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## Microbial community composition and function beneath temperate trees exposed to elevated atmospheric carbon dioxide and ozone

Received: 1 August 2001 / Accepted: 13 December 2001 / Published online: 14 February 2002  
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**Abstract** We hypothesized that changes in plant growth resulting from atmospheric CO<sub>2</sub> and O<sub>3</sub> enrichment would alter the flow of C through soil food webs and that this effect would vary with tree species. To test this idea, we traced the course of C through the soil microbial community using soils from the free-air CO<sub>2</sub> and O<sub>3</sub> enrichment site in Rhinelander, Wisconsin. We added either <sup>13</sup>C-labeled cellobiose or <sup>13</sup>C-labeled *N*-acetylglucosamine to soils collected beneath ecologically distinct temperate trees exposed for 3 years to factorial CO<sub>2</sub> (ambient and 200 μl l<sup>-1</sup> above ambient) and O<sub>3</sub> (ambient and 20 μl l<sup>-1</sup> above ambient) treatments. For both labeled substrates, recovery of <sup>13</sup>C in microbial respiration increased beneath plants grown under elevated CO<sub>2</sub> by 29% compared to ambient; elevated O<sub>3</sub> eliminated this effect. Production of <sup>13</sup>C-CO<sub>2</sub> from soils beneath aspen (*Populus tremuloides* Michx.) and aspen-birch (*Betula papyrifera* Marsh.) was greater than that beneath aspen-maple (*Acer saccharum* Marsh.). Phospholipid fatty acid analyses (<sup>13</sup>C-PLFAs) indicated that the microbial community beneath plants exposed to elevated CO<sub>2</sub> metabolized more <sup>13</sup>C-cellobiose, compared to the microbial community beneath plants exposed to the ambient condition. Recovery of <sup>13</sup>C in PLFAs was an order of magnitude greater for *N*-acetylglucosamine-amended soil compared to cellobiose-amended soil, indicating that substrate type influenced microbial metabolism and soil C cycling. We found that elevated CO<sub>2</sub> increased fungal

activity and microbial metabolism of cellobiose, and that microbial processes under early-successional aspen and birch species were more strongly affected by CO<sub>2</sub> and O<sub>3</sub> enrichment than those under late-successional maple.

**Keywords** Soil microorganisms · Carbon-13-phospholipid fatty acid analysis · Elevated carbon dioxide · Elevated ozone · Soil carbon cycling

### Introduction

Human activity has increased the concentration of CO<sub>2</sub> and O<sub>3</sub> in the earth's troposphere (Barnola et al. 1995; Finlayson-Pitts and Pitts 1997), and each of these trace gases has the potential to modify photosynthesis and plant growth across broad geographic regions, albeit in opposing ways (Curtis 1996; Pye 1988). Understanding the response of forests to increasing concentrations of tropospheric CO<sub>2</sub> and O<sub>3</sub> is of particular importance, because these ecosystems contain a large proportion of the C stored on land (75%) and account for 42% of global net primary productivity (Schlesinger 1997). Elevated CO<sub>2</sub> often stimulates forest productivity, which is followed by greater soil C inputs through higher rates of litterfall, root turnover, and rhizodeposition (Pregitzer et al. 1995; DeLucia et al. 1999). In contrast, tropospheric O<sub>3</sub> is a phytotoxic pollutant detrimental to forest productivity (Pye 1988; Karnosky et al. 1996). An increase in this trace gas has the potential to diminish greater forest growth and C storage resulting from elevated atmospheric CO<sub>2</sub>. However, it is not known how CO<sub>2</sub> and O<sub>3</sub> interact to influence the C cycle of forest ecosystems.

Plant growth, belowground C allocation, and microbial metabolism are inextricably linked to C cycling within an ecosystem (Zak et al. 1993). Microorganisms mediate soil C cycling and rely on substrate inputs from plant detritus as sources of energy for cellular metabolism. Greater belowground C inputs beneath plants growing under elevated CO<sub>2</sub> are therefore expected to stimulate

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microbial metabolism of plant-derived substrates (Norby 1994; Pregitzer et al. 1995) and alter rates of C and N cycling in soil (Zak et al. 1993; Berntson and Bazzaz 1998). As elevated CO<sub>2</sub> increases the flux of C from roots to soil (Van Veen et al. 1991; Canadell et al. 1996), there are a number of accompanying responses by soil microbial communities. These include greater turnover of soil bacteria and fungi (Hungate et al. 2000), enhanced bacterial utilization of substrates (Rillig et al. 1997), higher levels of microbial biomass (Williams et al. 2000), more rapid rates of respiration (Hungate et al. 2000; King et al. 2001) and increased extracellular enzyme activity (Dhillon et al. 1996; Larson et al., in press). Additionally, soil microbial communities may also be affected beneath trees exposed to elevated O<sub>3</sub>. Damage to the photosynthetic system in O<sub>3</sub>-sensitive plant species could reduce productivity and soil C inputs, thus counteracting the effects of elevated CO<sub>2</sub> on microbial metabolism in soil.

We have been studying the influence of elevated CO<sub>2</sub> and O<sub>3</sub> on the growth of contrasting temperate tree species at the Rhinelander, Wisconsin, free-air CO<sub>2</sub>-O<sub>3</sub> enrichment (FACE) site (King et al. 2001; Dickson et al. 2000). Several pieces of evidence suggest that soil C cycling has been altered by changes in litter production, particularly litter originating from fine roots. We have observed that elevated CO<sub>2</sub> significantly increased fine root production, soil respiration (King et al. 2001), and the activity of enzymes involved with the degradation of plant and fungal cell wall (i.e., cellobiohydrolase and *N*-acetylglucosamidase, respectively). These effects were largely eliminated by elevated O<sub>3</sub> (King et al. 2001; Larson et al., in press). Greater rates of plant and fungal cell wall degradation under elevated CO<sub>2</sub>, along with greater soil C inputs and respiration, may be linked to changes in microbial community composition and rates of substrate metabolism (Sinsabaugh 1994; Carreiro et al. 2000).

