

# Extracellular Enzyme Activity Beneath Temperate Trees Growing Under Elevated Carbon Dioxide and Ozone

Jennifer L. Larson, Donald R. Zak,\* and Robert L. Sinsabaugh

## ABSTRACT

Soil microorganisms are limited by the amount and type of plant-derived substrates entering soil, and we reasoned that changes in the production and biochemical constituents of plant litter produced under elevated CO<sub>2</sub> and O<sub>3</sub> would elicit physiological changes in soil microbial communities. To test this idea, we studied microbial activity beneath trembling aspen (*Populus tremuloides* Michx.), paper birch (*Betula papyrifera* Marsh.), and sugar maple (*Acer saccharum* Marsh.) growing under experimental atmospheric CO<sub>2</sub> (ambient and 522.7 μL L<sup>-1</sup>) and O<sub>3</sub> (ambient and 54.5 nL L<sup>-1</sup>). To assess changes in microbial community function, we measured microbial biomass, respiration, and the metabolism of root-derived substrates using BIOLOG GN microplates. We also measured the activity of phosphatase, leucine aminopeptidase, α-glucosidase, N-acetylglucosaminidase, cellobiohydrolase, phenol oxidase, and peroxidase enzymes, which are involved in plant and fungal litter decomposition. Microbial biomass and respiration were not significantly altered by elevated CO<sub>2</sub> and O<sub>3</sub>. Cellobiohydrolase activity significantly increased under elevated CO<sub>2</sub>; however, this response was eliminated by elevated O<sub>3</sub>. N-acetylglucosaminidase activity also increased under elevated CO<sub>2</sub>, but elevated O<sub>3</sub> did not significantly alter this response. We found no difference in the metabolism of amino acids, organic acids, and simple carbohydrates, suggesting our experimental treatments did not alter the use of these substrates by soil microorganisms. Our analysis indicates that changes in plant growth in response to elevated CO<sub>2</sub> and O<sub>3</sub> alters microbial metabolism in soil.

THE INCREASING CONCENTRATION of atmospheric CO<sub>2</sub> has the potential to alter the cycling of C and N in terrestrial ecosystems, because it can modify the production and biochemistry of plant litter entering soil, which, in turn, controls microbial activity. Elevated CO<sub>2</sub> can increase photosynthesis (Kubiske et al., 1997; Curtis et al., 2000) and plant growth (Mikan et al., 2000; Zak et al., 2000a), as well as alter the biochemical composition of plant tissue produced above (Cotrufo et al., 1994) and belowground (Cotrufo and Ineson, 1995). Roots are an important source of organic substrates for microbial growth, and most studies suggest root production will increase as CO<sub>2</sub> accumulates in the Earth's atmosphere (Pregitzer et al., 2000). Moreover, elevated CO<sub>2</sub> can increase fine root C/N in some species (*Betula pendula* and *Picea sitchensis*; Cotrufo and Ineson, 1995) and alter concentrations of starch, sugar, and total nonstructural carbohydrates in others (*Pinus taeda*; Lewis et al., 1994). Therefore, the degree to which elevated CO<sub>2</sub> modifies the production and chemistry of root and leaf litter should have direct effects on soil microorganisms, be-

cause these plant tissues are the primary substrates for microbial metabolism in soil.

Ozone is a greenhouse gas that is accumulating in the lower atmosphere, and elevated O<sub>3</sub> has the potential to alter soil microbial communities through its influence on plant litter production. Elevated O<sub>3</sub> can decrease photosynthesis (Pye, 1988; Coleman et al., 1995a) and net C gain (Skärby et al., 1987; Reich et al., 1990) in many woody plants, which should modify litter production. For example, high levels of O<sub>3</sub> often decrease root growth to a greater extent than foliage or stem growth (Manning et al., 1971; Blum and Tingey, 1977; Hogsett et al., 1985). Elevated O<sub>3</sub> also can modify the biochemical composition of fine roots, wherein starch and soluble sugar concentrations can decline following exposure to elevated O<sub>3</sub> (Andersen et al., 1991). It is possible that decreased growth and biochemical changes under elevated O<sub>3</sub> could potentially mitigate increases in plant growth and changes in tissue biochemistry resulting from elevated atmospheric CO<sub>2</sub>. However, we do not understand how these atmospheric gases will interact to alter the input of organic substrates to soil, which could potentially modify microbial activity in soil.

In a recent experiment, we observed that elevated CO<sub>2</sub> significantly increased the biomass of living and dead fine roots, but the magnitude (83–113%) of this response differed between tree species and was eliminated by elevated O<sub>3</sub> (King et al., 2001). Because microbial growth is constrained by the type and amount of organic substrates entering soil (Babiuk and Paul, 1970; Smith and Paul, 1990), the aforementioned changes in belowground plant growth could potentially alter both substrate availability and microbial activity. The presence of substrates can induce the synthesis of specific extracellular enzymes (Paul and Clark, 1996), and we reasoned that the activity of key enzymes involved with plant litter decomposition should respond to changes in fine-root litter under elevated CO<sub>2</sub> and O<sub>3</sub>. We also reasoned that changes in microbial activity under elevated CO<sub>2</sub> and O<sub>3</sub> would differ among plant taxa. To test these ideas, we measured extracellular enzyme activities and the metabolism of labile, root-derived substrates beneath contrasting temperate tree species grown under experimental CO<sub>2</sub> and O<sub>3</sub> treatments.

## MATERIALS AND METHODS

### Experimental Design

Our study was conducted at a free-air CO<sub>2</sub> and O<sub>3</sub> enrichment (FACE) experiment located 25 km west of Rhinelander,

J.L. Larson and D.R. Zak, School of Natural Resources & Environment, Univ. of Michigan, Ann Arbor, MI 48109; R.L. Sinsabaugh, Dep. of Earth, Ecological and Environmental Sciences, Univ. of Toledo, Toledo, OH 43606-3390. Received 9 Mar. 2001. \*Corresponding author (drzak@umich.edu).

**Abbreviations:** FACE, free-air CO<sub>2</sub> and O<sub>3</sub> enrichment; MB<sub>c</sub>, microbial biomass C; MUB, methylumbelliferone; PCA, principal components analysis.

