Linking above- and belowground responses to global change at community and ecosystem scales

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Abstract

Cryptic belowground organisms are difficult to observe and their responses to global changes are not well understood. Nevertheless, there is reason to believe that interactions among above- and belowground communities may mediate ecosystem responses to global change. We used grassland mesocosms to manipulate the abundance of one important group of soil organisms, arbuscular mycorrhizal (AM) fungi, and to study community and ecosystem responses to CO_2 and N enrichment. Responses of plants, AM fungi, phospholipid fatty acids and community-level physiological profiles were measured after two growing seasons. Ecosystem responses were examined by measuring net primary production (NPP), evapotranspiration, total soil organic matter (SOM), and extractable mineral N. Structural equation modeling was used to examine the causal relationships among treatments and response variables. We found that while CO_2 and N tended to directly impact ecosystem functions (evapotranspiration and NPP, respectively), AM fungi indirectly impacted ecosystem functions by influencing the community composition of plants and other root fungi, soil fungi and soil bacteria. We found that the mycotrophic status of the dominant plant species in the mesocosms determined whether the presence of AM fungi increased or decreased NPP. Mycotrophic grasses dominated the mesocosm communities during the first growing season, and the mycorrhizal treatments had the highest NPP. In contrast, nonmycotrophic forbs were dominant during the second growing season and the mycorrhizal treatments had the lowest NPP. The composition of the plant community strongly influenced soil N, and the community composition of soil organisms strongly influenced SOM accumulation in the mesocosms. These results show how linkages between above- and belowground communities can determine ecosystem responses to global change.

Nomenclature:

CO_2	=	carbon dioxide
N	=	nitrogen
AM	=	arbuscular mycorrhizal
SEM	=	structural equation model
λ	=	Path coefficient in SEM
NPP	=	net primary productivity
SOM	=	total soil organic matter
CLPP	=	community-level physiological profiles
MRPP	=	multiple response permutation procedure
NMDS	=	nonmetric multidimensional scaling
PLFA	=	Phospholipid fatty acids

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Introduction

Arbuscular mycorrhizal (AM) fungi are dominant soil microorganisms in grassland ecosystems, comprising an estimated 30–60% of soil microbial biomass (Olsson *et al.*, 1999). Most grassland plants form nutritional symbioses with AM fungi in which the plant receives mineral nutrients and water in exchange for fixed carbon. A large proportion of plant photosynthate is allocated to AM symbionts (Peng *et al.*, 1993); consequently, AM fungi are important contributors to soil organic carbon (Rillig, 2004). Anthropogenic enrichment of CO_2 and N can influence the balance of trade between plants and AM fungi. If CO_2 enrichment increases photosynthetic rates, then mycorrhizal biomass is predicted to increase (Fitter *et al.*, 2000), and if N enrichment reduces mineral deficiency, then mycorrhizal biomass is predicted to decrease (Treseder & Allen, 2002). However, empirical studies show that these predictions are too simplistic, and community composition and edaphic conditions dictate whether or not AM biomass will increase with CO_2 enrichment and decrease with N enrichment (Johnson *et al.*, 2003a, 2005).

Plant community structure is influenced by AM fungi because mycorrhizal symbioses influence the outcome of plant interactions with competitors, parasites, pathogens and herbivores (Newsham *et al.*, 1995; Marler *et al.*, 1999; Linderman, 1988, 2000; Gehring & Whitham, 2002). Plant species vary in their mycotrophy, the degree to which they feed through mycorrhizas, so that feedbacks between communities of plants and AM fungi can either increase or decrease plant diversity (Bever *et al.*, 1997; Hartnett & Wilson, 1999; Urcelay & Diaz, 2003). Furthermore, AM fungi are known to influence the community composition of soil microbes (Schreiner *et al.*, 1997; Reynolds *et al.*, 2003), including populations of pathogenic soil bacteria and fungi (Linderman, 2000). The impact of AM fungi on the structure and functioning of above- and belowground communities is important to consider in global change scenarios, because there is increasing recognition that community structure may influence ecosystem properties (Luyssaert *et al.*, 2007; Bardgett *et al.*, 2008). Thus, to make effective global change predictions, ecosystem ecologists and community ecologists need to find ways to link their research metrics.

The species composition of plant communities is expected to change in response to CO_2 enrichment because of differences in photosynthetic pathway and life-history strategies (Bazzaz, 1990; Koch & Mooney, 1996; Reich *et al.*, 2001). In addition, transpiration is generally reduced because of lower stomatal conductance in CO_2-enriched systems (Field *et al.*, 1995). This change in plant water use efficiency can increase soil water, and change microbial processes and nutrient cycling (Hungate *et al.*, 1997). Plant species also respond differently to N enrichment; and, at the community level, as N becomes less limiting, plant productivity generally increases while plant diversity decreases (Tilman, 1987). All of these changes in community structure have the potential to alter ecosystem processes such as net primary productivity (NPP), N cycling, evapotranspiration and soil organic matter (SOM) accumulation. Because the availability of soil N may limit the capacity of primary producers to consume anthropogenic CO_2 (Hungate *et al.*, 2003; Luo *et al.*, 2004; Reich *et al.*, 2006), simultaneously addressing both above- and belowground responses to CO_2 and N enrichment is necessary to formulate realistic global change predictions (Wolters *et al.*, 2000).

We examined plant and soil communities in a 2-year mesocosm experiment that manipulated CO_2 and N availability in the presence or absence of AM fungi. Our first year focused entirely on mycorrhizal mediation of aboveground plant responses to CO_2 and N enrichment (Johnson *et al.*, 2003b). The present study from the second year of the experiment includes detailed analyses of belowground microorganisms and analyzes linkages among the composition of plant and soil communities with ecosystem responses. We addressed the following questions:

1. What are plant community and ecosystem responses to enrichment of CO_2 and N and the presence of AM fungi?
2. What are the responses of soil- and root-inhabiting microorganisms to the availability of CO_2, N and AM symbioses?
3. What is the relative importance of community structure on ecosystem responses to enrichment of CO_2, N and AM symbioses?

Materials and methods

Experimental design

Mesocoms used in this experiment were designed to replicate the plant communities, and N and CO_2 treatments used in the free-air CO_2 enrichment experiment in Cedar Creek, MN, USA (Reich *et al.*, 2001), with one additional factor that is difficult to manipulate in the field, the presence or absence of AM fungi. We used a three factor randomized block design with two levels of each factor: elevated and ambient atmospheric CO_2, high and low soil N, and live or dead AM fungal inoculum. Each factor combination was replicated six times for a total of 48 mesocosms. In the spring of 2001, each mesocosm (dimensions: 48 cm × 38 cm × 43 cm deep) was filled with 61 L of steam sterilized Cedar Creek soil.

Treatments

The CO_2 treatments were established in 12 greenhouse chambers (2.5 m × 1.3 m × 1.5 m tall) with randomly assigned ambient (368 ppm) and elevated (550 ppm) CO_2. The +N mesocosms were given 0.701 g NO_3NH_4 in 500 mL water three times during the growing season in each year of the experiment. This level was equivalent to 4 g N m^{-2} yr^{-1}. Mycorrhizal treatments were created in 2001 by adding 16 g fresh weight of AM colonized roots, spores and hyphae from fungal cultures established from Cedar Creek soil to +AM mesocosms and an equal amount of autoclaved inoculum to −AM mesocosms. To equalize the communities of soil microorganisms of the two treatments, each of the 24 −AM mesocosms received 175 mL of a microbial slurry that had been rinsed from the +AM inoculum and filtered through a 20 μm sieve to remove the AM fungal propagules. All 48 mesocosms received 250 mL of a second microbial slurry prepared from fresh Cedar Creek soil using the same method to equalize the background soil microbes [see Johnson *et al.* (2003b) for details]. The integrity of the AM treatment in each mesocosm was checked in April 2002 by analyzing random samples of roots for AM colonization as described below. Mycorrhizal contamination in one nonmycorrhizal mesocosm caused this experimental unit to be eliminated entirely from analysis.