Because plants are known to differ in their growth response to elevated CO<sub>2</sub> and O<sub>3</sub>, we reasoned that species-specific differences in litter inputs would control the magnitude of the microbial response beneath plants grown under elevated CO<sub>2</sub> and O<sub>3</sub> (Hungate et al. 1996; Hättenschwiler and Körner 2000). Quaking aspen (*Populus tremuloides* Michx.) and paper birch (*Betula papyrifera* Marsh.) are early-successional species that are adapted to high light conditions and grow more quickly than the late-successional sugar maple (*Acer saccharum* Marsh.). If microbial activity is primarily regulated by soil C inputs, then the effect of elevated CO<sub>2</sub> and O<sub>3</sub> on microbial metabolism would be greater for aspen and birch species than for maple.

We amended soils from our FACE experiment with <sup>13</sup>C-labeled cellobiose and *N*-acetylglucosamine to determine how changes in plant litter inputs under elevated CO<sub>2</sub> and O<sub>3</sub> have altered microbial metabolism and the flow of C through the heterotrophic microbial community. We hypothesized that microbial metabolism of these substrates would parallel previously observed changes in

cellobiohydrolase and *N*-acetylglucosamidase activities, with greater rates of metabolism occurring beneath plants growing under elevated CO<sub>2</sub>. We also reasoned that O<sub>3</sub> would dampen this effect, a pattern consistent with fine root litter production (King et al. 2001), soil respiration (King et al. 2001), and microbial enzyme activity (Larson et al., in press).

## Materials and methods

### Study site and sample collection

Our study was conducted at the FACE site located in Rhinelander, Wisconsin (45°40.5'N, 89°37.5'E) (Dickson et al. 2000). This 32-ha facility consists of 12 circular, 30-m-diameter rings that are spaced 100 m apart to minimize between-ring drift of CO<sub>2</sub> and O<sub>3</sub>. Each ring is split into three community types, and each ring section is planted with an equal density of trees. One half of each ring was planted with five aspen clones of varying sensitivity to O<sub>3</sub>; one quarter of each ring was planted with birch and aspen; the remaining quarter was planted with maple and aspen. A total of 670 trees (currently ~2 m in height) were planted in each ring in 1997. This is a split-plot, randomized, complete block design, in which there are three replicates of factorial atmospheric CO<sub>2</sub> and O<sub>3</sub> treatments; the three tree communities split the CO<sub>2</sub>-O<sub>3</sub> main plots.

The FACE system consists of a high-volume blower, a plenum pipe for air distribution and a circular arrangement of 32 vertical vent pipes that compose each FACE ring. CO<sub>2</sub> and O<sub>3</sub> treatments were applied during the growing season in 1998, 1999, and 2000. Elevated-CO<sub>2</sub> rings were fumigated with ~560 μl CO<sub>2</sub> l<sup>-1</sup>, which is 200 μl CO<sub>2</sub> l<sup>-1</sup> above ambient atmospheric CO<sub>2</sub>. Elevated-O<sub>3</sub> rings were fumigated with ~55 nl O<sub>3</sub> l<sup>-1</sup>, or 20 nl O<sub>3</sub> l<sup>-1</sup> above average ambient atmospheric O<sub>3</sub>. Monitoring and control equipment maintained target CO<sub>2</sub> and O<sub>3</sub> concentrations with real-time computer algorithms linked to analyzers by fiber-optic connections. A complete description of hardware and performance is found in Dickson et al. (2000).

Soils at the site are mixed, frigid, coarse loamy Alfic Haplorthods. The sandy loam A horizon (~15 cm thick) grades into a loamy B horizon (~30 cm thick) and then into a sandy loam C horizon containing stratified sand and gravel. Physical and chemical soil properties are summarized in Table 1. In general, soil properties varied little across the site, but total soil C and N averaged slightly higher in the elevated CO<sub>2</sub> and CO<sub>2</sub>+O<sub>3</sub> treatments (Dickson et al. 2000).

In September 2000, we collected six soil cores (3 cm in diameter and 10 cm in depth) at random locations from inside each ring section (i.e., split plot). Cores were composited by ring section, homogenized, transported to the laboratory on ice, and stored at 4°C prior to analysis.

### <sup>13</sup>C labeling and incubation experiment

Within 48 h of field collection, soil subsamples (60 g) were amended with tracer (10 μg <sup>13</sup>C g<sup>-1</sup>) amounts of <sup>13</sup>C-labeled substrates, which were delivered in 4 ml deionized water. Thirty-six samples (one from each CO<sub>2</sub>×O<sub>3</sub>×species treatment combination) were incubated with <sup>13</sup>C-cellobiose (100% <sup>13</sup>C), and a duplicate set of 36 subsamples was incubated with <sup>13</sup>C-labeled *N*-acetylglucosamine (100% <sup>13</sup>C). Amended soil was incubated at 19°C for 10 h, a time period we had previously determined to be sufficient for microbial assimilation of the added labeled compounds. We also added deionized water to one additional subsample from each FACE ring to determine the natural abundance of <sup>13</sup>C in soil pools. Field soil moisture was determined by oven drying (70°C) additional subsamples. All subsequent analyses were performed on these <sup>13</sup>C-labeled and unlabeled soils.