**Table 1.** Summary of physical and chemical soil properties beneath plants grown under experimental CO<sub>2</sub> and O<sub>3</sub> treatments. Soil samples were collected prior to planting within each FACE ring, and treatment means are presented with the standard deviations enclosed in parentheses. Additional detail can be found in Dickson et al. (2000).

	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>
<b>Soil texture</b>				
% sand	55.1 (3.58)	53.9 (2.60)	58.3 (1.98)	55.0 (2.94)
% silt	36.1 (3.15)	37.8 (2.30)	35.3 (3.74)	37.4 (2.68)
% clay	8.7 (1.31)	8.4 (1.04)	6.4 (1.87)	7.7 (0.72)
<b>Gravimetric moisture content: <math>\theta_{(WHC)}</math></b>	<b>0.10 (0.02)</b>	<b>0.11 (0.01)</b>	<b>0.10 (0.002)</b>	<b>0.11 (0.005)</b>
<b>D<sub>b</sub>, Mg m<sup>-3†</sup></b>	<b>2.37 (0.14)</b>	<b>1.30 (0.13)</b>	<b>1.32 (0.15)</b>	<b>1.43 (0.10)</b>
<b>Total C, %</b>	<b>1.54 (0.27)</b>	<b>1.68 (0.33)</b>	<b>1.60 (0.32)</b>	<b>1.31 (0.20)</b>
<b>Total N, %</b>	<b>0.12 (0.02)</b>	<b>0.13 (0.03)</b>	<b>0.12 (0.03)</b>	<b>0.10 (0.0)</b>
<b>C/N</b>	<b>12.9 (0.77)</b>	<b>12.4 (0.44)</b>	<b>13.5 (0.70)</b>	<b>12.8 (0.65)</b>
<b>Extractable P, g N g<sup>-1</sup></b>	<b>124 (28.1)</b>	<b>155 (23.5)</b>	<b>132 (22.4)</b>	<b>136 (17.3)</b>

† Bulk density.

WI. This experiment consists of twelve 30-m-diam. FACE rings, assigned to factorial treatments of atmospheric CO<sub>2</sub> (ambient and 522.7  $\mu\text{L L}^{-1}$ ) and O<sub>3</sub> (ambient and 54.5  $\text{nL L}^{-1}$ ; see Dickson et al., 2000 for details). Treatments were arranged in a complete block design, with three replications of each treatment combination. Each FACE ring was divided into three sections. In one-half of each ring, we planted five trembling aspen genotypes of differing O<sub>3</sub> and CO<sub>2</sub> responsiveness (Coleman et al., 1995a,b; Curtis et al., 2000). The other half of each ring was further divided into two quarters; one was planted with aspen and sugar maple and the other was planted with aspen and paper birch. In June 1997, trees were planted at a 1 by 1 m spacing in each ring section, and our sampling occurred during the second full growing season after planting. Mean soil properties for the CO<sub>2</sub> and O<sub>3</sub> treatment combinations are summarized in Table 1.

To measure microbial activity and biomass in soil, we collected six 2.5 by 10 cm soil cores from each section of the twelve FACE rings. Samples were collected at random locations between tree stems. These samples were composited by ring section in the field, stored on ice, and transported to our laboratory for analysis. Fine roots in our samples were removed using forceps, and rhizosphere soil was removed from the roots by shaking them vigorously inside a polyethylene bag. We used nonrhizosphere soil for our analyses of microbial biomass C (MB<sub>c</sub>), respiration, and extracellular enzyme activity, and we assayed the metabolism of labile, root-derived substrates using rhizosphere soil. The small amount of rhizosphere soil collected from our cores precluded measurements of MB<sub>c</sub>, respiration, and extracellular enzyme activity. Samples were collected in May, July, and October 1999 to quantify seasonal differences in the aforementioned attributes.

### Microbial Biomass Carbon and Respiration

Two 30-g subsamples of nonrhizosphere soil from each ring section were used to determine MB<sub>c</sub> and respiration using the CHCl<sub>3</sub> fumigation-incubation technique. One subsample was fumigated for 24 h with ethanol-free chloroform in a vacuum desiccator, while the second subsample was incubated without chloroform (control). Following the 24-h incubation, the desiccator containing the fumigated samples was evacuated eight times to remove remaining chloroform. Each fumigated sample was inoculated with 0.5 g of the corresponding control sample. Each fumigated and control sample was sealed in a 1-L mason jar and incubated at 25°C for 14 d. Following the 14-d incubation, the headspace gas of the jars was analyzed for CO<sub>2</sub> using a Tracor 540 gas chromatograph (Tremetrics Corp., Austin, TX). Microbial biomass was determined by subtracting the amount of CO<sub>2</sub> in the control sample from that in the fumigated sample. The difference was divided by a cor-

rection factor ( $K_c = 0.41$ ) to estimate MB<sub>c</sub> (Voroney and Paul, 1984). Microbial respiration was measured as the increase in CO<sub>2</sub> in unfumigated samples over the 14-d incubation.

### Enzyme Assays

Soil microorganisms synthesize extracellular enzymes based on the concentration of substrates present in the soil (Paul and Clark, 1996). Therefore, changes in plant-derived substrates entering soil under elevated CO<sub>2</sub> and O<sub>3</sub> should alter the activity of enzymes used in their degradation. We used methylumbelliferone (MUB) linked substrates to determine the rate at which the microbial community in nonrhizosphere soil metabolized six classes of plant-derived compounds (*sensu* Sinsabaugh et al., 1999). We assayed the activities of 1,4- $\alpha$ -glucosidase, cellobiohydrolase, phosphatase, 1,4- $\beta$ -N-acetylglucosaminidase, and leucine-aminopeptidase.