Planting

In the spring of 2001, three seedlings each of 14 species of grassland plants were randomly planted for a total of 42 plants per mesocosm. Plants in six different functional groups were included: (1) perennial C_3 grasses (*Agropyron repens* L., *Koeleria cristata* Pers. and *Poa pratensis* L.), (2) C_4 grasses [*Andropogon gerardii* Vitman, *Schizachyrium scoparium* (Michauux) Nash and *Bouteloua gracilis* (Willd. ex Kunth) Lag. ex Griffiths], (3) mycotrophic forbs (*Achillea millefolium* L., *Heliopsis helianthoides* L. and *Solidago rigida* L.), (4) nonmycotrophic forbs (*Berteroa incana* L. and *Salsola kali* L.), (5) mycotrophic legumes [*Lespedeza capitata* (Michaux) and *Petalostemum villosum* Nutt.] and (6) a nonmycotrophic legume (*Lupinus perennis* L.). Plant shoots were cut and aboveground biomass was measured at the end of the 2001 growing season (results published in Johnson *et al.*, 2003b). The mesocosms were moved out of the CO_2 chambers and into a cold warehouse for the winter and returned to the greenhouse in April 2002. Winter mortality of perennial species was nearly 100%, requiring each mesocosm to be reseeded with three seeds each from 10 of 12 of the plant species. Seeds were randomly scattered on the surface of each mesocosm. Seedling recruitment from *S. kali* and *B. incana* was high, so they were not reseeded. Thus, the experimental treatments and plant composition in each mesocosm maintained a legacy of the 2001 growing season.

Plant measurements

In September 2002, each plant was individually harvested and separated into roots and shoots, dried for a minimum of 72 h at 60 °C and weighed. Composite subsamples of roots were randomly collected from each mesocosm and analyzed for fungal colonization. Plant biomass was summed and analyzed by functional groups. NPP in each mesocosm was estimated by summing the oven-dried root and shoot biomass of all species and dividing by the mesocosm surface area (g m^{-2} yr^{-1}).

Abiotic measurements

Soil moisture levels were measured using time domain reflectometry (TDR; Campbell Instruments, Logan, UT, USA). Calibration curves were created using a simulated mesocosm to convert TDR voltage readings to volumetric water content. Four times weekly, we added the amount of water necessary to keep mesocosms at 4.9% moisture by mass based on TDR readings. This moisture content simulated 75% of field water-holding capacity, and thus precluded water loss through drainage holes in the bottom of mesocosms. This was done to eliminate soil moisture as a confounding variable and to determine how the treatments affected water usage. The total amount of water added across the entire 2002 growing season was summed as a proxy of seasonal evapotranspiration from each mesocosm.

Available soil NH_4 and NO_3 in the mesocosms were extracted with KCl and measured at the beginning (April) and the end (October) of the 2002 growing season. Concentrations of NH_4 and NO_3 in the soil extracts were measured using an autoanalyzer (Lachat Quickchem FIAþ8000; Lachat Instruments, Milwaukee, WI, USA). In October 2002, the amount of total SOM was estimated by loss on ignition (Schulte & Hopkins, 1996).

Fungal and bacterial community measurements

Five soil cores, 30 cm deep and 0.5 cm wide, were taken across the surface of each mesocosm in April and at harvest (October) to analyze the populations of AM fungal spores and densities of fungal hyphae. Spores were extracted using the sucrose centrifugation method and quantified to morphospecies using a compound microscope at $100–1000 \times$ magnification (Schenck & Perez, 1990; Johnson *et al.*, 1999). Fungal hyphae were extracted from soils using the methods of Abbott *et al.* (1984) and quantified using a gridded eyepiece graticule in a compound microscope at $250 \times$ magnification. AM hyphae were quantified in four discrete categories: <2.5, $2.5–6$, >6 and $>6 \,\mu m$ orange hyphae. From the same samples, we also quantified nonmycorrhizal (septate) hyphae. A composite root sample from all plant species was cleared with 5% KOH and stained with trypan blue (Koske & Gemma, 1989). Colonization by AM and other root endophytes was determined using the gridline intersect method at $200 \times$ magnification (McGonigle *et al.*, 1990). Mycorrhizal colonization was separated into arbuscules, vesicles and hyphae. Other (non-AM) endophytic fungi were separated into five groups based on distinctive morphological characteristics of septate or melanized hyphae and other intraradical structures. Root endophytes lacking distinctive morphological characteristics were quantified but were not assigned a unique category. We were able to identify two morphotypes to the genus level (Barr & Allan, 1982; Deacon & Saxena, 1997).

A community fingerprint of soil organisms was determined from the same soil samples using phospholipid fatty acid (PLFA) analysis (Olsson *et al.*, 1999). Lipids were extracted from soil that was collected at harvest and stored frozen. Soil samples were freeze-dried and then lipids were extracted from 5 g subsamples by vortex mixing (1 min) in a one-phase mixture of citrate buffer, methanol and chloroform (0.8 : 2 : 1, v/v/v, pH 4.0). The lipids were fractionated into neutral lipids, glycolipids and phospholipids on prepacked silica columns (100 mg sorbent mass; Varian Medical Systems, Palo Alto, CA, USA) by eluting with 1.5 mL chloroform, 6 mL acetone and 1.5 mL methanol, respec-

tively. The fatty acid residues in neutral lipids and phospholipids were transformed into free fatty acid methyl esters and analyzed by gas chromatography using a 50 m HP5 capillary fused silica column (Hewlett-Packard, Palo Alto, CA, USA) with H_2 as the carrier gas. The fatty acid methyl esters were identified and quantified in relation to the added internal standard (fatty acid methyl ester 19 : 0). These were compared with those identified earlier by gas chromatography–mass spectrometry. Biomass of AM fungi was estimated from the PLFA 16 : 1ω5 (Olsson, 1999). A community fingerprint of bacterial communities was determined from the following PLFAs: i15 : 0, a15 : 0, 15 : 0, i16 : 0, i17 : 0, a17 : 0, 17 : 0, cy17 : 0, 18 : 1ω7 and cy19 : 0 which are common signature fatty acids for bacteria (Frostegård *et al.*, 1993).

Bacterial and fungal community-level physiological profiles (CLPP) were measured using microtiter plates from Biolog Inc. (Hayward, CA, USA). CLPP plates contain C sources that are commonly used to metabolically fingerprint bacterial and fungal communities in ecosystems (e.g. Garland & Mills, 1991). Bacterial CLPPs were assessed using ECO plates that contain 31 environmentally relevant C substrates replicated three times per plate; fungal CLPPs were assessed using SFN-2 plates that consist of 95 unique C substrates per plate.

Bacterial and fungal plates were prepared by extracting 4 g of soil in 36 mL of 50 mm K_2HPO_4 buffer (pH 6). Soil slurries were allowed to settle for 30 min and then a 0.4 mL aliquot of this extraction was removed and diluted in 39.6 mL of inoculation solution for a final 1 : 1000 dilution (Classen *et al.*, 2003). Bacterial growth was prevented on fungal plates by adding 1 μg of streptomycin sulfate and 0.5 μg tetracycline per microtiter well (Dobranic & Zak, 1999; Classen *et al.*, 2003). Bacteria and fungal plates were inoculated with 100 μL of the 1 : 1000 dilution per well. Equipment, solutions and glassware were sterilized in an autoclave before use and plates were inoculated in a laminar-flow hood in order to reduce the risk of contamination.