**Table 1** Summary of physical and chemical soil properties prior to treatment (July 1997) at the free-air CO<sub>2</sub> and O<sub>3</sub> enrichment site in Rhinelander, Wisconsin. Values are means with SEMs in parentheses (King et al. 2000)

Treatment	Ambient O <sub>3</sub>		Elevated O <sub>3</sub>	
	Ambient CO <sub>2</sub>	Elevated CO <sub>2</sub>	Ambient CO <sub>2</sub>	Elevated CO <sub>2</sub>
Soil texture				
% Sand	55.1 (2.1)	53.9 (1.5)	58.3 (1.1)	55.0 (1.7)
% Silt	36.1 (1.8)	37.8 (1.3)	35.3 (2.1)	37.4 (1.6)
% Clay	8.7 (0.75)	8.4 (0.58)	6.4 (1.1)	7.7 (0.40)
Bulk density	1.27 (0.08)	1.30 (0.08)	1.32 (0.09)	1.43 (0.06)
Total C (g C kg <sup>-1</sup> )	15.4 (1.6)	16.8 (1.9)	16.0 (1.8)	13.1 (1.2)
Total N (g N kg <sup>-1</sup> )	1.2 (0.1)	1.3 (0.2)	1.2 (0.2)	1.0 (0.0)
C:N	12.9 (0.5)	12.4 (0.2)	13.5 (0.4)	12.8 (0.4)

#### Microbial respiration

Microbial respiration was determined 1 h after substrate addition by placing the amended soils in 0.95-l mason jars with airtight lids and rubber septa for gas sampling. Using a gas-tight syringe, we withdrew a 30-ml aliquot of air from the headspace of each incubation vessel. We determined headspace CO<sub>2</sub> concentration and the δ<sup>13</sup>C of CO<sub>2</sub> using a Finnigan Delta Plus isotope ratio mass spectrometer (IRMS) with a ConFlo II interface (ThermoFinnigan, Bremen). The headspace concentration of CO<sub>2</sub> and δ<sup>13</sup>C was measured 4 times at 1-h intervals during the first 4 h of the experiment. The rate of respiration was calculated using the time-linear change in headspace CO<sub>2</sub> (μg CO<sub>2</sub> h<sup>-1</sup>) divided by the dry mass of soil. We determined the amount of substrate <sup>13</sup>C respired by multiplying the mass of C respired by atom % excess <sup>13</sup>C; the natural abundance of <sup>13</sup>C in microbial respiration was determined using the samples amended with deionized water. Analytical error for the mass spectrometer, based upon replicate measurements of a single homogenized soil sample, had a 1% coefficient of variation. Analysis of internal standards indicated an analytical error of <5%.

#### Dissolved organic C and soil organic matter

Subsamples (20 g) from each of the 72 amended soils were extracted with 40 ml of 0.5 M K<sub>2</sub>SO<sub>4</sub> to determine dissolved organic C (DOC). We evaporated extracts and determined C and δ<sup>13</sup>C by mass-spectrometry using a CE Elantech NC2500 interfaced to a Finnigan Delta Plus IRMS. The remaining extracted soil was oven dried and pulverized with a ball mill. The C and δ<sup>13</sup>C of the extracted soil was determined by mass-spectrometry as described above. These values correspond to the amount of labeled substrate that had been incorporated into the soil organic matter (SOM).

The excess <sup>13</sup>C recovered in SOM and DOC was determined by multiplying the concentration of C (μg C g<sup>-1</sup>) by the mass of soil (g) and its atom % excess <sup>13</sup>C (measured atom % <sup>13</sup>C minus natural abundance atom % <sup>13</sup>C). The natural abundance of <sup>13</sup>C was determined from samples receiving only deionized water. Excess <sup>13</sup>C (μg <sup>13</sup>C) represents the total amount of <sup>13</sup>C recovered in SOM and DOC for each soil sample.

#### <sup>13</sup>C-phospholipid fatty acid analysis

At the end of the 10-h incubation, soil subsamples (10 g) were placed at -70°C overnight, and then freeze-dried for phospholipid fatty acid (PLFA) analysis. Lipids were extracted with a single-phase solvent system modified to include a PO<sub>4</sub><sup>3-</sup> buffer (Bligh and Dyer 1959; White et al. 1979). Silicic acid column chromatography was used to fractionate the total lipid extract into neutral, glyco- and polar lipids (Gehron and White 1983). Silicic acid columns were placed on a vacuum manifold system to facilitate elution (Burdick and Jackson, Muskegon, Mich.). Polar lipids were then subjected to a mild alkaline reagent that contained methanol to form fatty acid methyl esters (FAMES), which were separated and quantified using a Finnigan Delta plus mass spectrometer with a GC/C III interface (ThermoFinnigan) coupled to a HP 5973 GC (Agilent Technologies, Palo Alto, Calif.) (sensu Boschker et al. 1998). Chromatographic peaks were quantified based on a 19:0 standard, and double-bond locations in monounsaturated PLFA were confirmed by GC-MS analysis of their dimethyldisulfide adducts. A standard qualitative bacterial FAME mix (Matreya, Pleasant Gap, Pa.) also was used to identify and quantify FAMES by chromatographic retention time (Ringelberg et al. 1997). The abundance and δ<sup>13</sup>C of individual FAMES were expressed as μg PLFA C g<sup>-1</sup> dry soil and as μg excess <sup>13</sup>C. Fatty acid nomenclature follows Ratledge and Wilkinson (1988).

The polyenoic, unsaturated PLFAs (18:2ω6, 18:1ω9c, and 18:3ω3) are biomarkers for soil fungi (Federle et al. 1986), and the monoenoic and cyclopropane unsaturated PLFAs (16:1ω5c, 16:1ω7c, 16:1ω9c, a16:0, cy17:0, 18:1ω7c and cy19:0a) are characteristic of Gram-negative bacteria. Straight-chain PLFAs that are non-specific bacterial biomarkers include 14:0, 16:0, and 18:0. We used branched, saturated PLFAs (10me16:0, 15:0, i15:0, a15:0, i16:0, 17:0, i17:0 and a17:0) to indicate the presence of Gram-positive bacteria in soil (Frostegård and Bååth 1994; Grayston et al. 2000). We used total PLFA as an index of living microbial biomass.

