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Changes in microbial activity and composition in a pasture ecosystem exposed to elevated atmospheric carbon dioxide

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Abstract

Elevated atmospheric CO_2 increases aboveground plant growth and productivity. However, carbon dioxide-induced alterations in plant growth are also likely to affect belowground processes, including the composition of soil biota. We investigated the influence of increased atmospheric CO_2on bacterial numbers and activity, and on soil microbial community composition in a pasture ecosystem under Free-Air Carbon Dioxide Enrichment (FACE). Composition of the soil microbial communities, in rhizosphere and bulk soil, under two atmospheric CO_2 levels was evaluated by using phospholipid fatty acid analysis (PLFA), and total and respiring bacteria counts were determined by epifluorescence microscopy. While populations increased with elevated atmospheric CO_2 in bulk soil of white clover (*Trifolium repens* L.), a higher atmospheric CO_2 concentration did not affect total or metabolically active bacteria in bulk soil of perennial ryegrass (*Lolium perenne* L.). There was no effect of atmospheric CO_2 on total bacteria populations per gram of rhizosphere soil. The combined effect of elevated CO_2 on total root length of each species and the bacterial population in these rhizospheres, however, resulted in an 85% increase in total rhizosphere bacteria and a 170% increase in respiring rhizosphere bacteria for the two plant species, when assessed on a per unit land area basis. Differences in microbial community composition between rhizosphere and bulk soil were evident in samples from white clover, and these communities changed in response to CO_2 enrichment. Results of this study indicate that changes in soil microbial activity, numbers, and community composition are likely to occur under elevated atmospheric CO_2, but the extent of those changes depend on plant species and the distance that microbes are from the immediate vicinity of the plant root surface.

Introduction

The global atmospheric concentration of CO_2 is increasing primarily as consequence of anthropogenic production of CO_2, and a doubling of pre-industrial concentrations has been predicted to occur by the latter part of the next century (King et al., 1992; Rotty and Marland, 1986). One consequence of an increased concentration of atmospheric CO_2 is an increase in plant biomass production due to higher net C assimilation (Bazzaz, 1990; Kimbal et al., 1993; Mosseau and Saugier, 1992). The influence of elevated atmospheric concentrations of CO_2 on plants is not limited to aboveground plant growth, and belowground processes are also likely to be affected (Paterson et al., 1997; Rogers et al., 1994; Sadowsky and Schortemeyer, 1997). An increase in root biomass, total rhizodeposition, and changes in chemical composition of plant tissues and root exudates may occur under

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elevated CO_2 levels (Bazzaz, 1990; Cotrufo, 1994; Rattray et al., 1995; Rogers et al., 1994; Schortemeyer et al., 1996). Taken together, these alterations are likely to affect soil microbial populations.

Because roots are the major source of additional C input into the soil, it is expected that the first effects of elevated CO_2 on soil microbial populations would occur in the rhizosphere. Moreover, because of their intimate contact with roots, soil microbes colonizing the rhizosphere will be more affected by these changes (Sadowsky and Schortemeyer, 1997), than those microbes living in the bulk soil at some greater distance from plant roots. Schortemeyer et al. (1996) reported an increase in populations of *Rhizobium leguminosarum* bv. *trifolii* in the rhizosphere of white clover (*Trifolium repens* L.), but not in the rhizosphere of perennial ryegrass (*Lolium perenne* L.) or in bulk soil. Similarly, Marilley et al. (1999) reported an increased dominance of *Pseudomonas* sp. strains in the rhizosphere of perennial ryegrass, but not in the rhizosphere of white clover. These studies indicate that the rhizosphere is a selective habitat for specific microbial populations that vary by plant species. However, the effects of elevated atmospheric CO_2 on soil microbial population in the bulk soil cannot be overlooked, where long term soil C concentrations may be elevated due to changes in root turnover, and successional changes imparted by the movement and decomposition of soil microorganisms. Interestingly, many past studies done to examine the influence of CO_2 enrichment on soil microbial parameters failed to report whether rhizosphere or bulk soil was analyzed.

Results from studies examining microbial responses to elevated atmospheric CO_2 have been contradictory (O'Neill, 1994). Possible causes for these discrepancies include the use of different methodologies to measure microbial parameters, artificial plant and microbial growth conditions, the presence of different plant species, variation in bacterial populations colonizing the rhizosphere, and diverse soil conditions.