Structural equation model (SEM)

We created an *a priori* conceptual model of hypothesized causal relationships among our treatment variables (mycorrhiza, CO_2 and N), and community and ecosystem response variables (Fig. 1a; Table 1; Bollen, 1989; Shipley, 2000a; McCune & Grace, 2002). SEM is well suited for this purpose, because it allows analysis of net treatment effects on response variables and also interactions among response variables. SEM derives from a synthesis of path analysis and factor analysis, usually with a maximum likelihood-based goodness-of-
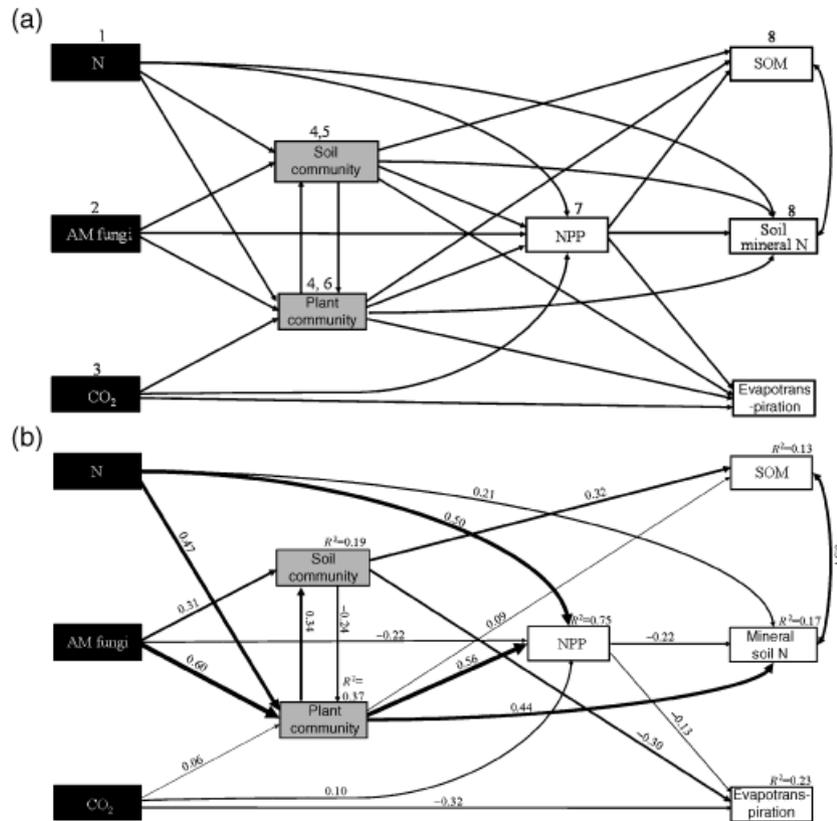
Fig. 1 (a) Our *a priori* hypothesized model of causal relationships among treatment and response variables. Arrows indicate hypothesized causal relationships. Black boxes indicate exogenous variables (treatments), gray boxes indicate endogenous community-level response variables and open boxes indicate endogenous ecosystem-level response variables. Predictions shown on the model as numbers above a box or arrow are described in Table 1a. (b) Our final structural equation model: width of arrows indicates the strength of the causal influence. Numbers above arrows indicate path coefficients; a measure of the strength of causal relationships and mathematically equal to either partial regression coefficients or correlation coefficients. R^2 values represent the proportion of variance explained for each endogenous variable.

fit test. By comparing the covariance structure of data that is implied by the model with the actual covariance structure of the data, we can test whether a model fits the data (Grace & Pugesek, 1998). Indirect path coefficients are mathematically derived using the path rules by quantifying the effects of all indirect paths to the variables of interest (Grace, 2006). Total effects are calculated by summing direct and indirect effects.

We chose to use a main effect model (no treatment interactions) to simplify interpretation and because few treatment interactions were observed in the data using ANOVA. We selected four measured variables describing key ecosystem functions for inclusion in our SEM: total available soil N ($NH_4 + NO_3$), total water use (evapotranspiration), SOM and NPP. Our *a priori* conceptual model included nearly all the possible interactions. We did not expect there to be important direct pathways between CO_2 and the soil community or between CO_2

and soil mineral N, because we reasoned that these effects would be strongly mediated through the plant community responses. Similarly, we anticipated that the effects of the N treatment on SOM would be mediated by the plant and soil communities, and we did not hypothesize a relationship between SOM and evapotranspiration (Fig. 1a; Table 1). We followed the recommendations of McCune & Grace (2002) and used ordination techniques to reduce data and synthesize informative single variables from multivariate datasets of plant community composition and soil community composition. We used nonmetric multidimensional scaling (NMDS) in conjunction with the Bray–Curtis distance measure to ordinate mesocosms based upon plant community composition (total biomass of plants by functional group) and soil community composition (PLFA data) independently. Axes were rotated to maximize correlation with downstream variables (i.e. NPP,

Table 1 Our expected (a) and observed (b) outcomes including our SEM direct, indirect and total effect coefficients for hypothesized causal relationships in the SEM

(a) Expected outcomes		(b) Observed outcomes (λ)		
		Direct path	Indirect paths	Total effect
Treatment effects				
*1. Enrichment of N will	affect soil community composition	**None**†	.15	.15
	affect plant community composition	.47	−.04	.44
	increase NPP	.50	.24	.74
	increase soil mineral N	.21	.03	.24
2. The presence of AM fungi will	affect soil community composition	.31	.16	.47
	affect plant community composition	.60	−.11	.48
	increase NPP	−**.22**	.27	.05
3. Elevated CO$_2$ will	affect plant community composition	.06	None	.06
	increase NPP	.10	.03	.13
	decrease evapotranspiration	−.32	−.02	−.34
Community-mediated effects				
4. Soil community will	affect plant community composition	−.34	.02	−.32
Plant community will	affect soil community composition	.24	−.02	.22
5. Soil community will	affect NPP	**None**	None	None
	affect SOM	.32	.05	.27
	affect soil mineral N	**None**	−.07	−.07
	affect evapotranspiration	−.30	.04	−.26
6. Plant community will	affect NPP	.56	.04	−.52
	affect SOM	.09	−.09	.19
	affect soil mineral N	.44	−.15	.29
	affect evapotranspiration	**None**	−.16	−.16
Ecosystem interactions				
7. NPP will	increase SOM	None	None	None
	decrease soil mineral N	−.22	None	−.22
	increase evapotranspiration	−**.13**	**None**	−**.13**
8. SOM and soil N will influence each other. Direction unresolved		.34	None	.34

*Numbers on the expected outcomes correspond to arrows in the *a priori* model (Fig. 1a).
†Bold text highlights instances where the expected effect is different from the observed effect.
SEM, structural equation model; AM, arbuscular mycorrhizal; SOM, soil organic matter; NPP, net primary production.

SOM, evapotranspiration and soil mineral N). The scores of the ordination axis, which maximized correlations with downstream variables, were used to represent the variables in the model. Higher values of plant community composition reflect greater mycotrophic forb abundance relative to C$_4$ grasses, C$_3$ grasses and nonmycotrophic forbs. Likewise, lower values for soil community composition reflect higher abundance of AM fungi and some bacteria relative to other soil organisms.

We used AMOS 5.0 (SPSS Inc., 2003) to formulate a SEM consistent with our *a priori* model. As a confirmatory test of our hypotheses, we tested absolute fit of our models using the Bollen & Stine (1992) bootstrap test, the maximum likelihood χ^2 goodness-of-fit test and Jöreskog's goodness-of-fit index (GFI). In these χ^2 tests, low *P*-values indicate lack of fit; therefore, contrary to

most statistical tests, high *P*-values are generally considered a desirable fit. Pathways whose presence did not improve or harm model fit (when path coefficients (λ) were ≤ 0.05) were removed from the model to conserve parameters and maintain statistical power (Fig. 1b). We confirmed the fit of the final model using the previously described model fit tests and also the Shipley's D-sep test, which is not asymptotic and can be used with small sample sizes (Shipley, 2000b).