We suspended 1.0 g of nonrhizosphere soil from each composite sample in 60 mL of 50 mM acetate buffer (pH 5.0). The slurry was mixed with a tissue homogenizer (Polytron Devices Inc., Paterson, NJ) and diluted with additional buffer to 125 mL. The suspensions were stored in 125-mL Nalgene screw-cap bottles for up to 30 min prior to analysis. Sixteen replicates of three separate enzyme assays were conducted on individual 96-well microplates. Each plate also contained eight replicates of a blank, a 4-MUB standard, a negative control, and a quench standard. Plates were incubated at 25°C and 25  $\mu\text{L}$  of 200 mM NaOH was added to each well to terminate the enzymatic reactions. Fluorescence resulting from the cleavage of 4-MUB from utilized substrate was determined with an *f*-Max fluorimeter (Molecular Devices Corp., Sunnyvale, CA). Excitation energy was 355 nm and emission was measured at 460 nm. Enzyme activities are reported as nmol 4-MUB g<sup>-1</sup> h<sup>-1</sup>.

We used colorimetric assays to determine the activity of phenol oxidase and peroxidase, both of which oxidize phenols and contribute to lignin degradation. A 25 mM *L*-3,4-dihydroxyphenylalanine (*L*-DOPA) solution was prepared in acetate buffer to assay the activity of these enzymes. The procedure for measuring the activity of these enzymes was similar to that described above. Clear microplates (LabSystems; Helsinki, Finland) were used for the colorimetric assay, and each contained sixteen replicates of each soil. There were eight blank replicates for each soil (soil with no substrate), as well as eight replicates of a reference standard. For the peroxidase assay, 25  $\mu\text{L}$  H<sub>2</sub>O<sub>2</sub> was added to each well. Following an 18 h incubation, absorbance was read on an EL-800 plate reader (Biotek Instruments Inc., Winooski, VT) at 450 nm. Activity was reported as  $\mu\text{mol L-DOPA}$  converted per gram per hour. The results of all enzymatic assays are expressed on a dry weight basis.

**Table 2. List of C sources on BIOLOG GN microplates found in root exudates (Campbell et al., 1997).**

Carbohydrates	Carboxylic acids	Amino acids
arabinose	acetic acid	<i>L</i> -alanine
<i>D</i> -fructose	citric acid	<i>L</i> -alanyl-glycine
<i>D</i> -galactose	$\alpha$ -keto valeric acid	<i>L</i> -asparagine
$\alpha$ - <i>D</i> -glucose	malonic acid	<i>L</i> -aspartic acid
maltose	propionic acid	hydroxy <i>L</i> -proline
<i>D</i> -raffinose	succinic acid	<i>L</i> -leucine
<i>L</i> -rhamnose		<i>L</i> -ornithine
sucrose		<i>L</i> -phenylalanine
		<i>L</i> -serine
		<i>L</i> -threonine
		$\gamma$ -amino butyric acid

### Metabolism of Root-Derived Substrates

We used a subset of BIOLOG GN substrates (BIOLOG, Hayward, CA) that are found as constituents of root exudate to assess their metabolism in rhizosphere soil (*sensu* Campbell et al., 1997; Table 2). Two 10-g samples of rhizosphere soil were diluted in 100 mL of a 0.85% (w/v) NaCl solution and placed on an electric shaker for 30 min at 250 rpm. Each sample underwent a second dilution; 10 mL from each sample were placed into 100 mL of NaCl solution. We did not adjust our dilutions for differences in inoculum density, because an initial survey indicated that microbial biomass was equivalent across treatments in both rhizosphere and nonrhizosphere soil (D.R. Zak, unpublished data, 1998). From the second dilution, 150  $\mu$ L was used to inoculate individual wells of BIOLOG GN plates, which were subsequently incubated at 25°C for 72 h; absorbance at 595 nm was measured following inoculation and after 72 h (EL-800; Biotek Instruments, Winooski, VT). At each measurement time, the absorbance value of the control well was subtracted from the absorbance value of all other wells. Using these corrected values, we subtracted the initial absorbance values from those after the 72-h incubation to calculate overall color development, a measure of substrate metabolism.

### Statistical Analyses

Analyses of microbial biomass, microbial respiration, and enzyme activity were performed using a mixed-model ANOVA for a randomized complete block design split by species and time. Block, CO<sub>2</sub>, O<sub>3</sub>, species, and time were fixed effects in this model. To test main effects (CO<sub>2</sub> and O<sub>3</sub>), split-plot effects (species), split-split plot effects (time), and their interactions, we derived expected mean squares and used the appropriate mean square for the denominator of each *F*-test. Significance for all statistical analyses was accepted at  $\alpha = 0.05$ .

We used an identical ANOVA model to test for significant differences in the use of root-derived substrates on BIOLOG GN plates. In addition, we conducted a principal components analysis (PCA) to ordinate CO<sub>2</sub>, O<sub>3</sub>, and species treatment combinations by microbial growth on amino acids, organic acids, and simple carbohydrates. Separate PCAs were performed on the corrected absorbance values obtained in May, July, and October.

## RESULTS

### Microbial Biomass Carbon and Respiration

Mean MB<sub>c</sub> in nonrhizosphere soil beneath plants grown under elevated CO<sub>2</sub> (90  $\mu$ g C g<sup>-1</sup>) was 18% greater than that beneath plants growing ambient CO<sub>2</sub> (76  $\mu$ g C g<sup>-1</sup>; main effect means), but this difference was not significant. Mean MB<sub>c</sub> beneath plants grown under elevated

O<sub>3</sub> (95  $\mu$ g C g<sup>-1</sup>) was greater than the mean value under ambient O<sub>3</sub> (70  $\mu$ g C g<sup>-1</sup>; main effect means); this difference also was not significant. Microbial respiration did not differ significantly among any treatment or treatment combination (data not shown). However, there were significant differences in both MB<sub>c</sub> and respiration between sampling dates (data not shown), wherein MB<sub>c</sub> and respiration declined over the growing season. Mean MB<sub>c</sub> in May, July, and October were 83, 18, and 15  $\mu$ g C g<sup>-1</sup>, respectively. Microbial respiration for May, July, and October averaged 193, 35, and 22  $\mu$ g CO<sub>2</sub>-C g<sup>-1</sup> d<sup>-1</sup>, respectively. We found no significant difference in mean MB<sub>c</sub> or respiration among the aspen, aspen–birch, and aspen–maple species treatments (species main effect).