The relative abundance of each PLFA (% of total PLFA C) was used to compare microbial community composition among treatments. The excess <sup>13</sup>C recovered in each PLFA was determined by multiplying the mass of PLFA C (μg C g<sup>-1</sup>) by the mass of soil (g) and its atom % excess <sup>13</sup>C (measured atom % <sup>13</sup>C minus natural abundance atom % <sup>13</sup>C). Natural abundance of <sup>13</sup>C in each PLFA was determined from samples receiving only deionized water. We used the ratio of <sup>13</sup>C in fungal versus bacterial PLFAs to assess whether our experimental treatments altered the metabolic activity of fungi, relative to bacteria (Bardgett et al 1996).

#### Statistical analyses

We used an ANOVA for a split-plot, randomized complete block design to test the influence of CO<sub>2</sub>, O<sub>3</sub> and species on the amount of <sup>13</sup>C recovered in soil respiration, PLFAs, DOC and SOM. Separate analyses were conducted for samples amended with cellobiose and *N*-acetylglucosamine. We used an identical ANOVA model to determine the influence of our experimental treatments on total PLFA and the proportion of PLFAs that are biomarkers for Gram-positive bacteria, Gram-negative bacteria, and fungi. Treatment effects were considered significant if *P*<0.05.

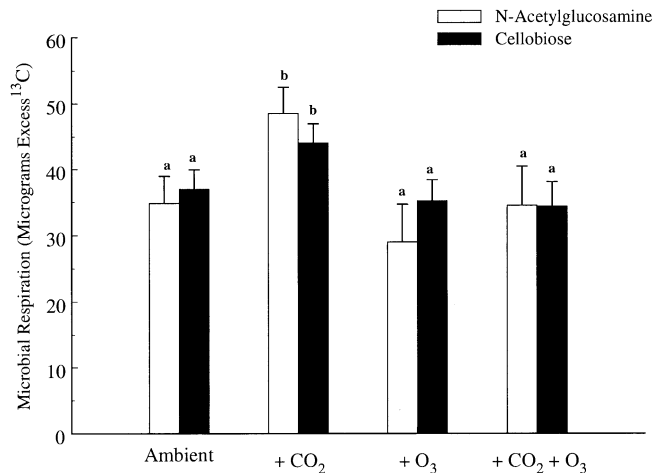
## Results

Total recovery of <sup>13</sup>C-cellobiose averaged 76% across CO<sub>2</sub>-O<sub>3</sub> treatment combinations, and average recovery of isotope in the <sup>13</sup>C-*N*-acetylglucosamine labeled samples averaged 85% (Table 2). Total recovery of isotope did not differ significantly among any treatment or treatment combination.

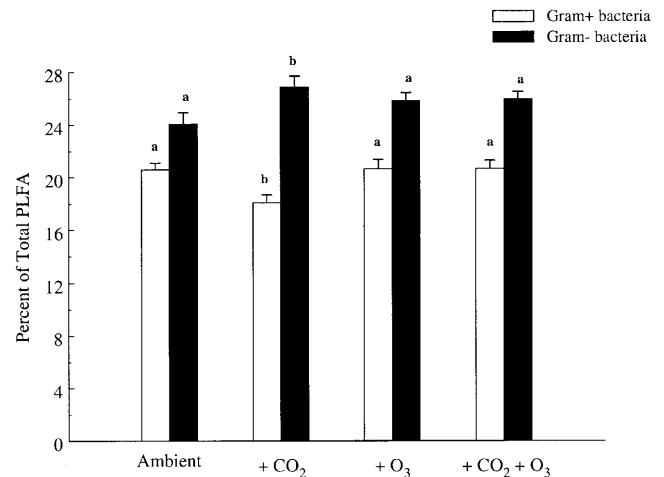
The addition of <sup>13</sup>C-labeled cellobiose or *N*-acetylglucosamine did not increase the rate of microbial respira-

**Table 2** Summary of  $^{13}\text{C}$  recovered from soils amended with cellobiose and *N*-acetylglucosamine by soil pool. Values are treatment means with SEMs in parentheses. Means in a row with different letters are significantly different. SOM Soil organic matter, DOC dissolved organic C, PLFA Phospholipid fatty acid

Treatment		Ambient	+CO <sub>2</sub>	+O <sub>3</sub>	+CO <sub>2</sub> +O <sub>3</sub>	<i>P</i>
Substrate		Mean (SEM)	Mean (SEM)	Mean (SEM)	Mean (SEM)	
		Recovery (%)				
Cellobiose	Respiration	6.16a (0.43)	7.34b (0.43)	5.87a (0.46)	5.74a (0.43)	0.0121
	SOM	60.55 (1.72)	66.35 (2.48)	60.16 (3.21)	66.02 (3.87)	0.0548
	DOC	4.08 (0.40)	3.00 (0.28)	3.64 (0.70)	5.13 (0.78)	0.3916
	PLFA	2.51(0.23)	3.22 (0.26)	2.79 (0.27)	3.28 (0.27)	0.1760
	Total	73.30 (1.69)	79.91 (2.60)	72.46 (3.78)	80.16 (4.05)	0.1107
<i>N</i> -Acetylglucosamine	Respiration	5.81a (2.10)	8.09b (0.57)	4.84a (0.82)	5.75a (0.86)	0.0021
	SOM	46.26 (1.29)	44.14 (1.57)	45.79 (1.51)	44.06 (1.28)	0.1507
	DOC	7.94 (0.50)	8.62 (0.20)	7.25 (0.37)	8.52 (0.16)	0.3535
	PLFA	27.51(0.59)	26.48 (1.78)	25.28 (2.10)	25.83 (2.13)	0.4588
	Total	86.25 (2.39)	85.38 (3.06)	82.57 (1.77)	82.31 (1.13)	0.2531



**Fig. 1** Mean excess  $^{13}\text{C}$ -cellobiose and *N*-acetylglucosamine recovered ( $\mu\text{g}$ ) in microbial respiration ( $n=9$ ). The length of each bar is 1 SE, and treatment means for each substrate that have the same letter are not significantly different