Several studies have reported increases in soil microbial biomass under elevated CO_2 (Berntson and Bazzas, 1997; Hungate et al., 1996; Rice et al., 1994). Zak et al. (1993) observed that microbial biomass C in the rhizosphere and bulk soil of *Populus grandidentata* was greater under elevated than ambient CO_2. Similarly, Diaz et al. (1993) reported an increase in microbial biomass C and N at elevated CO_2 in two herbaceous communities. However, no evidence of a significant response of microbial populations to elevated CO_2 levels in rhizosphere and bulk soil has been found in studies examining maize, yellow-poplar, and cotton (O'Neill et al., 1987; Rogers et al., 1992; Whipps, 1985). Schortemeyer et al.(1996) also reported that the size of the total heterotrophic microbial populations in the rhizosphere of white clover or perennial ryegrass did not change when subjected to elevated CO_2. In a more recent study, Schortemeyer et al. (2000) reported that microbial activity, microbial biomass, and bacterial numbers did not increase in a natural Florida scrub ecosystem after 2 years of CO_2 enrichment. In a study conducted for 3 years in the foothills of the Swiss Jura Mountains, Niklaus (1998) reported that microbial biomass C and microbial activity was not significantly influenced by elevated CO_2, although microbial N increased by 18%. Similarly, Zak et al. (2000) found that after 2.5 growing seasons, microbial biomass, gross N mineralization, microbial immobilization, and nitrification were equivalent in bulk soils under ambient and elevated CO_2.

Increases in soil microbial activity, however, have been reported in several studies. Rogers et al. (1992) and Runion et al. (1994) observed an increase in microbial activity in the rhizosphere of cotton exposed to CO_2 enrichment. Rice et al. (1994) reported greater microbial activity in a tallgrass prairie under elevated atmospheric CO_2 than under ambient conditions, butthe difference was significant only when N fertilizer was added. Similarly, Niklaus and Körner (1996) observed a significant increase in soil basal respiration in a late successional alpine grassland exposed to elevated atmospheric CO_2, but only when fertilizer was added.

While changes in the soil microbial community structure also have been predicted to occur under elevated atmospheric CO_2, probably due to alterations in substrate availability for microbial biosynthesis caused by greater plant productivity and changes in tissue chemistry, definitive studies have yet to be done under a variety of environmental conditions. Populations of *Rhizobium leguminosarum* bv. *trifolii* increased in the rhizosphere of white clover under CO_2 enrichment in a FACE grassland ecosystem (Schortemeyer et al., 1996). In addition, Montealegre et al. (2000) reported a shift in the community composition, and an increase in the competitiveness, of *R. leguminosarum* bv. *trifolii* as a result of an increased atmospheric CO_2 concentration.

Phospholipid fatty acid (PLFA) and fatty acid methyl ester (FAME) analyses have been used to describe changes in soil microbial community structure

(Tunlid and White, 1991). The fatty acid portion of these molecules serves as a marker for different soil microorganisms (Frostegard et al., 1993; Vestal and White, 1989). Phospholipid fatty acid and fatty acid methyl ester analyses allow determination of soil microbial community composition without the need for time-consuming culturing in selective growth media, which severely underestimate and biases community diversity. Zak et al. (1996, 2000) and Ringelberg et al. (1997), used PLFA analysis to determine the composition of soil microbial communities beneath *Populus grandidentata*, *Populus tremuloides*, and *Quercus alba*, respectively, growing under ambient and elevated atmospheric CO_2. Zogg et al. (1997) also used PLFA analysis to gain insight into changes in microbial community structure following soil warming related to simulated global climate change.

The objectives of this study were to evaluate the influence of elevated atmospheric CO_2on bacterial population size and its relation to microbial activity in bulk soil- and rhizosphere-associated microorganisms under established stands of white clover and perennial ryegrass. These are two of the most extensively grown cool season forage plant species, which are known to respond differently to elevated CO_2 because of their growth habit and sources of N. We anticipated that the two species would, therefore, have differential effects on microbial populations in the rhizosphere and in bulk soil. The effect of CO_2 enrichment on soil microbial community structure for white clover was also evaluated. These objectives were addressed by using the Swiss FACE facility. In this type of system, elevated CO_2 concentrations are maintained in large, open-field rings, without disturbing the microclimate of the plant–soil system. This field-level system does not suffer from the numerous shortcomings associated with plant growth chambers, or open- and closed-top chamber studies.