### Enzyme Activities

The effect of elevated CO<sub>2</sub> on phosphatase activity in nonrhizosphere soil varied significantly among species; however, date, CO<sub>2</sub>, and O<sub>3</sub> were not significant as main effects. Phosphatase activity was not influenced by CO<sub>2</sub> in nonrhizosphere soil beneath aspen and aspen–birch, but activity was significantly lower beneath aspen–maple (Fig. 1; Table 3).

Leucine aminopeptidase activity did not differ significantly between ambient and elevated CO<sub>2</sub> treatments (Fig. 2A). Ozone, time, and species also were not significant as main effects, nor were any interactions significant (Table 3).

In May and October,  $\alpha$ -glucosidase activity did not differ in the ambient and elevated CO<sub>2</sub> treatment. However, activity in July was significantly higher in the elevated CO<sub>2</sub> treatment (Fig. 3A). There were no significant effects of time or O<sub>3</sub> on  $\alpha$ -glucosidase activity, nor was there a significant interaction between these factors and species (Table 3). Species also was not significant as a main effect.

Time and CO<sub>2</sub> interacted to significantly influence *N*-acetylglucosaminidase (Table 3). On each sampling date, *N*-acetylglucosaminidase activity was significantly greater under elevated CO<sub>2</sub>, compared with the rate at ambient CO<sub>2</sub> (Fig. 3B); this response was disproportionately greater in July. Averaged across sampling date, soils beneath plants grown under elevated CO<sub>2</sub> had more rapid rates of *N*-acetylglucosaminidase activity than the soils under ambient CO<sub>2</sub> (Fig. 2C). Ozone and species were not significant as main effects, nor were there any significant interactions (Table 3).

On each sampling date, cellobiohydrolase activity was significantly greater under elevated CO<sub>2</sub> compared with the rate at ambient CO<sub>2</sub> (Table 3; Fig. 3C). Ozone and CO<sub>2</sub> also interacted to significantly influence cellobiohydrolase activity. Activity under elevated CO<sub>2</sub> and ambient O<sub>3</sub> was 43.4% greater than activity under elevated CO<sub>2</sub> and elevated O<sub>3</sub> (Fig. 4). Carbon dioxide, O<sub>3</sub>, and species were not significant as main effects (Table 3).

Activities of phenol oxidase and peroxidase did not differ significantly under ambient and elevated CO<sub>2</sub> treatments (Figs. 2E, F). Ozone and species also were not significant as main effects, nor was the activity of

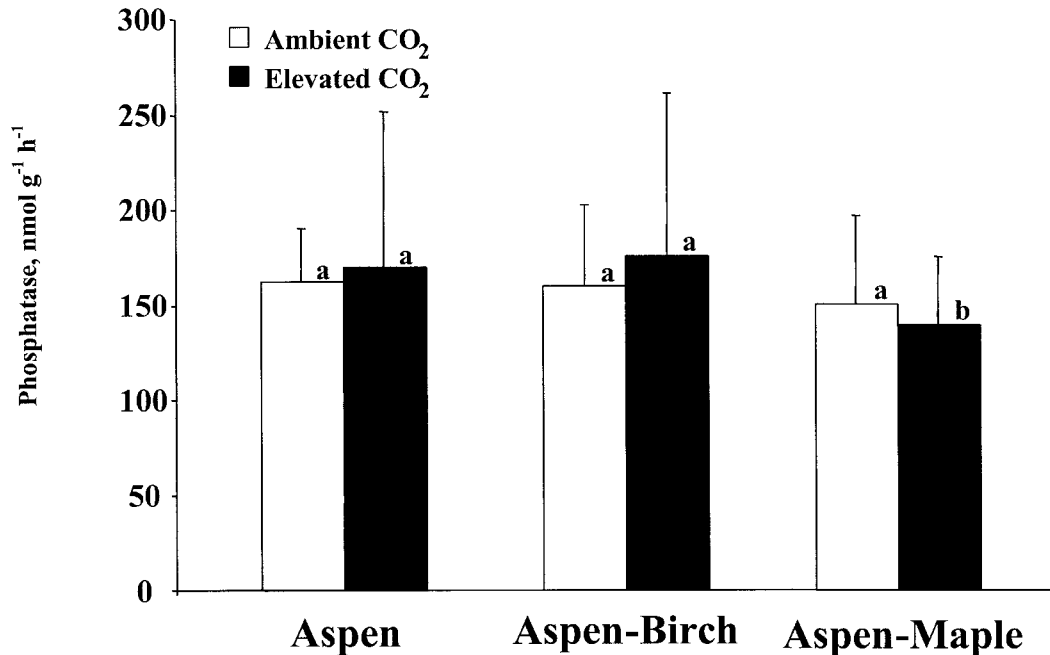


Fig. 1. Mean phosphatase activity in nonrhizosphere soil collected beneath aspen, aspen-birch, and aspen-maple growing under ambient and elevated CO<sub>2</sub>. Means with the same letter are not significantly different, and one standard deviation is indicated by the length of each error bar.

these soil enzymes influenced by sampling date or the interaction of main factors (Table 3).

### Metabolism of Root-Derived Substrate

Metabolism of labile, root-derived substrates in rhizosphere soil (Table 2) was little affected by CO<sub>2</sub>, O<sub>3</sub>, species, and their interactions (data not shown). However, time was a significant main effect in our ANOVA, with greater metabolism of most substrates occurring in July. Nonetheless, we did find that acetic acid, citric acid, and malonic acid were used to a significantly greater extent in May than the other sampling dates. Principal components analysis of the metabolism (color develop-

ment) of root-derived substrates on BIOLOG GN plates provided no separation among CO<sub>2</sub> and O<sub>3</sub> treatments for the aspen and aspen-maple ring sections on all sampling dates. However, in October, PCA of growth on root exudates showed marginal separation along PC 1 for rhizosphere soil collected beneath the aspen-birch species combination grown under elevated CO<sub>2</sub> (Fig. 5A). In this analysis, PC 1 accounted for 36% of the total variance and PC 2 accounted for 20%. The loadings computed for each substrate showed that *L*-serine, *L*-aspartic acid, *L*-asparagine, and  $\gamma$ -amino-butyric acid weighed most heavily on the positive segment of PC 1. Citric acid also received a high score on this axis.

