</td>
<td>14.2 ± 1.3</td>
<td>17.2 ± 1.3</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>536.2 ± 44.5</td>
<td>566.8 ± 42.4</td>
</tr>
<tr>
<td>Phenol oxidase</td>
<td>208.4 ± 29.5</td>
<td>167.2 ± 17.3</td>
</tr>
</tbody>
</table>
Figure 4.5. Main effect of plant species richness treatment on extracellular enzyme activity. Enzyme activity was averaged across N treatments. Error bars indicate one standard error of the mean. Means with the same letter are not significantly different at $\alpha = 0.05$, as determined by Tukey’s HSD test.
cellobiohydrolase activity in species-rich plant communities together indicate that higher plant species richness increased microbial metabolism of new photosynthate. This implies that greater plant species richness can enhance microbial activity in grassland ecosystems.

Nitrogen addition eliminated the positive effect of plant species richness on 18:2\(\omega 6\) relative abundance, but it is not clear what the underlying mechanism for this observation may be. Nitrogen fertilization can lower soil fungal abundance (Bardgett et al., 1996; Bardgett et al., 1999; Boussuyt et al., 2001), and N addition as a main effect had the tendency to reduce fungal abundance in this study (data not shown). Inhibitory effect of N addition on white-rot basidiomycetes and mycorrhizae has been well studied (Fog et al., 1988; Treseder, 2004), but it is not clear why the inhibitory effect of added N on fungi was most obvious on species-rich plant communities. If white-rot basidiomycetes that produce ligninolytic enzymes were repressed by N addition, phenol oxidase and peroxidase activity could show similar trend with 18:2\(\omega 6\) relative abundance, but this was not the case (data not shown). The relative abundance of 18:2\(\omega 6\) did not show significant correlation with any of the extracellular enzymes but 18:2\(\omega 6\) concentration had a weak, but significant correlation with N-acetylglucosaminidase activity (\(P < 0.01, r^2 = 0.11, n = 73\)). This is consistent with Miller et al. (1998)’s study, where they have shown that the activity of N-acetylglucosaminidase, an enzyme that degrades chitin has significant positive correlation with fungal PLFA concentration, and concluded that N-acetylglucosaminidase activity can be an indicator of fungal biomass. However, it is difficult to infer the ecological consequence of the change in 18:2\(\omega 6\)
relative abundance due to the interactive effects of N addition and plant species richness with current results.

Total microbial biomass was lowered (-27%; $P = 0.02$) in N amended soil, which implies decreased substrate availability for microbial communities. The influence of N addition on soil microbial communities is likely to be direct, and not mediated through an increase in organic substrate input, because litter production was enhanced in response to N amendment in this experiment. Current evidence suggests that N addition inhibited microbial access to older forms of soil organic matter, rather than new photosynthate. Although not statistically significant, a decrease in all of the $\delta^{13}C$ of microbial PLFAs under N addition indicates that new photosynthate that is more depleted in $^{13}C$ was preferentially incorporated over soil organic matter. Marginal increase in $\beta$-glucosidase activity ($P = 0.06$) in N amended soil also suggests that degradation of cellulose from new substrate was modestly enhanced, and this is consistent with other studies where N addition enhanced cellulose degradation (Fog, 1988; Careirro et al, 2000; Compton et al., 2004; Frey et al., 2004).

Nitrogen addition lowered phenol oxidase activity by 20%, and although the result was not significant, this may explain how soil organic matter degradation was inhibited. Keyser et al. (1978) found that excess $NH_4^+$ can inhibit the synthesis of lignin-degrading enzymes by white-rot basidiomycetes. Through an extensive review of studies on the effect of N amendment on organic matter decomposition, Fog (1988) concluded that N addition in organic or inorganic form can repress lignin degradation by white-rot basidiomycetes, and in a few cases, actinomycetes and bacteria. Phenol oxidase degrades
phenolic structures that make up 10-15% of lignin, and cannot attack recalcitrant nonphenolic units of lignin that can only be degraded by peroxidase (Hofrichter and Steinbüchel, 2001). Therefore, N addition could have repressed soil organic matter decomposition by inhibiting phenol oxidase activity. Peroxidase activity was not lowered by N addition, which indicates that degradation of intact lignin was unaffected in N amended soil. Altogether, these results suggest that N addition likely inhibited the decomposition of by-products of lignin predominant in soil organic matter, rather than intact lignin associated with plant litter. Other N deposition studies also have found reductions in ligninolytic enzyme activities (Carreiro et al., 2000; Saiya-Cork et al., 2002; DeForest et al., 2004a; Frey et al., 2004; Sinsabaugh et al., 2004), and in some cases there were simultaneous decreases in microbial biomass (DeForest et al., 2004a; DeForest et al., 2004b; Frey et al., 2004), further lending support to my argument.