Materials and methods

Description of experimental site

This study was done using a field-scale FACE facility (Hendrey et al., 1993; Lewin et al., 1994), established in Eschikon, Switzerland (47° 27′ N and 8° 41′ E, elevation 550 m above sea level) on a fertile clay loam soil. The soil consisted of 27% clay, 32% silt, 37% sand, and 4% organic matter. The experiment contained three fumigated (600 ppm CO_2) and three control (ambient CO_2, 350 ppm) rings, each 18 m in diameter, and situated at least 100 m apart. Each FACE ring (main plot) contained a 1.8 m × 2.8 m subplot consisting of white clover (*Trifolium repens* L. cultivar Milkanova) and a 1.8 m × 2.8 m subplot containing ryegrass (*Lolium perenne* L. cultivar Bastion), each grown as monocultures. The plants were established in fall of 1992. Fumigation with CO_2 into the open rings began on May 1993, and was maintained since that time during the growing season. The herbage of clover and ryegrass plants in each plot were cut and removed four or five times a year.

Microbial activity and total cell counts

Soil sampling

Soil samples were taken from white clover and ryegrass plots in all six rings (fumigated and control) in June 1996, immediately after the second cutting. Eight soil cores (2 cm diam.) were taken at random from each subplot within a FACE ring, to a depth of 10 cm (most of the root mass was present in the upper 10 cm of soil), and mixed to form two composite samples, one for clover and one for ryegrass from each FACE ring. The average gravimetric soil moisture at sampling was 28.5%. Samples were stored at 4 °C for up to 1 month until processed.

Extraction and processing of soil bacteria

Numbers of total and actively respiring (viable) bacteria present in the rhizosphere soil (soil that adhered to the roots) and bulk soil (non-rhizosphere, root-free soil) were determined by epifluorescence microscopy based on the method described by Rodriguez et al. (1992). Total cell counts were determined by treating the samples with the DNA-specific fluorochrome 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO). Actively respiring bacteria were detected by the reduction of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Polyscience, Inc.,Warrington, PA). Each sample was stained with both CTC and DAPI. All solutions used for epifluorescence microscopy were filtered (0.22 μM pore-size filter, Nucleopore, Livermore, CA) prior to use, and CTC solution was prepared immediately before use.

Immediately before analysis, each soil sample was passed through a 2-mm sieve to remove gravel and large organic material. Bacteria were extracted from rhizosphere- and bulk-soil using the method of Kingsley and Bohlool (1981). Rhizosphere soil was obtained by collecting all visible roots, with the adhering soil,

from the composite soil samples and shaking on a wrist action shaker for 30 min in 30 mL of buffer (0.05 *M* $NH_4H_2PO_4$, pH 7.0, and 0.1% gelatin). Small roots were removed from soils using a dissecting microscope. After shaking, the soil suspension was separated from the roots. This soil suspension contained rhizosphere soil. Dilutions of the rhizosphere-soil suspension were made in half-strength (autoclaved and filtered) R2A medium, pH 7.3 (Reasoner and Geldreich, 1985). A 1 mL aliquot of a 10^{-1} soil dilution was incubated with 0.3 mL of 3 m*M* CTC at room temperature for 4 h in the dark on a horizontal shaker (150 rpm). Reactions were stopped by the addition of formaldehyde to a final concentration of 3.7%. Three subsamples from each sample were incubated with CTC. Killed controls were prepared by incubating cell suspensions with formaldehyde (3.7%) for 5 min before the addition of CTC. Controls containing R2A media, but no soil suspension were also included. Aliquots of the rhizosphere-soil suspension (before incubation with CTC) were dried for 48 h at 105 °C to determine soil mass.

For bulk soil, a 10 g sample of soil, remaining after all roots and adhering soil had been removed, was extracted in 95 mL of buffer (0.05 *M* $NH_4H_2PO_4$, pH 7.0, and 0.1% gelatin). Soil suspensions were shaken for 30 min on a wrist action shaker and allowed to settle for 30 min. A 30 mL aliquot of the upper, soil-free, liquid phase containing the bacteria was collected and diluted in half-strength (autoclaved and filtered) R2A medium. A 1 mL aliquot of a 10^{-2} dilution of the soil suspension was incubated with CTC as described above. Soil moisture for bulk soil was determined after drying subsamples at 105 °C for 48 h.