Table 3. Analysis of extracellular enzyme activities in nonrhizosphere soil collected beneath contrasting tree species grown under experimental CO<sub>2</sub> and O<sub>3</sub> treatments. Listed are *P* values for the significance of each *F* test in a mixed-model for a randomized complete block design, split by species and time.

	Phosphatase	1,4- $\alpha$ -glucosidase	Leucine aminopeptidase	1,4- $\beta$ -N-acetylglucosaminidase	Cellobiohydrolase	Phenol Oxidase	Peroxidase
	<i>P</i> value						
Block	0.954	0.874	0.770	0.982	0.678	0.892	0.874
CO <sub>2</sub>	0.662	0.280	0.554	0.019	0.068	0.898	0.606
O <sub>3</sub>	0.394	0.771	0.355	0.109	0.065	0.243	0.631
CO <sub>2</sub> $\times$ O <sub>3</sub>	0.916	0.320	0.611	0.213	0.043	0.720	0.686
Error a: Block(CO <sub>2</sub> $\times$ O <sub>3</sub> )							
Species	0.197	0.689	0.668	0.129	0.058	0.303	0.408
Species $\times$ CO <sub>2</sub>	0.017	0.234	0.190	0.231	0.242	0.757	0.466
Species $\times$ O <sub>3</sub>	0.988	0.893	0.735	0.940	0.167	0.188	0.616
Species $\times$ CO <sub>2</sub> $\times$ O <sub>3</sub>	0.847	0.187	0.854	0.733	0.554	0.813	0.616
Error b: Species $\times$ Block(CO <sub>2</sub> $\times$ O <sub>3</sub> )							
Time	0.001	0.001	0.052	0.001	0.008	0.001	0.089
Time $\times$ CO <sub>2</sub>	0.124	0.035	0.511	0.028	0.050	0.537	0.647
Time $\times$ O <sub>3</sub>	0.125	0.751	0.431	0.292	0.842	0.410	0.124
Time $\times$ Species	0.915	0.750	0.666	0.832	0.140	0.791	0.552
Time $\times$ CO <sub>2</sub> $\times$ O <sub>3</sub>	0.204	0.262	0.940	0.187	0.512	0.743	0.599
Time $\times$ Species $\times$ CO <sub>2</sub>	0.165	0.125	0.512	0.209	0.995	0.461	0.803
Time $\times$ Species $\times$ O <sub>3</sub>	0.804	0.100	0.210	0.409	0.795	0.519	0.227
Time $\times$ Species $\times$ CO <sub>2</sub> $\times$ O <sub>3</sub>	0.473	0.074	0.269	0.030	0.803	0.551	0.863
Error c: Time $\times$ Species $\times$ Block(CO <sub>2</sub> $\times$ O <sub>3</sub> )							

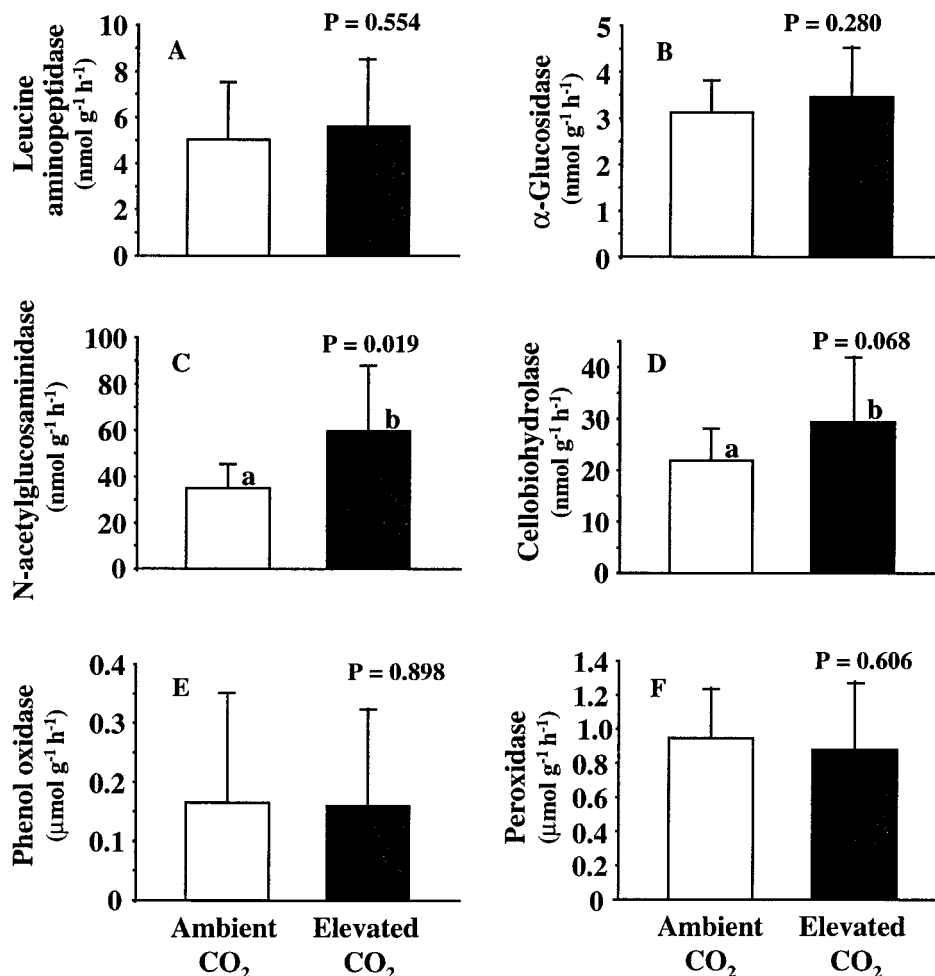


Fig. 2. The mean activity of extracellular enzymes involved in the degradation of plant litter under ambient and elevated CO<sub>2</sub>. Values are the means in nonrhizosphere soil averaged across ozone, species, and sampling dates. One standard deviation is indicated by the length of each error bar.