Statistical analyses

Mycorrhizal response variables (i.e. spores, root colonization and hyphal densities) were examined using two-way analysis of variance (ANOVA) with the nonmycorrhizal mesocosms removed from the model. All other response variables were analyzed using three-way ANOVA

with the blocking variable, chamber, nested within CO_2 level and modeled as a random effect (SAS-JMP for Windows, version 4.04, 2000). A paired *t*-test was conducted to compare mineral N measurements from April and October. The assumptions of normality and homogeneity of variances were tested by examination of the residuals and using the Shapiro Wilk's goodness-of-fit test and Levene's test, and appropriate transformations were used when necessary.

Plant community, spore community, PLFA and CLPP data were analyzed using multiple response permutation procedure (MRPP) and NMDS (McCune & Medford, 1999; PC-ORD for Windows, version 4.4, 1999). MRPP is a nonparametric method for testing multivariate differences among *a priori* groups (McCune & Medford, 1999). We used Bray–Curtis distance because it is most appropriate for ecological community data, and because it does not assume normality and does not interpret zeros in the data as similarity (Minchin, 1987). The reported test statistic is an 'A-value', which is the effect size describing within-group homogeneity, where zero indicates that heterogeneity within a sample is equal to that expected by chance. Positive values indicate within sample heterogeneity is higher than expected by chance, and negative values indicate lower within sample heterogeneity than expected by chance. Values above 0.1 are generally considered acceptable for ecological community data (McCune & Grace, 2002). The *P*-value is the probability that an observed difference is due to chance alone. To visualize differences among plant communities by treatment, we used NMDS. We calculated species richness, evenness and Shannon's diversity for plant and AM fungal communities using PC-ORD.

Results

SEM

Our *a priori* model satisfactorily fit the data (Fig. 1a and b; maximum likelihood $\chi^2 = 15.23$; $P = 0.71$; Bootstrap $P = 0.687$; D-sep $\chi^2 = 4.65$; $P > 0.995$; GFI = 0.939). The only alteration to the *a priori* model was the removal of four paths that did not contribute to the fit of the model and explained little or no variance. The omitted paths were from N treatment to soil community, from soil community to soil mineral N and NPP, and from plant community to evapotranspiration (Fig. 1b). Variance explained was relatively high for NPP ($R^2 = 0.75$), and plant community composition ($R^2 = 0.37$). Table 1 lists the observed path coefficients (λ) for direct, indirect and total effects for each of the hypothesized paths in our *a priori* model. Individual paths are discussed in the appropriate sections below.

Plant responses

Biomass responses of plant functional groups were generally consistent with expectations. There was an interaction between CO_2 and N, where C_3 grasses were smaller with N fertilization under ambient CO_2, but larger with N under elevated CO_2 ($F = 5.70$, $P = 0.02$; Fig. 2a). Biomass of C_3 grasses increased with elevated CO_2 ($F = 4.93$, $P = 0.03$) and N fertilization ($F = 7.03$, $P = 0.01$) and decreased with AM fungi ($F = 4.52$, $P = 0.04$; Fig. 2a). Biomass of C_4 grasses was relatively unresponsive to treatments, increasing slightly with AM fungi ($F = 3.58$, $P = 0.07$), but not changing in response to any other treatments (Fig. 2b). Mycotrophic forbs had higher biomass in both $+N$ ($F = 30.90$, $P < 0.0001$) and $+AM$ treatments ($F = 13.85$, $P = 0.0008$; Fig. 2c). Nonmycotrophic forbs had higher biomass with $+N$ treatments ($F = 114.73$, $P < 0.0001$), but lower biomass in $+AM$ treatments ($F = 16.73$, $P = 0.0003$; Fig. 2d). Mycotrophic legumes had higher biomass with AM fungi ($F = 5.42$, $P = 0.03$; Fig. 2e), whereas the nonmycotrophic legume had lower biomass in the $+N$ treatments ($F = 5.55$, $P = 0.02$; Fig. 2f).

Plant community composition as a whole was strongly altered by N fertilization ($A = 0.216$, $P < 0.0001$; Fig. 3a) and AM fungi ($A = 0.053$, $P = 0.01$; Fig. 3b) but did not change with elevated CO_2 ($A = -0.004$, $P = 0.64$; Fig. 3c) or with treatment interactions. These results are further illustrated by our causal SEM (Fig. 1a and b; Table 1), which shows that plant community composition responded to N fertilization (Fig. 1a, path 1; $\lambda = 0.47$) and AM fungi (Fig. 1a, path 2; $\lambda = 0.60$), whereas CO_2 had a minor direct influence on plant community composition (Fig. 1a, path 3; $\lambda = 0.06$).

Soil organisms

AM fungi. A total of 14 morphospecies of AM fungal spores were observed, all consistent with species previously found in Cedar Creek soils. As a whole, the AM spore community was unaltered by CO_2 ($A = -0.01$, $P = 0.95$), N treatments ($A = 0.01$, $P = 0.62$) or their interactions ($A = -0.03$, $P = 0.97$). Spore diversity measured by Shannon's diversity index was not affected by any treatment, but tended to decrease with N fertilization ($F = 3.46$, $P = 0.07$). Spore abundance of only one morphospecies, *Glomus clarum*, responded to treatment with reduced abundance under N fertilization ($F = 5.95$, $P = 0.03$). Root colonization by AM fungi in $+AM$ and $-AM$ mesocosms was $21.8 \pm 1.3\%$ and $1.5 \pm 0.6\%$, respectively. Within the mycorrhizal mesocosms, total root colonization ($F = 2.79$ $P = 0.10$) and number of vesicles ($F = 2.98$, $P = 0.10$) tended to decrease with N fertilization, and percent colonization
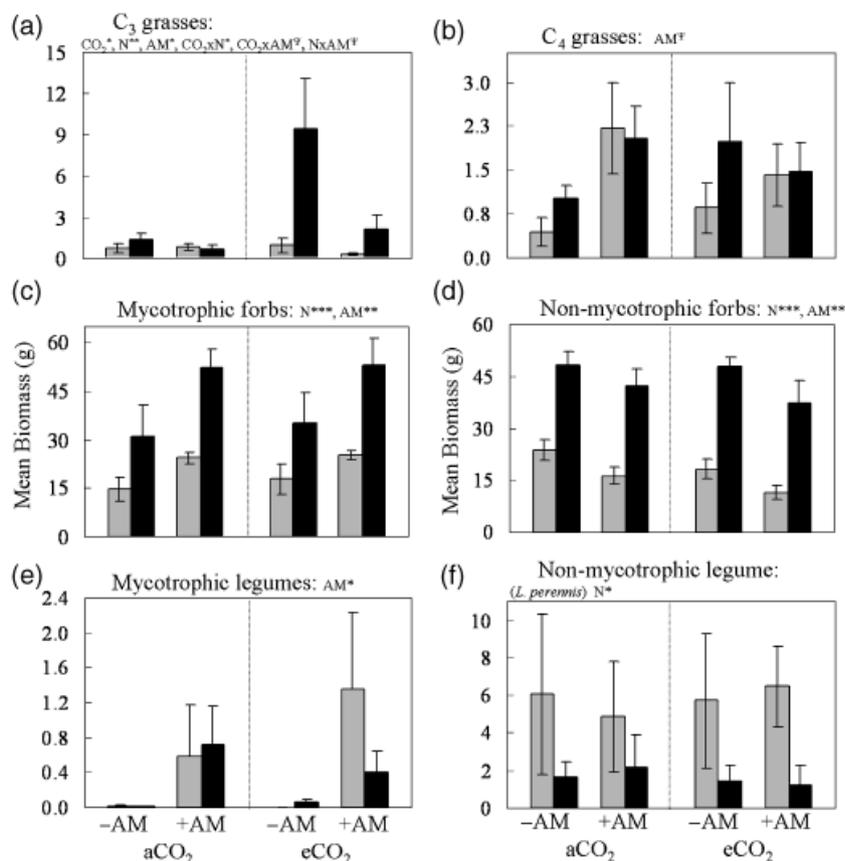
Fig. 2 Mean biomass of plants by functional group (mean $\pm$ 1 standard error, $n = 6$): (a) C_3 grasses, (b) C_4 grasses, (c) mycotrophic forbs, (d) nonmycotrophic forbs, (e) mycotrophic legumes and (f) nonmycotrophic legumes (in this case, only one species was present, *Lupinis perennis*). The graphs are split, with ambient CO_2 (aCO_2) on the left and elevated CO_2 (eCO_2) on the right. Each side is further split, with $-AM$ on the left and $+AM$ on the right. The $-N$ treatment is represented by gray bars and $+N$ treatment is represented by black bars. The significance level of treatments and interactions as tested by ANOVA are indicated as: $\Psi 0.10 \geq P > 0.05$, *$0.05 \geq P > 0.01$, **$0.01 \geq P > 0.001$, ***$0.0001 \geq P$.
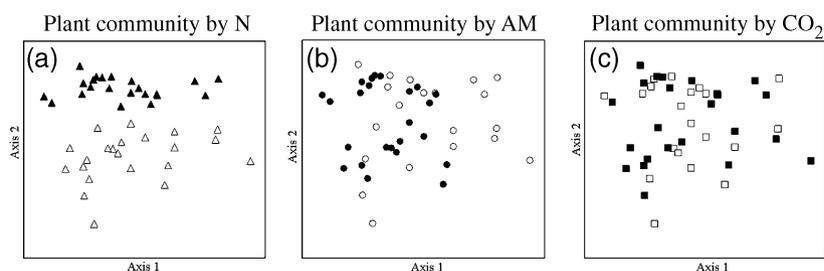
Fig. 3 Nonmetric multidimensional scaling ordinations of the response of the plant community to (a) N treatment, (b) arbuscular mycorrhizal (AM) treatment and (c) CO_2 treatment. Open symbols represent ambient N and CO_2 and the absence of AM fungi. Closed symbols represent elevated N and CO_2 and the presence of AM fungi.