A decrease in concentration of all microbial PLFAs by N addition indicates that the impact of N addition is not confined to a narrow range of microbial groups. Nitrogen deposition could have directly affected all of the microbial groups, or selectively reduced the white-rot basidiomycetes, and the subsequent decrease in phenol oxidase activity could have reduced microbial access to substrates protected by partially degraded lignin. However, this cannot be discerned with PLFA analysis, and further investigation would be needed to understand how increased N availability affected white-rot basidiomycetes.

Ammonium nitrate addition may have lowered soil pH and this could have decreased microbial biomass; however, soil pH was not measured in my study and this remains to be determined. Soil pH in a neighboring plant diversity study in this site was
6.2 (Zak et al., 2003), which leads me to think soil pH in the BioCON experiment would be similar. In a calcareous grassland study, soil pH was 6.5 under control treatment and NH₄NO₃ addition at the level of 7 g N m⁻² yr⁻¹ decreased the soil pH by a modest degree (to 6.2) (Johnson et al., 1998). Therefore, relatively low level of N added in my study (4 g N m⁻² yr⁻¹) may not have lowered soil pH to a degree where it may affect microbial communities.

Higher levels of N applied to soil can chemically bind with plant litter during decomposition and form complexes that are highly recalcitrant (Berg and McLaugherty, 2003), another mechanism by which substrate availability could have been reduced. Nitrogen addition to bare plots increased the mass of light and heavy soil fractions (Dijkstra et al., 2004), and this suggests that N can directly bind with soil OM and increase C sequestration. In the BioCON experiment, the amount of soil C was positively correlated with the amount of isotope-labelled N fertilizer residing in soil (Dijkstra et al., 2004), which indicates that organic compounds in soil could have formed complexes with added N that are resistant to microbial degradation.

The lack of significant N effect on soil microbial communities other than decreasing total microbial biomass could have been due to greater plant production in response to N addition. Because N addition rate (4 g N m⁻² yr⁻¹) is comparable to other studies that test the effects of atmospheric N deposition where they have added N in the range of 3 to 15 g N m⁻² yr⁻¹ (Johnson et al., 1998; Ajwa et al., 1999; DeForest et al., 2004a; Frey et al., 2004; Sinsabaugh et al., 2004), the differences in microbial community responses among these studies can be attributed to ecosystem characteristics such as plant
production, plant litter biochemistry, and soil microbial community composition.
Enhanced plant production under N addition in this experiment indicates that N is a
limiting nutrient in this ecosystem (Tilman, 1987). Because plant production increased in
response to N addition, the inhibitory effect of N amendment on microbial communities
may have been smaller than found in other studies where plant production was not altered
by N addition. For example, NO₃⁻ deposition study in a sugar maple forest found that
ligninolytic enzyme activities were significantly suppressed by NaNO₃, and there was no
simultaneous increase in plant production (Saiya-Cork et al., 2002; DeForest et al.,
2004a). Chronic NH₄NO₃ addition in Harvard forest significantly decreased microbial
biomass and phenol oxidase activity, and increased β-glucosidase activity (Compton et
al., 2004; Frey et al., 2004). Nitrogen addition decreased plant growth in this ecosystem
(Compton et al., 2004; Frey et al., 2004), which could have amplified direct effect of N
addition on microbial communities because plants were obviously unable to absorb
excess N. A cross-site study that has compared the effect of N amendment among
grassland ecosystems that differ in their degree of N limitation, microbial response was
significantly related to plant response (Johnson et al., 1998). In two grassland ecosystems
where N addition decreased vegetation cover, N addition lowered microbial biomass;
however, in a heathland N addition increased vegetation cover and this was accompanied
by higher microbial biomass (Johnson et al., 1998). These studies support the idea that
the effect of N addition on heterotrophic microbial communities can be determined only
in conjunction with associated plant response to N addition, because of microbial
dependence on plants for energy.
Decrease in $\delta^{13}$C of microbial PLFAs in species-rich plant communities indicates that soil microorganisms preferentially incorporated new photosynthate over older forms of organic matter with increasing plant species richness. Microorganisms in soil could have assimilated recent labile photosynthate that increased with greater plant species richness, especially fine roots, which are the largest constituents of the total plant biomass with plant species richness (Reich et al., 2004). Significantly lower microbial $\delta^{13}$C in species-rich plant communities suggests that it is the new photosynthate that enhances microbial metabolism with greater plant species richness.