**Fig. 2** The abundance of Gram-positive (10me16:0, 15:0, i15:0, a15:0, i16:0, 17:0, i17:0, a17:0) and Gram-negative (16:1w5c, 16:1w7c, 16:1w9c, a16:0, cy17:0, 18:1w7c, cy19:0a) bacterial phospholipid fatty acids (PLFAs) in  $^{13}\text{C}$ -cellobiose-amended soil. The length of each bar is 1 SE, and treatment means for each bacterial group that have the same letter are not significantly different

tion ( $2 \mu\text{g C-CO}_2 \text{ h}^{-1} \text{ g}^{-1}$ ) relative to samples receiving deionized water, indicating that addition of the  $^{13}\text{C}$ -labeled substrates did not stimulate microbial activity. The amount of  $^{13}\text{C}$  recovered in microbial respiration increased beneath plants exposed to elevated CO<sub>2</sub> by 29%, but elevated O<sub>3</sub> dampened this response (Fig. 1); this was true for samples amended with cellobiose or *N*-acetylglucosamine. Although the magnitude of the CO<sub>2</sub> effect varied among species, we found no significant effect of species on rates of microbial respiration in soil amended with either substrate (data not shown). Nonetheless, microbial communities beneath aspen and aspen-birch exposed to elevated CO<sub>2</sub> respired greater amounts of  $^{13}\text{C-CO}_2$  than those beneath aspen-maple; this was true for both cellobiose and *N*-acetylglucosamine. Average recovery of both substrates in microbial respiration beneath aspen-maple ranged from 28 to 34  $\mu\text{g } ^{13}\text{C}$ , whereas the range broadened for aspen (34–49  $\mu\text{g}$ ) and aspen-birch (40–50  $\mu\text{g}$ ).

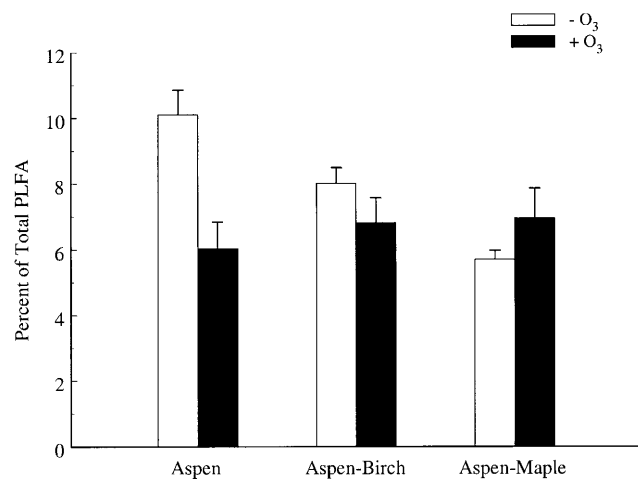
The greatest proportion of both labeled substrates was recovered in SOM (Table 2). Recovery of  $^{13}\text{C}$  in SOM

averaged 63% in cellobiose-amended soil and 45% in soil amended with *N*-acetylglucosamine; values were not significantly influenced by CO<sub>2</sub>, O<sub>3</sub>, species, or their interactions. Recovery of  $^{13}\text{C}$ -cellobiose in DOC ranged from 3 to 5% among treatments, and mean recovery varied from 7 to 9% in soils labeled with  $^{13}\text{C}$ -*N*-acetylglucosamine (Table 2). For each substrate,  $^{13}\text{C}$  recovery in DOC did not differ significantly among treatments.

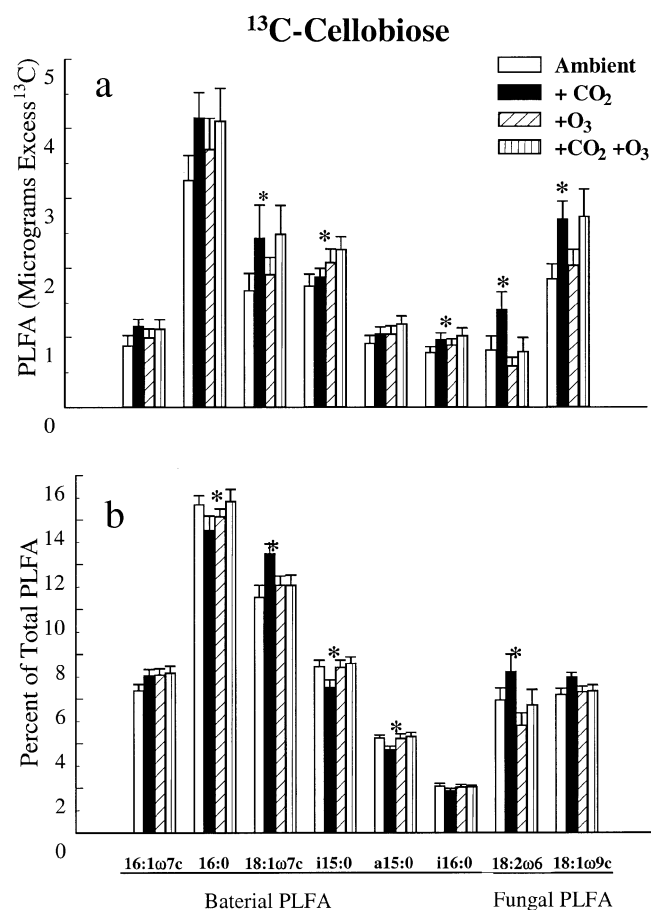
#### Community composition and substrate metabolism

In  $^{13}\text{C}$ -cellobiose-amended soil, the relative proportion of Gram-positive bacterial PLFAs decreased, whereas the relative proportion of Gram-negative bacterial PLFAs increased beneath plants fumigated with CO<sub>2</sub> (Fig. 2). This effect was dampened by elevated O<sub>3</sub>, because the relative proportion of Gram-positive and Gram-negative bacterial PLFAs beneath plants fumigated with CO<sub>2</sub> and O<sub>3</sub> was similar to the ambient treatment. We also found that reductions in soil fungi beneath ele-