by arbuscules was lower in N fertilized mesocosms ($F = 5.74$, $P = 0.03$; Fig. 4a and b). Total density of AM fungal hyphae in the soil was not changed by any treatment (CO_2; $F = 0.79$, $P = 0.40$, N: $F = 1.19$,

$P = 0.28$; Fig. 4c); however, the thick $> 6\,\mu m$ diameter) orange colored AM fungal hyphae, which may be indicative of the genus *Scutellospora* (Wolf, 2001; INVAM, 2007), were twice as abundant in ambient
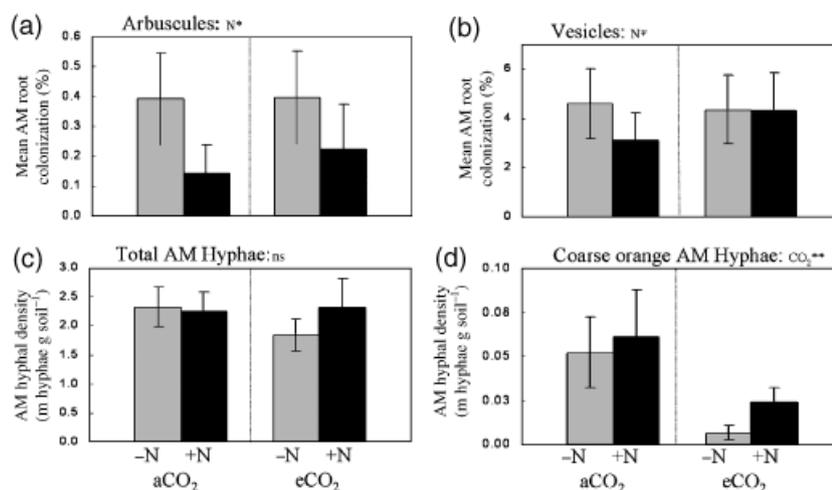
Fig. 4 Arbuscular mycorrhizal (AM) root colonization and density of AM fungal hyphae in the mesocosm soil (mean $\pm$ 1 standard error, $n = 6$): (a) arbuscules (b) vesicles, (c) total density of AM fungal hyphae in the soil and (d) density of coarse orange AM fungal hyphae in the soil. AM treatment effect was not tested here. Ambient CO_2 (aCO_2) is on the left and elevated CO_2 (eCO_2) on the right. The $-N$ treatment is represented by gray bars and $+N$ treatment is represented by black bars. The significance levels as tested by ANOVA are given in accordance with Fig. 2.

compared with elevated CO_2 ($F = 12.91$, $P = 0.005$; Fig. 4d).

Non-AM fungi. Septate fungal hyphae in the soil, produced by fungi that do not form AM symbioses, had greater length in elevated CO_2 ($F = 8.33$, $P = 0.01$) and $+$ AM mesocosms ($F = 4.49$, $P = 0.04$; Fig. 5a). Colonization of plant roots by non-AM fungi was 29% lower in ambient CO_2 (mean: 25.40, SE: 2.03) than in elevated CO_2 (mean: 17.81, SE: 1.42; $F = 11.81$, $P = 0.002$). A higher Shannon's diversity of morphotypes of non-AM fungi was found in the presence of AM fungi ($F = 4.37$, $P = 0.04$; Fig. 5b). There was also a significant interaction with CO_2 and N, where diversity was higher without fertilization at ambient CO_2 levels, but not at elevated CO_2 levels ($F = 4.09$, $P = 0.05$). Morphotypes responded differently to the treatments. Of five morphotypes found, three responded to treatments, one did not and one was too rare to test (Fig. 5c–f; photographs in Fig. 6). Morphotype 1 had higher colonization in $+$ AM mesocosms ($F = 5.66$, $P = 0.02$; Fig. 5c). Morphotype 2 had lower colonization in elevated CO_2 treatments ($F = 4.93$, $P = 0.03$) and higher colonization in $+$ AM treatments ($F = 4.63$, $P = 0.04$; Fig. 5d). *Olpidium* sp. had lower colonization in both elevated CO_2 ($F = 9.93$, $P = 0.004$) and $+$ AM treatments ($F = 6.39$, $P = 0.02$; Fig. 5e). The *Polymyxa*-like morphotypee was equally common in all treatments (Fig. 5f) and morphotype 3 was too rare to test (mean frequency: 0.92%).

Soil community. The community composition of soil microorganisms was affected by the presence or absence of AM fungi ($A = 0.10$, $P < 0.0001$) but not CO_2 ($A = -0.004$, $P = 0.67$) or N ($A = 0.002$ $P = 0.32$). An NMDS ordination of all PLFAs minus the indicator for AM fungi ($16:1\omega5$) showed a strong separation of the soil community by AM treatment (Fig. 7a). The PLFA indicators of the bacterial community also showed that AM fungi were affecting the bacterial community composition (Fig. 7b). However, the CLPP method, using ECO plates, showed no treatment effects on the metabolic activity of the culturable bacterial community (CO_2: $A = -0.006$, $P = 0.643$, N: $A = 0.02$, $P = 0.02$, AM: $A = -0.002$, $P = 0.52$; Fig. 7c). The same was true for the CLPP method for culturable fungi (SFN plates; CO_2: $A = -0.02$, $P = 0.004$, N: $A = -0.002$, $P = 0.58$, AM: $A = 0.001$, $P = 0.33$ Fig. 7d). Our SEM illustrates that the AM treatment was the only experimental factor to directly affect the soil community composition (Fig. 1a, path 2; $\lambda = 0.31$); however, N fertilization indirectly influenced it through the plant community (Fig. 1a, paths 1 and 4; $\lambda = 0.15$; Fig. 1b; Table 1).