Greater assimilation of new photosynthate, total microbial biomass, and cellbiohydrolase activity with greater plant species richness indicate that plant species richness increased the rates of C cycling in the soil food web, most likely due to greater organic substrate availability. The marginally significant increase in $\beta$-glucosidase activity ($P = 0.08$) with higher plant species richness also supports the idea that cellulose degradation is enhanced in species-rich plant communities. Root biomass was not a significant covariate for any of the extracellular enzymes, and this could indicate that plant species richness per se, independent of plant production, can influence microbial degradative potential. Higher plant species richness could have provided soil microorganisms with organic substrates at a more consistent level due to complementary resource use in time and space by species-rich plant communities (Craine et al., 2003a; Craine et al., 2003b). It could also be the case that root biomass is not a good surrogate for belowground production. Although standing root biomass does reflect belowground production, the production and death of fine roots and root exudation cannot be
estimated, and soil microorganisms would preferentially feed on these labile parts of plants. Therefore, current evidence suggests that greater plant species richness, likely through combination of higher substrate input and complementary resource use, promotes faster rates of microbial metabolism in soil.

My study demonstrates that plant species richness can determine changes in microbial community composition in response to N deposition, but the exact mechanism for this needs to be elucidated. Decreases in total microbial biomass in N amended soil indicate that N addition can inhibit microbial access to organic substrates. Significantly lower δ^{13}C of microbial PLFAs in species-rich plant communities suggest that increases in microbial biomass and cellobiohydrolase activity with higher plant species richness result from greater plant production. Altogether, my results demonstrate that N deposition and plant species loss may interact to alter microbial community composition, and that greater plant species richness promotes higher rates of microbial metabolism in soil.
LITERATURE CITED


CHAPTER 5
SUMMARY AND CONCLUSIONS

My dissertation demonstrates that elevated CO₂, elevated O₃, atmospheric N deposition, and declining plant species richness individually are potent modifiers of soil microbial community composition and function, and that these global change components are not highly interactive. Elevated CO₂ and plant species richness as main effects increased cellulolytic and chitinolytic potential of microbial communities in soil, which was accompanied by an increase in fungal relative abundance. These results indicate that CO₂ enrichment and higher plant species richness could increase belowground C cycling rates through altering organic matter decomposition via changes in microbial community composition. Elevated O₃ decreased cellulolytic capacity and changed fungal community composition, but did not significantly interact with elevated CO₂. Experimental N deposition decreased total microbial biomass, which could be due to decrease in ligninolytic enzyme activity and the suppression of white rot basidiomycetes, or the repression of mycorrhizal infection. These results suggest that elevated CO₂, elevated O₃, atmospheric N deposition, and decreases in plant species richness as main effects exert stronger influences on microbial community composition and function than through their interactions.
Changes in microbial metabolism under elevated CO₂ in my study (Chapters 2 and 3) closely reflected the pattern of plant production (King et al., 2001a; Reich et al., 2001a). The metabolism of microbial communities under northern hardwood trees were stimulated by elevated CO₂ (Chapter 2), whereas CO₂ enrichment had little effect on soil microbial communities associated with grassland plant species that displayed a smaller growth response to elevated CO₂ (Chapter 3). These differences are likely due to the distinct response of plants in the two ecosystems. For example, in the Aspen FACE experiment, elevated CO₂ increased fine root biomass by 96% (King et al., 2001a). This was accompanied by a 39% increase in soil respiration (King et al., 2001a), which indicates that the rate of belowground C cycling significantly increased due to greater root production. Enhanced cellulolytic potential in this experiment also supports this idea (Chapter 2). On the other hand, CO₂ enrichment increased belowground biomass by 14% (Reich et al., 2001a) and soil CO₂ flux by 13% (Craine et al., 2001) in the BioCON experiment. This increase is smaller than that observed for the northern hardwood ecosystem, and it is likely that the extent of changes in microbial metabolic rate follows that of changes in plant production.

In both the Aspen FACE and BioCON experiments, fungi, rather than bacteria were more responsive to elevated CO₂, elevated O₃, and declining plant species richness. For example, in the Aspen FACE experiment, elevated CO₂ enhanced the activity of cellulolytic and chitinolytic enzymes mainly produced by fungi, and elevated O₃ counteracted this. Although the result was not significant, fungal relative abundance was 30% higher under elevated CO₂ than under ambient CO₂ (Chapter 2). In the BioCON
experiment, fungal relative abundance increased with greater plant species richness (Chapter 3). Moreover, this was accompanied by increases in cellulose- and chitin-degrading enzyme activity (Chapter 3). Fungi have a higher substrate use efficiency than bacteria (Paul and Clark, 1996), and fungal abundance can increase with greater plant litter production. Because fungi are major cellulose-decomposers (Lynd et al., 2002), an increase in cellulose input to soil via enhanced plant production could also promote fungal abundance. Moreover, fungi are more adapted to using plant litter of lower litter quality (i.e. lower lignin:N ratio) (Berg and McLaugherty, 2003), and more recalcitrant litter may further favor fungal abundance.