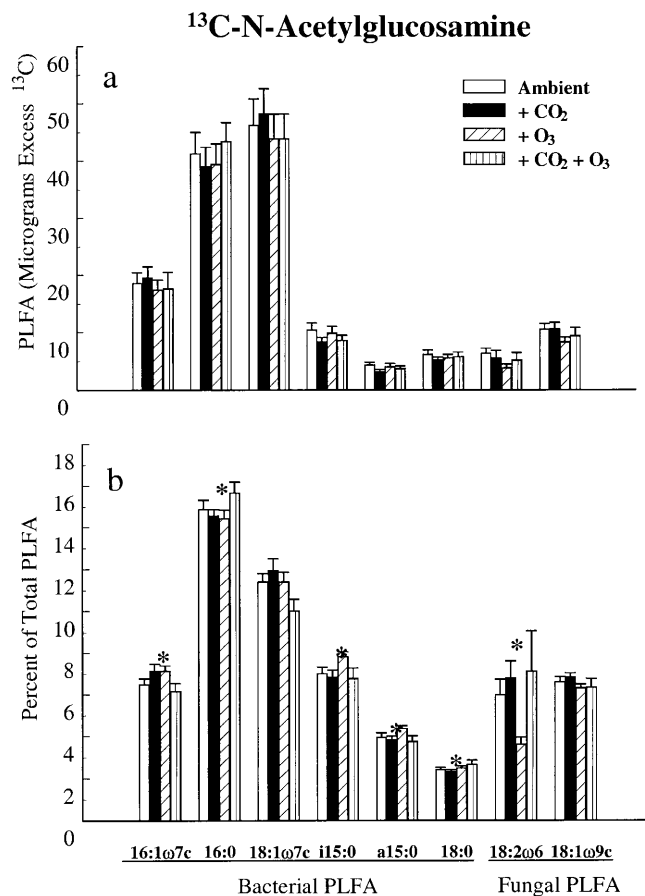




**Fig. 3** The influence of O<sub>3</sub> and species on the abundance of fungal PLFA in <sup>13</sup>C-cellobiose-amended soil. Values are treatment means and bars represent 1 SE. Elevated O<sub>3</sub> significantly lowered fungal PLFA in the aspen and birch-aspen treatments, but elevated O<sub>3</sub> had no effect on fungal PLFA in the maple-aspen treatment



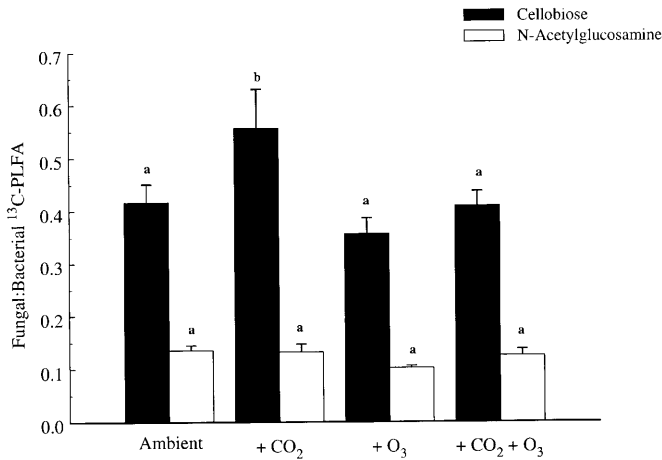
**Fig. 4** The amount of excess <sup>13</sup>C recovered in bacterial and fungal PLFA in soil labeled with <sup>13</sup>C-cellobiose (a), and the relative proportion of bacterial and fungal PLFAs in <sup>13</sup>C-cellobiose-labeled soil (b). Individual values are CO<sub>2</sub>×O<sub>3</sub> interaction means. Each PLFA identified with an asterisk contained significantly different amounts of <sup>13</sup>C (a) or differed in their proportion among the CO<sub>2</sub>×O<sub>3</sub> treatment combinations (see text for details)



**Fig. 5** The amount of excess <sup>13</sup>C recovered in bacterial and fungal PLFA in soil labeled with <sup>13</sup>C-N-acetylglucosamine (a), and the relative proportion of bacterial and fungal PLFAs in <sup>13</sup>C-N-acetylglucosamine-labeled soil (b). Individual values are CO<sub>2</sub>×O<sub>3</sub> interaction means. Each PLFA identified with an asterisk contained significantly different amounts of <sup>13</sup>C (a) or differed in their proportion among the CO<sub>2</sub>×O<sub>3</sub> treatment combinations (see text for details)

ated O<sub>3</sub> were evident for some tree species, as there was less fungal PLFA beneath aspen and aspen-birch growing under elevated O<sub>3</sub> than ambient O<sub>3</sub> (Fig. 3). Fungal PLFA in the aspen-maple treatment, on the other hand, was not affected by O<sub>3</sub> (Fig. 3). In contrast, we did not observe treatment differences in the relative proportion of fungal, Gram-positive, or Gram-negative bacterial PLFA in soils amended with <sup>13</sup>C-N-acetylglucosamine (data not shown).