Ecosystem responses

NPP. Nitrogen fertilization effectively doubled NPP from 144.57 to 303.26 g m^{-2} yr^{-1} ($F = 229.44$, $P < 0.0001$), but ANOVA results indicate that neither CO_2 enrichment nor AM fungi had a significant effect on NPP. The direct and indirect components of this response are elucidated by our SEM, where N enrichment led to a large direct increase in NPP (Fig. 1a, path 1; $\lambda = 0.50$) and CO_2 led to a minor direct increase in NPP (Fig. 1a, paths 3, 4 and 6;
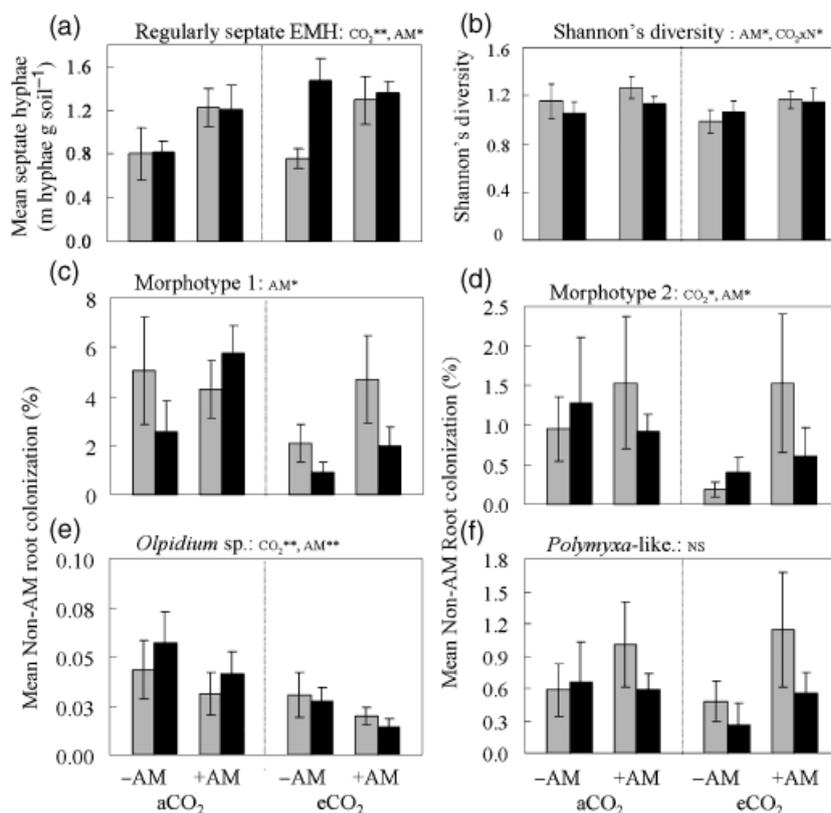
Fig. 5 Abundance and diversity of fungi other than arbuscular mycorrhizal (AM) fungi (mean ± 1 standard error, $n = 6$): (a) density of regularly septate hyphae in the soil, (b) Shannon's diversity of non-AM morphotypes. Percent root length colonized by (c) morphotype 1, (d) morphotype 2, (e) *Olpidium* sp. and (f) *Polymyxa*-like. The figure format follows that of Fig. 2.

$\lambda = 0.10$). There was a negative direct effect of AM fungi on NPP (Fig. 1a, paths 2, 4–6; $\lambda = -0.22$) related to reduced biomass of nonmycotrophic forbs in $+$ AM mesocosms (Fig. 2d). When the indirect effect of AM fungi on NPP through plant composition (Fig. 1a; $\lambda = 0.27$) is combined with the direct effect, the total effect of AM fungi on NPP becomes negligible (Fig. 1a, paths 2, 4 and 5; $\lambda = 0.05$). Similarly, when direct and indirect effects are combined, the total effect of CO_2 on NPP is small (Fig. 1a, paths 2, 4–6; $\lambda = 0.13$; Fig. 1b; Table 1). In contrast, N fertilization had a larger total effect when both direct and indirect influences were considered (Fig. 1a, paths 1, 4–6; $\lambda = 0.74$; Fig. 1b; Table 1).

Soil mineral nitrogen. Soil mineral nitrogen availability was different at the beginning (April) and end (October) of the growing season (NH_4; $t = 1.80$, $P = 0.04$, NO_3; $t = 1.66$, $P = 0.05$). In April, NH_4 was highest in N-fertilized mesocosms, but was not affected by other treatments ($F = 7.30$, $P = 0.01$; Fig. 8a). April NO_3 was lower with elevated CO_2 ($F = 16.00$, $P = 0.0003$) and higher with N fertilization ($F = 8.56$, $P = 0.006$; Fig. 8b).

In addition, there was a CO_2 by AM interaction; NO_3 was higher with mycorrhizas at ambient CO_2, but lower with mycorrhizas at elevated CO_2 ($F = 3.35$, $P = 0.07$; Fig. 8b). October NH_4 and NO_3 were only affected by AM fungi, with higher soil N in $+$ AM mesocosms ($F = 3.45$, 4.51; $P = 0.07$, 0.04, respectively; Fig. 8c and d). Indirect effect calculations in our SEM show the same pattern and provide details about causal relationships (Table 1). Final mineral N was affected similarly by AM fungi (Fig. 1a, paths 2, 4–8; indirectly; $\lambda = 0.20$) and N fertilization (Fig. 1a, path 1; directly; $\lambda = 0.21$). Plant community composition (Fig. 1a; path 4; $\lambda = 0.44$) and NPP (Fig. 1a, path 7; $\lambda = -0.22$) also affected final N as hypothesized, whereas the soil community did not affect soil mineral N as expected (Fig. 1a, path 5). Soil mineral N covaried with SOM as expected (Fig. 1a, path 8; $\lambda = 0.34$; Fig. 1b; Table 1).

Evapotranspiration. Soil water content was affected by all treatments, but there were no interactions (Fig. 8e). Less water was added to mesocosms with elevated CO_2 ($F = 5.57$, $P = 0.04$) and mycorrhizas ($F = 3.28$, $P = 0.08$). More water was added to $+$ N mesocosms ($F = 70.80$,
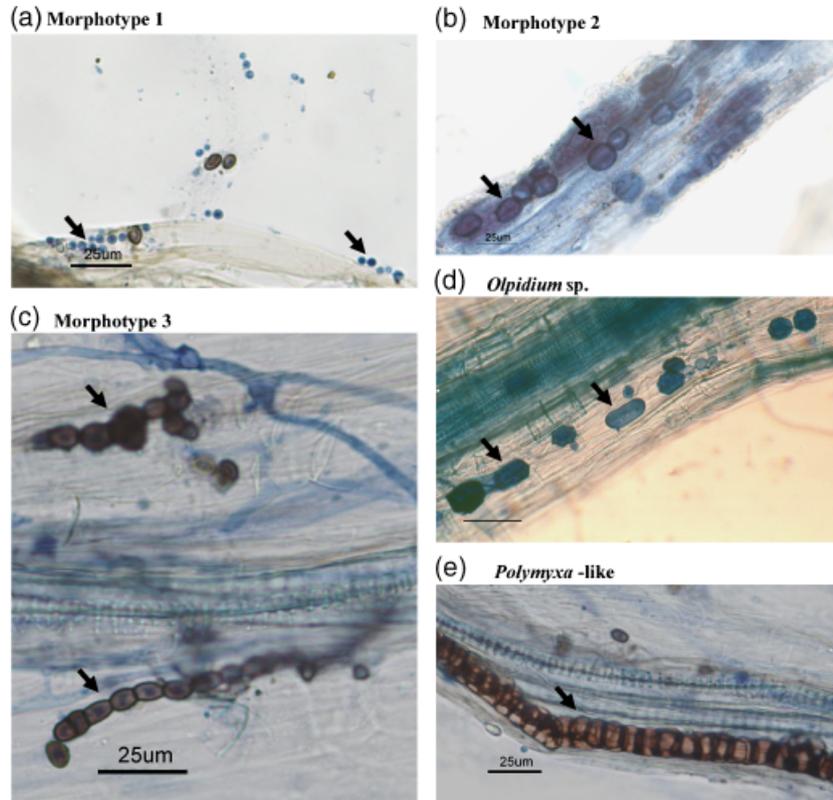
Fig. 6 Photographs of the five morphotypes of root endophytic fungi: (a) morphotype 1 (blue spheres), (b) morphotype 2 (blues spheres with double wall layer), (c) morphotype 3 (small blue-brown oblong spheres, often connected), (d) *Olpidium* sp. (double-walled hexagonal spheres or oblong thalli with opening at top or bottom) and (e) *Polymyxa*-like (small brown spheres with cells connected in three dimensions). All scale bars are 25 μm.