When all BIOLOG GN substrates were used, PCA provided virtually no separation among treatments for the aspen and aspen–maple ring sections in May and July. However, in October we did observe marginal separation along PC 1 for aspen–birch grown under elevated CO<sub>2</sub> (Fig. 5B). PC 1 accounted for 31% of the total variance in the model, and PC 2 accounted for 20%. Component loadings computed for each C source showed that *L*-ornithine, an amino acid found in root exudates and adonitol, a carbohydrate not present in root exudates, received high weights on PC 1. This analysis provided no discrimination of microbial metabolism of root-derived substrates beneath aspen and aspen–maple species combination grown under the CO<sub>2</sub> and O<sub>3</sub> treatment combinations on the three sampling dates.

## DISCUSSION

Increases in photosynthesis and plant growth, along with altered chemical composition of above and below-ground plant tissue, will likely accompany a rise in atmospheric CO<sub>2</sub> (Cotrufo et al., 1994; Cotrufo and Ineson, 1995; Kubiske et al., 1997; Curtis et al., 2000; Mikan et al., 2000; Zak et al., 2000a). In our experiment, elevated

CO<sub>2</sub> significantly increased the mass of dead fine roots (>0.5 mm) entering soil and rates of soil respiration; these responses were dampened by the presence of elevated O<sub>3</sub> (King et al., 2001). Changes in fine-root litter and soil respiration in response to our experimental treatments were identical to that of cellobiohydrolase activity; it was stimulated by elevated CO<sub>2</sub>, but elevated O<sub>3</sub> diminished this response. We also observed a stimulation of *N*-acetylglucosaminidase activity under elevated CO<sub>2</sub>; however, elevated O<sub>3</sub> did not significantly influence the activity of this enzyme. Taken together, changes in the input of dead fine roots to soil and changes in the rate of the degradative enzymes suggests that elevated CO<sub>2</sub> and O<sub>3</sub> have the potential to alter microbial activity by influencing root litter production. Further support for this contention come from the observation that elevated CO<sub>2</sub> significantly enhanced the metabolism of <sup>13</sup>C-labeled cellobiose and *N*-acetylglucosamine, a response that also was eliminated by elevated O<sub>3</sub> (Phillips et al., 2002). Although our sampling occurred after only two growing seasons, changes in belowground plant growth in response to atmospheric CO<sub>2</sub> and O<sub>3</sub> exerted a detectable influence on microbial activity that was

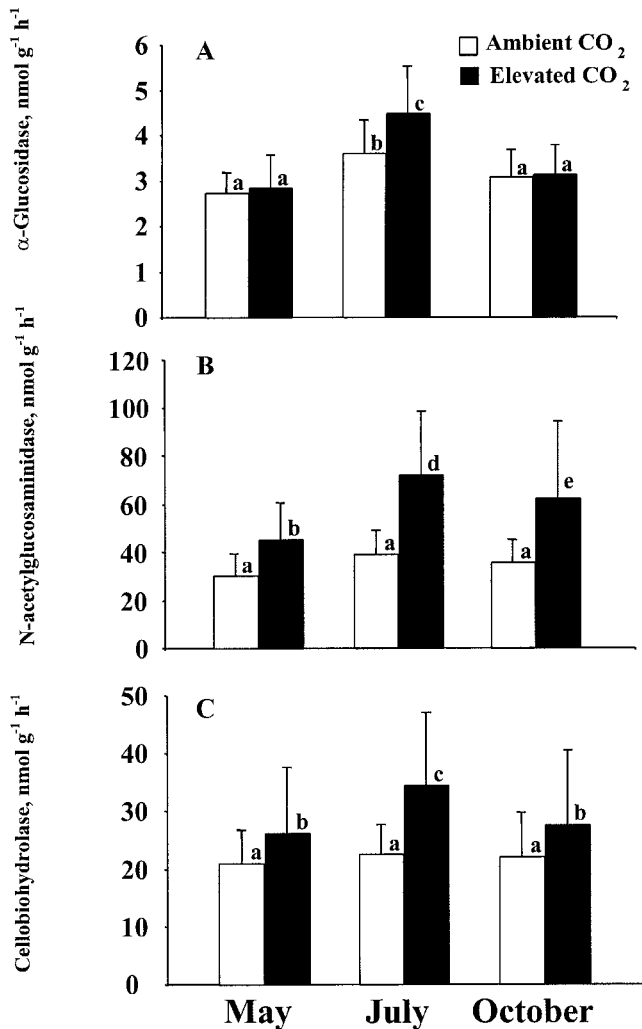


Fig. 3. Mean  $\alpha$ -glucosidase, *N*-acetylglucosaminidase, and cellobiohydrolase activity in nonrhizosphere soil under ambient and elevated CO<sub>2</sub> for May, July, and October sampling dates. Means with the same letter are not significantly different and one standard deviation is indicated by the length of each error bar.

consistent in subsequent growing seasons (Phillips et al., 2002).

Many studies have observed increases in below-ground plant productivity under elevated CO<sub>2</sub> (Poorter, 1993; Rogers et al., 1994; Curtis and Wang, 1998), a finding which has been hypothesized to foster a larger or more active microbial population in soil (Zak et al., 2000b). However, our results, along with numerous others (Jones et al., 1998; Kampichler et al., 1998; Niklaus, 1998; Hungate et al., 2000), have found no significant response of microbial biomass to elevated CO<sub>2</sub>. A review of 47 published reports on responses of soil C and N cycling to elevated CO<sub>2</sub> concluded that microbial biomass can exhibit both large increases and declines beneath woody taxa (i.e., 52% decline to a 121% increase), even though microbial respiration generally increases in response to elevated CO<sub>2</sub> (Zak et al., 2000b). After 2 yr of CO<sub>2</sub> and O<sub>3</sub> enrichment of the atmosphere, we were not able to detect a significant response of microbial biomass and respiration to elevated CO<sub>2</sub>, nor

did we find significant species-specific responses to either CO<sub>2</sub> or O<sub>3</sub>. This could be due, in part, to the relatively young age of the plants (i.e., 2 yr old) and the fact that they likely had not fully colonized both above- and belowground growing space. Several observations made in the subsequent growing season support this idea: elevated CO<sub>2</sub> significantly increased fungal activity and the metabolism of <sup>13</sup>C-cellobiose and <sup>13</sup>C-*N*-acetylglucosamine; moreover, the response of microbial metabolism to CO<sub>2</sub> and O<sub>3</sub> was greatest beneath aspen and aspen–birch (Phillips et al., 2002). Given the aforementioned results, soil microbial activity appears to be responding in concert with plant growth in our experiment, even though we initially could not detect a significant increase in microbial biomass.