Enumeration using epifluorescence microscopy

After incubation with CTC, 0.14 mL of each subsample was mixed with 1.86 mL of saline solution (0.85% NaCl) and filtered through a black, 0.2-μM-pore-size, 25 mm in diameter, polycarbonate membrane filter (Nucleopore, Livermore, CA). Filters were counterstained with 0.5 mL DAPI (10 μg/mL) for 10 min in the dark. Filters were washed with 0.85% saline solution, air dried, and stored in the dark for less than 24 h at 4 °C before examination. DAPI-stained cells were enumerated to determine total cell number, and CTC-positive cells were counted to determine the number of actively respiring cells. Ten to 15 fields of view per filter were counted at random to obtain a minimum count of 100 cells. Three filters were counted per sample. Data were expressed on the basis of soil dry weight.

To evaluate the ecosystem level effect of CO_2 enrichment on bacterial populations, we calculated rhizosphere bacterial populations per m^2 in the field using population numbers per cm root length, and root length density data (cm root/cm^3 soil, based on the top 10 cm of soil) for clover and ryegrass grown in the Swiss FACE Facility (kindly provided by Dr. Urs Zimmerman). The proportion of CTC-positive cells (active) was calculated as a percentage of the DAPI-stained (total) cells.

Microbial community structure

Soil sampling and cell extraction

Soil samples were taken from white clover plots from 4 FACE rings (two fumigated and two controls) in September 1996. Eight soil cores (2 cm diameter) were taken at random to a depth of 10 cm from each subplot and were mixed together to form a composite subplot sample. Each soil sample was passed through a 2-mm sieve to remove gravel and large organic material immediately before cell extraction. A 1 g portion of roots was collected per sample for extraction purposes. Soil and root samples were kept cold during the time when roots were picked from soils, and roots were kept in sealed plastic bags at 4°C for 1–2 h before cell extraction. Soil organisms present in the rhizosphere soil were extracted according to the method of Kingsley and Bohlool (1981). A 10 g portion of bulk (root-free) soil was also collected for analysis. Samples were immediately frozen at −80 °C until processed.

Phospholipid fatty acid (PLFA) analysis

Phospholipid fatty acid analysis was used to evaluate the composition of soil microbial communities for rhizosphere and bulk soil of white clover. Only white clover plants were examined because there were few reported effects of elevated atmospheric CO_2 on bacteria in ryegrass plots (Schortemeyer et al., 1996). Phospholipid fatty acids were extracted and analyzed by Microbial Insights, Inc. (Knoxville, TN). The mole fraction (in percent) of each PLFA in rhizosphere and bulk soil was used to determine the relative proportion of different groups of bacteria (Gram negative, Gram positive), actinomycetes, and eukaryotes at ambient and elevated atmospheric CO_2.

The fatty acid nomenclature used was as follows: phospholipid fatty acids are designated by the ratio

of total number of C atoms to the number of double bonds, followed by the position of the double bond from the methyl end (w) of the molecule. *Cis* and *trans* configurations are indicated by *c* and *t*, respectively. Methyl branches in the iso- (first C) or anteiso (second C) positions are indicated by the prefixes 'i' and 'a', respectively. The position of a methyl branch that was not in the iso or anteiso positions was determined by its distance from the carboxyl terminal end of the molecule.

Statistical analysis

Microbial cell counts were subjected to analysis of variance using the General Linear Models procedure of SAS, after evaluating variables for normality using the Shapiro-Wilk test in the UNIVARIATE procedure of SAS and for linear relationships between treatment means and standard deviation by regression (SAS, 1987). These analyses indicated the need for log transformation of five measured variables: respiring rhizosphere cell counts (per gram soil); bulk soil respiring cell counts (per gram soil); bulk soil total counts (per gram soil); respiring cell counts (per cm root length); and total cell counts (per cm root length). These variables were log-transformed before subsequent statistical analysis, and the resulting means were back-transformed for presentation.