$P < 0.0001$), but this was confounded with increased plant biomass in the $+N$ treatment. When we divided water use by plant biomass, we found that $+N$ mesocosms actually consumed less when measured as water used per gram of plant biomass ($F = 24.42$, $P < 0.0001$; Fig. 7f). Our SEM showed that CO_2 (Fig. 1a, path 3; $\lambda = -0.32$), soil community (Fig. 1, path 5; $\lambda = -0.30$) and NPP (Fig. 1a, path 6; $\lambda = -0.13$) have direct impacts on evapotranspiration (Fig. 1b; Table 1).

SOM. The AM and N treatments did not directly affect SOM; however, elevated CO_2 was associated with reduced SOM ($F = 3.90$, $P = 0.05$). Our SEM showed that soil community composition is strongly related to SOM (Fig. 1, path 5; $\lambda = 0.32$) whereas plant community composition had a minor effect (Fig. 1; path 6; $\lambda = 0.09$). In addition, indirect paths from AM fungi (Fig. 1a, paths 2, 4–8; $\lambda = 0.20$) and N (Fig. 1a, paths 1, 4–8; $\lambda = 0.09$) also led to higher SOM (Fig. 1b; Table 1).

Discussion

Plant community and ecosystem responses to CO_2 and N enrichment and AM fungi

Our study demonstrated that AM fungi have impacts far beyond the immediate symbiosis, affecting the composition of both above- and belowground communities (Figs 3 and 7), and available soil N (Fig. 8). Biomass of five of our six plant functional groups was influenced by the presence of AM fungi (Fig. 2). Finding mycorrhizal effects on plant community composition corroborate with our first year results (Johnson *et al.*, 2003b) and also the results of other studies (van der Heijden *et al.*, 1998; Hartnett & Wilson, 1999). However, the nature of the mycorrhizal effect on plant community composition differed between the 2 years of our study and this generated a reversal of outcomes on NPP. In both years, AM fungi increased the biomass of C_4 grasses and mycotrophic forbs, and decreased the biomass of C_3 grasses. In contrast, the effect of mycorrhizas on the nonmycotrophic forb *S. kali* was opposite in
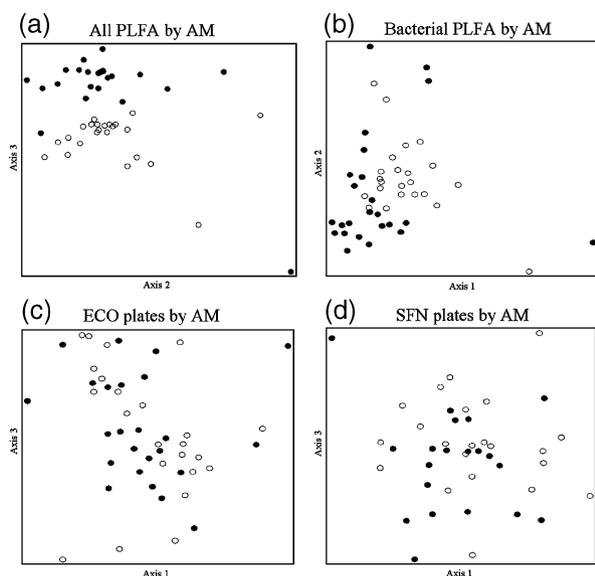
Fig. 7 Nonmetric multidimensional scaling ordinations of the response of various microbial community components to arbuscular mycorrhizal (AM) treatment: (a) phospholipid fatty acid (PLFA) fingerprint, (b) bacterial PLFA fingerprint, (c) ECO (bacterial) plates and (d) SFN (fungal) plates. Open circles indicate -AM mesocosms and closed circles indicate +AM mesocosms.

years 1 and 2. In the first year, biomass of *S. kali* was higher in mycorrhizal mesocosms but in the second year, it was reduced by AM fungi. This resulted in a reversal of AM effects on NPP in the second year. It is likely that several factors contributed to this result. By the second year, the network of AM fungal hyphae was more thoroughly established compared with the first year, so the detrimental effects of AM fungi on nonmycotrophic forbs were probably stronger (Allen *et al.*, 1989; Johnson, 1998). Also, plant communities in the second year were strongly impacted by winter mortality and subsequent reseeding. Grasses that were planted as seedlings dominated the plant communities during the first year, whereas forb species that set seed or produced tillers in year 1 dominated the communities in year 2. Finally, we increased the frequency of watering in the second year which may have favored forbs over grasses.

In April, N fertilization increased NH_4 and NO_3 availability as expected (Fig. 8a and b). In contrast, at the end of the second growing season, the N availability of fertilized and unfertilized mesocosms did not differ; instead, mycorrhizal mesocosms had higher available soil NH_4 and NO_3 compared with nonmycorrhizal mesocosms (Fig. 8c and d). This response is likely related to mycorrhizal effects on plant community composition. Mesocosms without mycorrhizas had a high biomass of *S. kali* and *B. incana*, two nitrophilic, nonmycotrophic forbs, whereas plant communities in

mesocosms with AM fungi were dominated by slow growing, mycotrophic forbs and C_4 grasses. It is likely that the ruderal plants in the nonmycorrhizal mesocosms sequestered more N in aboveground tissues and this reduced soil N availability to a greater degree than in the mycorrhizal mesocosms with lower populations of these nitrophilic plants.

As expected, N enrichment doubled NPP and strongly influenced plant community composition (Fig. 3). Biomass of C_3 grasses and forbs was strongly enhanced by N while the biomass of the legume *Lupinus* was reduced by N (Fig. 2). Also, CO_2 enrichment reduced evapotranspiration as expected. However, compared with the strong effects of AM fungi and N, CO_2 enrichment had a relatively small impact on community structure and ecosystem functions. Except for a biomass response by C_3 grasses, there were no CO_2 main effects or interactions in the second year of this experiment. During the first year, plant species richness was highest in the mycorrhizal mesocosms at elevated CO_2 due to higher survival of slow growing prairie forbs in this treatment (Johnson *et al.*, 2003b); however, this effect was not present in the second year. Other researchers have also observed that CO_2 effects diminish with time (Comins & McMurtrie, 1993; Klironomos *et al.*, 2005). Several factors may have generated this result in our system. In addition to the differences in plant community composition caused by differential recruitment described previously, we suspect that fewer CO_2 effects were observed in the second year because mesocosms received an average of 7 L (17%) more water. During the first year, mesocosms were watered 3 days each week, but during the second year, they were watered 4 days each week. Consequently, the mesocosms were less water stressed during the second year. Plants generally benefit less from elevated CO_2 when water is not limiting and stomatal water loss is less problematic (Field *et al.*, 1995). We expect that an interaction between CO_2 availability and mycorrhizas is also more likely to occur in water limited conditions, because for plants to maintain higher photosynthetic rates under elevated CO_2 requires more mineral nutrients and water supplied by AM symbioses (Treseder, 2004).