Nine PLFAs contained 80% of excess <sup>13</sup>C recovered in PLFAs in both cellobiose- and N-acetylglucosamine-amended soil. These PLFAs included biomarkers for Gram-positive bacteria (a15:0, i15:0, i16:0), Gram-negative bacteria (16:1ω7c, 18:1ω7c), non-specific bacteria (16:0, 18:0), and fungi (18:2ω6, 18:1ω9c). In cellobiose-amended soil, five of these PLFAs (18:1ω7c, i15:0, i16:0, 18:2ω6, 18:1ω9c) contained a greater proportion of <sup>13</sup>C beneath plants exposed to elevated CO<sub>2</sub> (Fig. 4a). Lower recovery of <sup>13</sup>C-cellobiose in 18:2ω6 was found beneath plants exposed to elevated O<sub>3</sub>, compared to



**Fig. 6** The ratio of <sup>13</sup>C recovered in fungal versus bacterial PLFAs. Values are CO<sub>2</sub>×O<sub>3</sub> interaction means for soil labeled with <sup>13</sup>C-cellobiose (closed bars) or <sup>13</sup>C-N-acetylglucosamine (open bars). Bars are 1 SE, and means for each substrate that have the same letter are not significantly different

ambient O<sub>3</sub>, and O<sub>3</sub> also dampened the positive effect of CO<sub>2</sub>. O<sub>3</sub> had a contrasting effect on i15:0; more <sup>13</sup>C-cellobiose was metabolized beneath plants exposed to elevated O<sub>3</sub>. In *N*-acetylglucosamine-amended soil, identical analyses performed on the eight individual PLFAs representing 80% of total PLFA indicated that CO<sub>2</sub> and O<sub>3</sub> did not affect the recovery of <sup>13</sup>C in any individual PLFA (Fig. 5a).

Microbial community composition was significantly influenced by a CO<sub>2</sub>×O<sub>3</sub> interaction (Figs. 4b, 5b). In general elevated CO<sub>2</sub> increased the abundance of fungal PLFAs in both cellobiose- and *N*-acetylglucosamine-amended soil, but some increases were not statistically significant. Bacterial PLFAs exhibited mixed responses, wherein the elevated-CO<sub>2</sub> treatment increased (16:1ω7c, 18:1ω7c) and decreased (16:0, i15:0, a15:0) their relative abundance (Figs. 4b, 5b). The response of PLFAs to the O<sub>3</sub> and CO<sub>2</sub>+O<sub>3</sub> treatments was variable.

The ratio of fungal to bacterial <sup>13</sup>C-PLFA indicated that fungi metabolized proportionately more cellobiose than *N*-acetylglucosamine, despite similarities in the relative abundance of total fungal PLFA. We found that the fungal:bacterial <sup>13</sup>C-PLFA ratio for cellobiose-amended soil was greater beneath plants growing under elevated CO<sub>2</sub>, and that this effect was also dampened by elevated O<sub>3</sub> (Fig. 6). Treatment differences in fungal:bacterial <sup>13</sup>C-PLFA did not occur in *N*-acetylglucosamine-amended soil, and may in part be due to lower fungal:bacterial activity (Fig. 6). Mean fungal:bacterial <sup>13</sup>C-PLFA in *N*-acetylglucosamine-amended soil (~0.12) was much lower than recovery fungal:bacterial <sup>13</sup>C-PLFA (~0.44) in cellobiose-amended soil. Along with microbial respiration, the magnitude of these responses varied with species, with early-successional species exhibiting greater fungal:bacterial <sup>13</sup>C-PLFA than the late-successional maple.

## Discussion

Our study demonstrates that microbial communities beneath plants growing under elevated CO<sub>2</sub> respired greater amounts of <sup>13</sup>C-labeled cellobiose and *N*-acetylglucosamine, indicating altered rates of microbial metabolism driven by changes in litter production. Greater plant growth under elevated CO<sub>2</sub> is likely to provide more organic substrates for microbial metabolism in soil, fueling more rapid rates of soil C cycling (Zak et al. 2000). Higher rates of soil respiration in forests and grasslands exposed to elevated CO<sub>2</sub> are frequently reported (Hungate et al. 1997; Edwards and Norby 1998; Pregitzer et al. 2000), and signal increases in root biomass (Edwards and Norby 1998), fine root production production/mortality (Matamala and Schlesinger 2000; King et al. 2001), and microbial activity (Zak et al. 1993; Hungate et al. 1997). We observed that microbial respiration was 29% greater beneath plants growing under elevated CO<sub>2</sub>. Moreover, the effect of CO<sub>2</sub> was similar among plant species, yet the magnitude of the CO<sub>2</sub> effect varied with species. Production of <sup>13</sup>C-CO<sub>2</sub> was greatest beneath aspen and aspen-birch growing under elevated CO<sub>2</sub>, compared with aspen-maple, but this was not statistically significant. Moreover, the effect of elevated CO<sub>2</sub> was generally dampened by elevated O<sub>3</sub>, a response identical to that of soil enzyme activity (Larson et al., in press) and fine root production/mortality in our experiment (King et al. 2001).

*N*-acetylglucosamine was utilized for biosynthesis to a greater extent than cellobiose, as evidenced by greater incorporation of <sup>13</sup>C into PLFA in soil amended with *N*-acetylglucosamine than in soil amended with cellobiose. We amended soils with *N*-acetylglucosamine because we had previously observed greater *N*-acetylglucosaminidase activity beneath plants grown under elevated CO<sub>2</sub>, which indicated higher rates of fungal or bacterial cell wall degradation (Larson et al., in press). Although microbial respiration of *N*-acetylglucosamine increased beneath plants growing under elevated CO<sub>2</sub>, the amounts of <sup>13</sup>C incorporated into fungal and bacterial PLFAs increased proportionately in *N*-acetylglucosamine metabolism by these organisms. This was not the case for cellobiose metabolism, because fungal PLFAs contained more <sup>13</sup>C than bacterial PLFAs. We cannot determine how the metabolism of *N*-acetylglucosamine differed physiologically from cellobiose; however, it is clear that changes in the amounts and types of substrates entering soil under elevated CO<sub>2</sub> or O<sub>3</sub> influenced microbial metabolism and soil C flow.