The experiment was analyzed as a completely randomized design in a split plot arrangement of treatments, with atmospheric CO_2 concentration being the main plot factor, and plant species being the subplot factor. Only two FACE ring replicates were used in the analysis of variance, because other research done at the Eschikon FACE site has shown that the third FACE ring replicates of each CO_2 treatment had different soil characteristics than the other two, including lower pH, organic C concentration, extractable P concentration, and higher dry bulk density (Nitschelm et al., 1997 and Dr. Ueli Hartwig, pers. comm.). These differences resulted in differential plant growth, even in the absence of CO_2 enrichment. Since relying on only two replicates increases the likelihood of missing important treatments effects, mean comparisons are presented for those situations in which the probability of obtaining a greater F statistic based on chance alone (Type I error) was <0.10. F statistics for all significant comparisons are given in the text. Differences in the mean proportion of PLFA profiles were tested using Two-Sample t-test with Statistix (Tallahassee, FL).

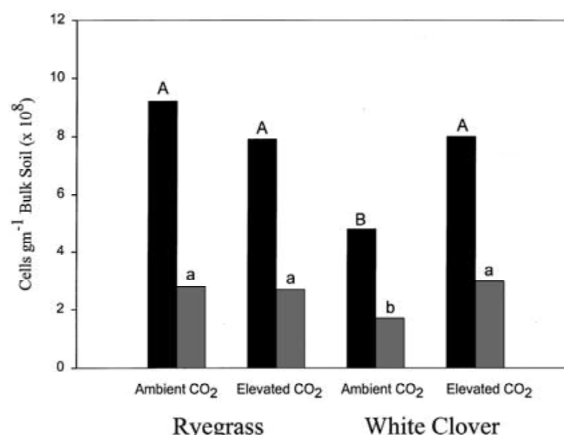

Figure 1. Bacterial populations per gram bulk soil for perennial ryegrass and white clover grown under ambient (350 ppm) and elevated (600 ppm) atmospheric CO_2. Bars followed by different letters indicate significant differences in total (upper case) or respiring (lower case) bacterial numbers, respectively ($P < 0.05$). Legend: ■ total bacteria; ■ respiring bacteria.

Results

Microbial activity and total cell counts

The total number of bacteria in bulk soil from ryegrass plots was 23% greater (8.5×10^8 cells/g dry soil) than in white clover plots (6.9×10^8 cells/g dry soil), averaged over the atmospheric CO_2 concentration treatment (P=0.079). There was no effect of atmospheric CO_2 concentration on total bacterial numbers in bulk soil, when averaged over plant species. However, there was a significant (P=0.069) treatment by species interaction (Figure 1). This interaction arose because there was no impact of CO_2 concentration on bacterial numbers in bulk soil under perennial ryegrass, but bacterial populations increased about 1.4 fold under white clover.

As with the total bacteria, we also found a significant CO_2 treatment by plant species interaction (P=0.056) for the number of active, respiring bacteria in bulk soil. There was no effect of either CO_2 treatment or plant species, averaged across the other main treatment. Reflecting the trend seen with total bacteria, the number of respiring bacteria did not change under ryegrass, but increased 1.7-fold under white clover (Figure 1). Treatments did not alter the proportion of respiring bacteria to total bacteria in the bulk soil (mean = 0.33, SE = 0.04).

In contrast, there were no detectable effects of CO_2 treatment on the total number of bacteria in rhizosphere soil (mean = 117×10^8 cells/g dry soil, SE