Cellulose comprises up to 40% of plant tissue (Wilke et al., 1983), and greater root-litter production in response to our experimental treatments should increase cellulose input to soil. In our study, cellobiohydrolase activity increased under elevated CO<sub>2</sub>, but this increase was only marginally significant 2 yr after initiation of our experimental treatments (Fig. 2D), a response that was consistent in subsequent growing seasons (H. Chung and D.R. Zak, unpublished data, 2001). Concurrent research at our FACE experiment has found a 113% increase in fine-root biomass for aspen, and an 84% increase in fine-root biomass in the aspen–birch species combination (King et al., 2001). These results suggest a greater amount of cellulose entered soil under elevated CO<sub>2</sub>, and this is consistent with the finding that elevated CO<sub>2</sub> significantly increased the metabolism of <sup>13</sup>C-cellobiose by soil fungi in our experiment (Phillips et al., 2002). In combination, increases in root-litter input, greater rates of cellobiohydrolase activity, and more rapid metabolism of labeled cellobiose all indicate that elevated CO<sub>2</sub> has enhanced cellulose degradation in soil. In a similar study, Mayr et al. (1999) found a significant increase in cellobiohydrolase activity beneath plants growing under elevated CO<sub>2</sub>, whereas Dhillon et al. (1996) reported a nonsignificant increase in the activity of this enzyme. These results all suggest an overall increase in cellulose metabolism by microbial communities in non-rhizosphere soil beneath plants exposed to elevated CO<sub>2</sub>.

In our experiment, greater cellobiohydrolase activity under CO<sub>2</sub> was mitigated by elevated O<sub>3</sub> (Fig. 4), a response consistent with that of fine-root litter production (King et al., 2001). These observations suggest that changes in root-litter production altered substrate availability and hence the activity of this soil enzyme. The fact that elevated O<sub>3</sub> also eliminated enhanced rates of <sup>13</sup>C-cellobiose metabolism under elevated CO<sub>2</sub> further supports this contention (Phillips et al., 2002). We are not aware of other studies that have directly evaluated changes in root-litter production and cellobiohydrolase activity under elevated O<sub>3</sub>. Nonetheless, ample evidence indicates that elevated O<sub>3</sub> can decrease photosynthesis and impair stomatal function (Tjoelker et al., 1995), which can lower the allocation of C to root growth (Scagel and Andersen, 1997). Moreover, Kress and Skelly (1982) reported a 41% decrease in biomass of *Acer saccharum* seedlings, and Wang et al. (1986) reported a 17% de-

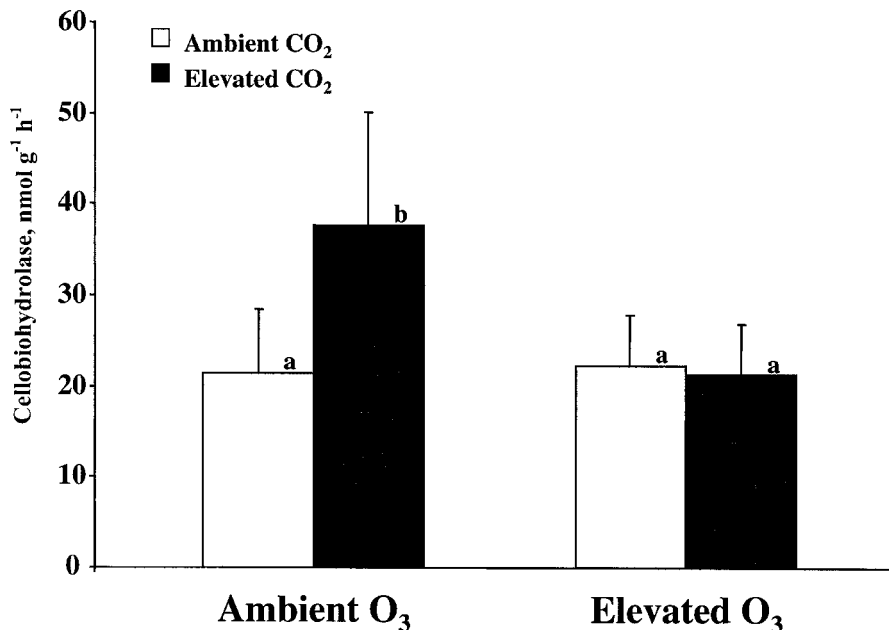


Fig. 4. The interaction of CO<sub>2</sub> and O<sub>3</sub> on cellulohydrolase activity in nonrhizosphere soil. Values are means across sampling dates and species treatments, and the standard deviation is indicated by the length of each error bar. Means with the same letter are not significantly different.

crease in biomass of *Populus tremuloides* seedlings subjected to elevated O<sub>3</sub>. A decrease in plant growth and allocation to roots under elevated O<sub>3</sub> should, in turn, lead to a decline in cellulose input to soil. Such a response

would be consistent with our observations of fine-root litter production, cellulohydrolase activity, and the metabolism of <sup>13</sup>C-cellobiose (King et al., 2001; Phillips et al., 2002).