The ratio of <sup>13</sup>C-cellobiose recovered in fungal:bacterial <sup>13</sup>C-PLFA increased from 0.5 to 0.7 in soils beneath plants growing under elevated CO<sub>2</sub>. Like microbial respiration, this response was dampened by elevated O<sub>3</sub>. In cellobiose-amended soil, higher fungal:bacterial <sup>13</sup>C-PLFA ratios corroborate reports that arbuscular mycorrhizal fungal biomass increases with CO<sub>2</sub> enrichment (Rillig et al. 2000), that fungal species composition shifts in favor of fungi that decompose cellulose (Jones

et al. 1998), and that fungi are more strongly stimulated by altered plant growth under elevated CO<sub>2</sub> than bacteria (Rillig 2000). Our experiment complements these data by demonstrating that the proportion of cellobiose metabolized by fungi increased to a greater extent than bacteria in soil beneath plants exposed to elevated CO<sub>2</sub>.

We also found that elevated CO<sub>2</sub> altered the composition of microbial communities without changing microbial biomass (i.e., total PLFA). In cellobiose-amended soil, PLFA biomarkers beneath plants growing under elevated CO<sub>2</sub> exhibited a proportional decrease in Gram-positive PLFAs and a proportional increase in Gram-negative PLFAs. Bruce et al. (2000) used a different technique to assess microbial community composition (i.e., DGGE) and did not detect a change in bacterial communities from CO<sub>2</sub> exposure. However, these investigators used a model ecosystem that was exposed to CO<sub>2</sub> for a short period (i.e., 38 weeks), thus making direct comparison with a FACE experiment difficult. A greater abundance of Gram-negative bacteria could occur if processes such as nitrification (Hungate et al. 1996; Holmes and Zak, in review) or symbiotic N fixation (Hungate et al. 1999), or decomposition by Gram-negative bacteria are enhanced when plants are grown under elevated CO<sub>2</sub>. It is interesting that a greater Gram-negative bacterial biomass was balanced by a lower Gram-positive biomass, resulting in no net change in total bacterial PLFA. Other field experiments also have shown that elevated CO<sub>2</sub> does not alter total microbial biomass in pine forest (Allen et al. 2000), tallgrass prairie (Rice et al. 1994), and annual grassland (Hungate et al. 1997) ecosystems, although these researchers did not employ PLFAs to measure (live) microbial biomass.

Microbial response to elevated CO<sub>2</sub> was attenuated by elevated O<sub>3</sub>, most strongly evidenced by the response of microbial respiration to our experimental treatments. We previously observed that elevated O<sub>3</sub> dampened the effect of elevated CO<sub>2</sub> on fine root biomass, soil respiration, and extracellular enzyme activity (King et al. 2001; Larson et al., in press). These responses may be linked to a decline in C flow from the rhizosphere to fungi and bacteria when plants are exposed to high concentrations of O<sub>3</sub> (Yoshida et al. 2001). Elevated O<sub>3</sub> has been observed to reduce litter quality and rates of decay (Findlay et al. 1996), microbial biomass (Islam et al. 2000), and fungal biovolume (Carreiro et al. 1999). These impacts on microbial community composition and activity could lead to changes in plant nutrition, plant competition, and species composition (Yoshida et al. 2001). We suggest that greater soil C inputs beneath plants exposed to elevated CO<sub>2</sub> resulted in greater microbial activity, and that elevated O<sub>3</sub> eliminated this effect across species by attenuating the positive effect of CO<sub>2</sub> on soil C inputs.

Aboveground responses to CO<sub>2</sub> and O<sub>3</sub> are often species specific (Broadmeadow and Jackson 2000; DeLucia and Thomas 2000; Hättenschwiler and Körner 2000; Medlyn et al. 2001). Thus, we studied how forest species influenced belowground responses to these trace gases. We found that CO<sub>2</sub> and O<sub>3</sub> altered microbial community

composition and function beneath aspen and aspen-birch. The effect of treatments on microbial respiration was similar for all species, yet recovery of <sup>13</sup>C-CO<sub>2</sub> beneath aspen and aspen-birch was greater than beneath aspen-maple. Similarly, beneath aspen and aspen-birch, fungal abundance declined with elevated O<sub>3</sub>, whereas O<sub>3</sub> did not reduce fungal abundance beneath aspen-maple. Our results demonstrate that 3 years of O<sub>3</sub> exposure reduced the relative abundance of fungi and attenuated the positive effect of CO<sub>2</sub> on microbial respiration beneath early-successional tree species. Therefore, the microbial community beneath forests dominated by early-successional tree species may be more sensitive to elevated atmospheric CO<sub>2</sub> and O<sub>3</sub> than forests dominated by late-successional tree species.

In summary, changes in plant growth in response to our experimental treatments elicited clear changes in the metabolism and composition of soil microbial communities. For example, elevated atmospheric CO<sub>2</sub> increased the rates at which cellobiose and *N*-acetylglucosamine were metabolized by soil microorganisms, especially beneath early-successional forest species. Changes in cellobiose metabolism resulted from an increase in fungal metabolism, whereas increases in *N*-acetylglucosamine metabolism likely resulted from a proportional increase in the activity of both soil bacteria and fungi. Our results indicate that increases in plant litter production under elevated CO<sub>2</sub> may be counterbalanced by more rapid rates of litter degradation, which ultimately return photosynthetically fixed C to the atmosphere. Consistent with changes in fine root litter and the activity of cellobiohydrolase and *N*-acetylglucosamidase, enhanced rates of substrate metabolism under elevated CO<sub>2</sub> were eliminated by elevated O<sub>3</sub>. Moreover, we underscore the importance of understanding belowground responses to elevated O<sub>3</sub>. Our results demonstrate that enhanced fungal activity beneath plants growing under elevated CO<sub>2</sub> is not evident when plants are also exposed to high concentrations of O<sub>3</sub>.

**Acknowledgements** The authors gratefully acknowledge financial support by the Department of Energy Program for Ecosystem Research (DE-FG02-93ER6166), the USDA-FS Northcentral Forest Experiment Station, and USDA Soil Biology Program. Matt Tomlinson, Matthew Bokach, and Haegeun Chung provided field and laboratory assistance. We also thank Aaron Peacock and associates at the Center for Environmental Biotechnology for their helpful instruction with PLFAs.

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