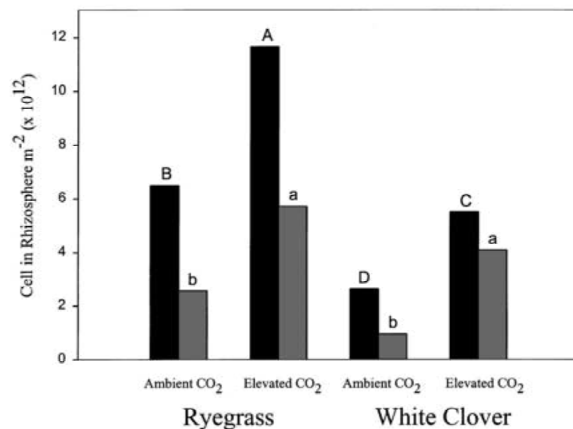

Figure 2. Bacterial numbers expressed on a per unit land area basis (m^2) in rhizosphere soil under perennial ryegrass and white clover grown under ambient (350 ppm) and elevated (600 ppm) atmospheric CO_2. Bars followed by different letters indicate significant differences in total (upper case) or respiring (lower case) bacterial numbers, respectively (P < 0.05). Legend: Bacterial populations per gram bulk soil for perennial ryegrass and white clover grown under ambient (350 ppm) and elevated (600 ppm) atmospheric CO_2. Bars followed by different letters indicate significant differences in total (upper case) or respiring (lower case) bacterial numbers, respectively (P < 0.05). Legend: ■ total bacteria; ■ respiring bacteria.

= 26.4 × 10^8 cells/g dry soil) or on the number of active, respiring soil bacteria near the roots (mean = 42 × 10^8 cells/g dry soil, SE = 1.2 × 10^8 cells/g dry soil). Higher atmospheric CO_2 concentration, however, increased the proportion of respiring:total bacteria by 1.4-fold (from 0.41 to 0.59, P = 0.034) in the rhizosphere soil.

The ecosystem level effect of CO_2 and plant species on rhizosphere bacterial populations is best assessed per unit land area, rather than per unit mass of rhizosphere soil. We found that both atmospheric CO_2 concentration and plant species affected total numbers of bacteria (number/m^2)present in the rhizosphere (Figure 2). There were about twice as many total rhizosphere bacteria in ryegrass as in white clover (9.1 *versus* 4.1 × 10^{12} cells/m^2), when averaged over CO_2 treatments (P=0.067). This difference was considerably smaller that the 5-fold higher root length density for ryegrass as compare to clover (Urs Zimmerman, unpublished data). Furthermore, increasing atmospheric CO_2 concentration resulted in an 85% increase in total rhizosphere bacterial numbers from 4.6 to 8.6 × 10^{12} cells/m^2, when averaged over the two plant species (P=0.033). Higher atmospheric CO_2 concentration also increased the number of respiring rhizosphere bacteria by 170% from 1.8 × 10^{12} cells/m^2 under ambient conditions to 4.9 × 10^{12} cells/m^2 under 600 ppm (P = 0.018), when averaged over plant species. There was no interaction between CO_2 and plant species on the number of respiring bacteria expressed on a unit land area basis.

Soil microbial community structure

Rhizosphere and bulk soil samples of white clover contained diverse microbial communities as indicated by PLFA analysis. Clear differences were observed between microbial communities in rhizosphere and bulk soil samples (Table 1). Bulk soil samples contained a greater percentage of PLFA profiles representative of Gram positive bacteria (terminally branched saturated PLFAs), actinomycetes (mid-chain branched saturated PLFAs), and obligate anaerobes (branched monoenoic PLFAs) than did rhizosphere soil samples. However, the percentage of Gram negative bacteria (represented by monoenoic PLFAs) detected in the rhizosphere soil was 1.7 times greater than that of bulk soil (Table 1).

The effect of CO_2 level on microbial community composition differed also between rhizosphere of white clover and bulk soil. The proportion of Gram negative bacteria in the bulk soil increased under CO_2 enrichment, while no effect was evident in the rhizosphere soil (Table 1). In contrast, the proportion of PLFA profiles representative of eukaryotes increased under high CO_2 levels in the rhizosphere soil. No effect of CO_2 level was observed for these PLFA profiles in bulk soil samples.

The microbial communities present in rhizosphere of white clover were primarily composed of monoenoic PLFA, generally found in Gram negative bacteria. A similar result was found for bulk soil. Rhizosphere samples contained the highest relative proportions of monoenoic PLFA (approximately 76%), while bulk soil samples contained approximately 46% of monoenoic PLFA (Table 1).

The CO2 level in the bulk soil affected the abundance of specific fatty acid biomarkers. For example, the relative proportion of the monoenoic PLFA 18:1ω7c was higher under CO_2 enrichment, while other PLFA biomarkers, such as 16:1ω7t and 19:1ω6c, remained unchanged. No effect of the CO_2 level was evident for the rhizosphere samples (Figure 3).