*N*-acetylglucosaminidase is a soil enzyme involved in chitin degradation (Alexander, 1977), and we observed a significant increase in the activity of this enzyme under elevated CO<sub>2</sub> (Fig. 2C). Similar results were found in alpine grassland in which *N*-acetylglucosaminidase activity under elevated CO<sub>2</sub> increased by nearly 30% (Mayr et al., 1999). Although we presently do not have data on specific fungal communities in our experiment, an increase in *N*-acetylglucosaminidase activity under elevated CO<sub>2</sub> may result from a greater production of fungal cell wall litter (Miller et al., 1998). This would be consistent with other studies that have observed greater mycorrhizal infection (%), and higher levels of mycorrhizal and saprophytic fungal biomass under elevated CO<sub>2</sub> (Rillig et al., 1998; Klironomos et al., 1996). *N*-acetylglucosaminidase in our study displayed a nearly two-fold increase with elevated CO<sub>2</sub> (Fig. 2C), and the magnitude of this response was similar in the presence and absence of elevated O<sub>3</sub> (i.e., no CO<sub>2</sub> × O<sub>3</sub> interaction). The relative response of *N*-acetylglucosaminidase to our experimental treatments was similar to that of <sup>13</sup>C-*N*-acetylglucosamine metabolism, which significantly increased under elevated CO<sub>2</sub> (Phillips et al., 2002). In combination, these results indicate that chitin degradation was enhanced by elevated CO<sub>2</sub>, but further work will be required to determine if increases in fungal-litter production are responsible for this observation.

Leucine aminopeptidase is an enzyme involved in the degradation of proteins, and we found no significant difference in the activity of this enzyme under ambient and elevated CO<sub>2</sub> (Table 3, Fig. 2A). This result is consistent with Mayr et al. (1999), who found leucine aminopep-

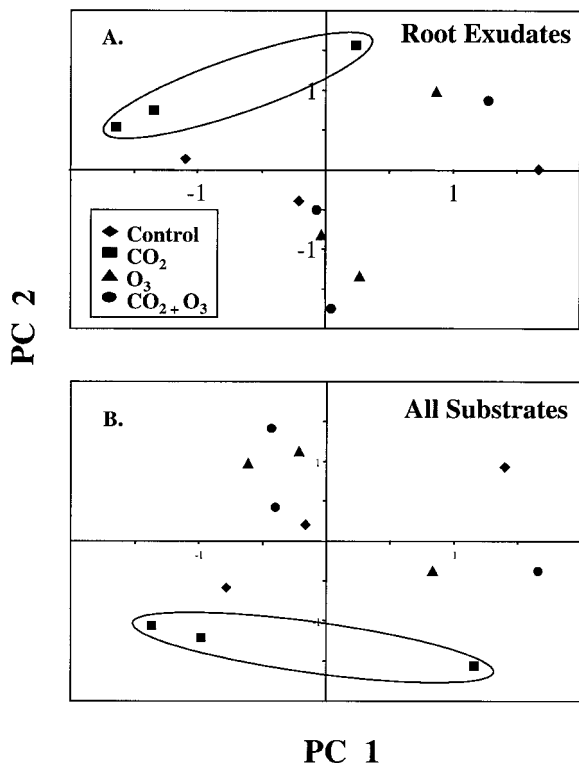


Fig. 5. Principal components analysis of microbial growth on BIOLOG GN microplates for (A) root exudates and for (B) all substrates. Results are from rhizosphere soil collected beneath the aspen-birch species combination during the October sampling date, which illustrates the poor discrimination among treatments based on substrate use.

tidase was unaffected by elevated CO<sub>2</sub>. We also found no significant influence of CO<sub>2</sub>, O<sub>3</sub>, or species combination on the activity of phenol oxidase, an enzyme involved with lignin degradation (Table 3). In the context of our experiment, this implies that protein and lignin inputs from root mortality were unchanged across all treatments. Clearly, chemical analyses of root tissue are needed to support to this contention.

Assaying the metabolism of simple substrates is one approach of assessing physiological capabilities of microbial communities under experimental conditions (Zak et al., 1994). Carbon dioxide, O<sub>3</sub>, sampling date, and species had no influence on the metabolism of amino acids, organic acids, and simple carbohydrates by microbial communities inhabiting rhizosphere soil. These results are consistent with elevated CO<sub>2</sub> studies in Mediterranean (Dhillion et al., 1996) and model tropical ecosystems (Insam et al., 1999), which observed no change in labile substrate metabolism under elevated CO<sub>2</sub>. In contrast, Mayr et al. (1999) found differences in the metabolic capabilities in an undisturbed, late-successional alpine grassland exposed to elevated CO<sub>2</sub>. In our experiment, rhizosphere microbial communities equivalently metabolized a range of amino acids, carbohydrates, and organic acids found as constituents of root exudates across all treatments. However, the activity of enzymes involved with plant and fungal cell wall degradation responded significantly to our experimental treatments in nonrhizosphere soil and were consistent with the responses of fine-root litter production.

In summary, we found that greater belowground plant growth under elevated CO<sub>2</sub> significantly increased the activities of cellobiohydrolase and *N*-acetylglucosaminidase. In contrast, elevated O<sub>3</sub> counteracted the CO<sub>2</sub> effect on cellobiohydrolase activity, but elevated O<sub>3</sub> had no significant influence on *N*-acetylglucosaminidase activity. Because these enzymes play a key function in the degradation of plant and fungal litter in soil, their response to our experimental treatments may presage a change in decomposition and the flow of substrates through soil food webs. This is consistent with greater rates of <sup>13</sup>C-cellobiose and *N*-acetylglucosamine beneath plants growing under elevated CO<sub>2</sub> (Phillips et al., 2002). Although the tree taxa we studied broadly differ in life-history traits, we have no initial evidence to suggest that changes in belowground growth in response to elevated CO<sub>2</sub> and O<sub>3</sub> will elicit fundamentally different responses by soil microbial communities (Phillips et al., 2002). Clearly, further work would be required to understand the long-term implication of our observations on the microbial processing of organic matter and its storage in soil. Notwithstanding this caveat, our observations suggest that changes in plant growth induced by high concentrations of CO<sub>2</sub> and O<sub>3</sub> in the Earth's atmosphere can mediate physiological changes in soil microbial communities, which, in turn, have the potential to alter soil C and N cycling in forests. Understanding how changes in plant growth in response to elevated CO<sub>2</sub> and O<sub>3</sub> impact the long-term dynamics of C and N in soil remains an important challenge.

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