Soil community responses to mycorrhizas, CO_2 and N

Microscopy of plant roots and PLFA analyses of soils indicate that the belowground community was strongly impacted by the AM fungal treatment (Figs 5–7). Little is known about the ecology of non-AM root endophytes, but recent molecular studies suggest that these cryptic fungi are remarkably abundant and diverse in grassland ecosystems (Vandenkoornhuyse *et al.*, 2003,
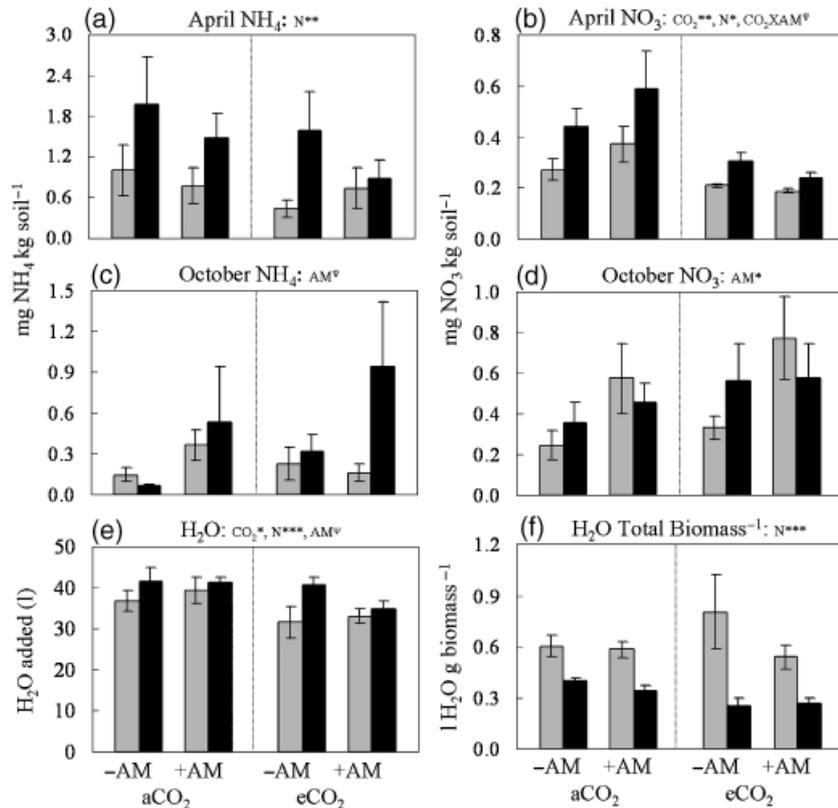
Fig. 8 Mean mineral nitrogen and water usage (means $\pm$ 1 standard error): (a) April NH_4, (b) April NO_3, (c) October NH_4, (d) October NO_3, (e) liters of water added and (f) liters of water added per gram plant biomass. The figure format follows that of Fig. 2.

2007). We found that three out of five morphotypes of root-inhabiting fungi were influenced by AM fungi and two morphotypes were influenced by CO_2 levels (Fig. 5). These fungi are not known to be pathogens and no root necrosis was observed. The density of non-AM (septate) hyphae in the soil was also responsive to AM and CO_2 treatments. Morphotypes of root fungi were individualistic in their responses to AM fungi and CO_2, so it is not possible to make generalizations about the effects of these treatments. However, the interaction between CO_2 and N levels on the diversity of root endophytes suggests that C : N ratios may influence endophypte community composition. These results warrant further experimentation to better understand the ecology of endophytic root fungi. This is especially important because of the possible role of dark septate endophytes (DSEs) in influencing ecosystem processes and interacting with other soil community members, particularly AM fungi (Jumpponen & Trappe, 1998; Jumpponen, 2001; Mandyam & Jumpponen, 2005). Although we do not know definitively, many of our non-AM endophytes may be classified as DSEs (Hasel-wandter & Read, 1982; Yu *et al.*, 2001).

Our PLFA data indicate that the composition of soil bacteria in our mesocosms was influenced by our AM treatment regardless of CO_2 or N treatments (Fig. 7). Bacteria and AM fungi are known to compete for resources (Christensen & Jakobsen, 1993) and potentially influence decomposition (Hodge, 2001; Langley & Hungate, 2003). Alternatively, it is possible that the initial AM fungal inoculum could have differentially introduced certain microorganisms despite our efforts to equalize the communities using microbial washes. Regardless of their initial origins, our SEM indicates that the species composition of soil organisms has ramifications on ecosystem properties, most notably the accumulation of SOM (Fig. 1b). The absence of significant CLPP responses to our experimental treatments is curious (Fig. 7c and d). This finding may be interpreted as functional redundancy in utilization of carbon substrates by the different microbial communities (Andrén *et al.*, 1995); or alternatively, our CLPP assay was not sensitive enough to distinguish differences among our treatments (Garland, 2000).

Insights from the SEM

Our SEM (Fig. 1; Table 1) shows that enrichment of CO_2 and N affect ecosystem processes both directly and indirectly through their influence on community com-

position. Sometimes, the community-mediated effects are greater than the direct effects. For example, plant community composition had a stronger influence on soil mineral N availability than the application of N fertilizer; and soil community composition influenced evapotranspiration to almost the same degree as the CO_2 treatment. Contrary to our *a priori* expectations, SOM was not related to NPP; instead, it was strongly related to the community of soil organisms. It is not unrealistic to expect NPP and SOM to be poorly related over a short-term experiment. However, finding such a strong effect of the composition of soil organisms to SOM accumulation is noteworthy and further study is needed to elucidate the mechanisms for this observed effect.

The SEM provided a means to model feedbacks between plant and soil community composition, where plant community positively affected soil community, and soil community negatively affected plant community. In our mesocosms, as mycotrophic forbs increased, so did the relative abundance of $16:1\Omega5$, a signature fatty acid for AM fungi, and as the abundance of AM fungi increased, the relative abundance of nonmyco-trophic forbs decreased. This finding is in accordance with the work by Bever *et al.* (1997) showing the importance of positive and negative feedbacks between communities of plants and AM fungi.

SEM helps synthesize disparate data and visualize complex relationships among biotic and abiotic factors. Combining traditional ANOVA methods with a SEM provided us many insights that would have been missed if only one of these approaches had been used. We were able to identify and classify all of the plants in our experimental system, but the identities of the microorganisms in the soil communities are largely unknown or simply coarse descriptions based upon their root colonization morphology, their fatty acid profile or their use of carbon substrates. Despite our limited knowledge of soil organisms, our study has shown a high degree of interconnectedness of above- and belowground communities. We also have shown that the composition of these communities can impact ecosystem responses to global change.

Realistic predictions of community and ecosystem responses to global change are challenging because of complex interactions among a myriad of above- and belowground factors that are difficult to experimentally control or measure at a large scale (Wolters *et al.*, 2000). Our study shows that mesocosms provide a means to manipulate some of these factors, and SEM provides a method to test hypotheses about the relative strength of direct and indirect relationships among community and ecosystem responses. We acknowledge that the dynamics of model ecosystems are contingent on their components and on the environmental conditions under which they were maintained. Similarly, the predictive value of a SEM is contingent on the causal structure imposed by the modelers. In constructing our mesocosms, we recreated Cedar Creek grasslands to the best of our ability, but concede that they are not perfect recreations, and that extrapolation from mesocosm experiments to the field should be made with caution. Nevertheless, we are encouraged by the fact that many of our findings corroborate with the findings of field studies (Reich *et al.*, 2001; Wolf *et al.*, 2003; Treseder, 2004) and the predictions of our *a priori* SEM. Also, the hypothesized causal relationships that were not supported by our data are insightful as warnings that our current concepts of interrelationships may be inadequate. In this regard, our final SEM should be considered an *a priori* model to be confirmed with future experiments.

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