In the Aspen FACE experiment and in other elevated CO$_2$ studies on northern hardwood trees, CO$_2$ enrichment increased plant production and leaf tannin concentration, and decreased leaf N concentration (Mansfield et al., 1999; Isebrands et al., 2001; King et al., 2001a; King et al., 2001b). Therefore, increase in plant production and change in litter biochemistry could have promoted fungal abundance, leading to observed increase in fungal metabolism (Chapter 2). In the BioCON experiment, greater fungal relative abundance and metabolism in species-rich plant communities (Chapter 3) may be due to a similar mechanism, because plant production increased and whole plant N concentration decreased with higher plant species richness (Reich et al., 2001a). Plant lignin content was not measured in the BioCON experiment, but if lignin:N ratio increased with greater plant species richness, this change in litter biochemistry together with higher plant production could have favored fungal abundance. Although I have observed significantly enhanced cellulolytic potential under elevated CO$_2$ and higher
Plant species richness, if plant litter quality decreases, C cycling rates may decline in the long-term. However, this cannot be discerned from my results, and can only be elucidated through a long-term plant litter decomposition study.

Plant species richness had significant positive effects on microbial biomass and cellulolytic capacity in the BioCON experiment (Chapters 3 and 4). More depleted δ^{13}C of microbial PLFAs in species-rich plant communities (Chapter 4) suggests that new photosynthate supports greater microbial biomass and fuels higher metabolic rates. Because plant production increased with higher plant species richness in the BioCON experiment (Reich et al., 2001a), it is very likely that greater substrate availability promoted higher microbial metabolism. However, plant species richness had a significant influence on microbial community composition and function even after the effects of legume abundance, total plant biomass, root biomass, and soil C content were taken into account (Chapters 3 and 4). This indicates that higher substrate availability cannot solely explain why microbial biomass and cellulolytic potential increased with greater plant species richness. Complementary resource use in time and space in species-rich plant communities (Reich et al., 2001b; Craine et al., 2003) may also help to explain the positive effect of plant species richness on soil microbial communities. Because species-rich plant communities could potentially provide organic substrates to soil microorganisms at a more consistent level, they could support higher microbial biomass and greater degradative potential. Therefore, it is likely that higher plant production and complementary resource use in species-rich plant communities together promote greater microbial biomass and cellulolytic capacity.
Experimental N deposition appears to have reduced substrate availability for microorganisms and lowered their microbial biomass through direct inhibition of ligninolytic enzymes. Increased N availability in N amended soil could also have reduced mycorrhizal infection (Johnson et al., 2003; Frey et al., 2004; Treseder, 2004), leading to a decreased microbial biomass. Although the results were not significant, repressed ligninolytic capacity in N amended soil (Chapters 3 and 4) may indicate that the activity of white-rot basidiomycetes could be inhibited due to N addition. In cases where interactive effects of N deposition, elevated CO₂, and plant species richness altered microbial biomass and fungal relative abundance, the pattern of changes was rarely similar to what has been observed for plant production (Chapters 3 and 4). These results suggest that when N deposition and interactions among other global change components on microbial communities are being studied, considering the direct inhibitory effect of N on soil microbial communities may be important. To determine the specific microbial populations affected by N deposition and how this will propagate to alter the rates of C and N cycling, it will be helpful to study microbial community composition and function using molecular tools that can discern changes in microbial communities at a finer level. Because the pattern of changes in microbial community composition was not immediately reflected in changes in degradative potential (Chapters 3 and 4), further long-term study also is warranted to determine the ecological consequences of alterations in microbial community composition.

Altogether, my dissertation demonstrates that the main effects of global environmental changes have a stronger influence on soil microbial communities than
their interactive effects. In most cases, increases or decreases in microbial activity closely followed the trend of plant production under global change. This was especially the case for cellulolytic potential, and these results partially support my hypothesis that global change will manifest its effect on soil microbial communities through an alteration in plant production and litter biochemistry. However, the main effects of experimental N deposition and plant species richness on soil microbial communities could not be solely explained by changes in plant production. Moreover, the interactions among elevated CO₂, N deposition, and plant species richness on microbial community composition were more difficult than the main effects to interpret. Therefore, further investigation should focus on plant litter biochemistry and microbial groups that can be directly affected by N deposition. My study demonstrates that soil microbial communities may not be strongly affected by interactions among global environmental changes that alter plant production and litter biochemical constituents. Another alternative is that the interactive effects will only be apparent in a long-term study due to a lag effect, and further study is required to determine how interactive effects of global change will alter microbial community composition and function.
LITERATURE CITED