Terminally branched saturated PLFAs, such as i15:0 i16:0 that are commonly found in Gram positive bacteria (O'Leary and Wilkinson, 1988), were also detected in the samples. The highest relative propor-

Table 1. Community structure analysis based on phospholipid fatty acids (PLFA) for rhizosphere and bulk soil of white clover grown under two CO_2 levels

PFLA Profiles	Percentage of Total PFLA[a]			
	Rhizosphere Soil		Bulk Soil	
	Ambient CO_2	Elevated CO_2	Ambient CO_2	Elevated CO_2
Monoenoics	76.2 ± 0.8	75.8 ± 0.7	45.4 ± 0.1**	46.5 ± 0.1**
Normal saturates	11.9 ± 0.8	11.9 ± 0.6	11.5 ± 0.2	11.5 ± 0.2
Terminally branched saturates	4.0 ± 0.0	4.0 ± 0.1	18.4 ± 0.1	18.3 ± 0.1
Mid-chain branched saturates	0.6 ± 0.0	0.7 ± 0.0	13.0 ± 0.5	12.2 ± 0.4
Branched monoenoics	0.5 ± 0.1	0.5 ± 0.1	6.8 ± 0.2	6.6 ± 0.1
Other Fatty Acids	0	0	0.2 ± 0.1	0.2 ± 0.1
Eukaryotes	6.8 ± 0.1*	7.2 ± 0.1*	4.8 ± 0.1	4.8 ± 0.1

[a]Values are means of two replications ± standard errors on mean.
*Denotes significant CO_2 effect at $P<0.1$.
**Denotes significant CO_2 effect at $P<0.05$.

tions these PFLAs (approximately 18%) were detected in bulk soil samples. In contrast, the proportion of terminally branched saturated PLFA for rhizosphere samples was only 4%. The fatty acids i15:0 and i17:0 were less abundant at elevated CO_2 levels for the bulk soil samples, while elevated CO_2 did not alter the composition of these terminally branched saturated PLFA in the rhizosphere (Figure 3).

Branched monoenoic PLFA were detected in all samples, with the highest relative proportion in the bulk soil (approximately 7%). Branched monoenoic PLFA are usually found in the cell membranes of obligate anaerobes. The branched monoenoic PLFA i17:1ω7c is a biomarker for the anaerobic sulfate/iron reducing bacterium *Desulfovibrio*, and was detected in all samples. This fatty acid was less abundant at elevated CO_2 levels in the bulk soil (data not shown).

Mid-chain branched PLFA biomarkers, such us tuberculostearic acid 10me18:0 which is found in *Actinomyces* spp. (Federle, 1986; Kroppenstedt, 1985), were detected in rhizosphere and bulk soil samples. Bulk soil samples contained approximately 13% of this PLFA type, while only 0.7% of mid-chain branched PLFA were detected in the rhizosphere.

Eukaryote PLFAs, which are signatures for fungi, protozoa, algae, and higher plants, were also detected. Rhizosphere samples contained 7% of eukaryote PLFAs and bulk soil samples contained almost 5%. Although the relative proportions of eukaryotes in the bulk soil under ambient and elevated CO_2 concentrations were not significantly different, the biomarker 20:4ω6, which is found in protozoa (Vestal and White, 1989), was more abundant under elevated CO_2 (Figure 3).

Discussion

In this study, we observed a significant increase in total and metabolically active bacterial populations in the bulk soil of white clover plots exposed to elevated atmospheric CO_2. Although total populations of rhizosphere bacteria did not change when these two plant species were grown under elevated CO_2, a much greater proportion of the total bacterial population was present as active, respiring cells. When population levels were expressed on a land area basis they increased in the rhizosphere of both white clover and perennial ryegrass under elevated CO_2. This is the first report, to our knowledge, of ecosystem-level microbial population responses to CO_2. Other studies have also reported an increase of microbial activity as a result of elevated CO_2 concentrations (Dhillion et al., 1996; Rice et al., 1994; Rogers et al., 1992; Runion et al., 1994), however, in some cases it is difficult to determine if the authors used rhizosphere soil or bulk soil in their analyses.

The concentration of CO_2 in the soil is much greater than above ground levels, mainly due to microbial respiration (Lamborg et al., 1983). Therefore, effects of elevated atmospheric CO_2 on soil microbial population size, activity, and composition most likely occur by indirect means through changes in the quantity and quality of plant-derived inputs into the

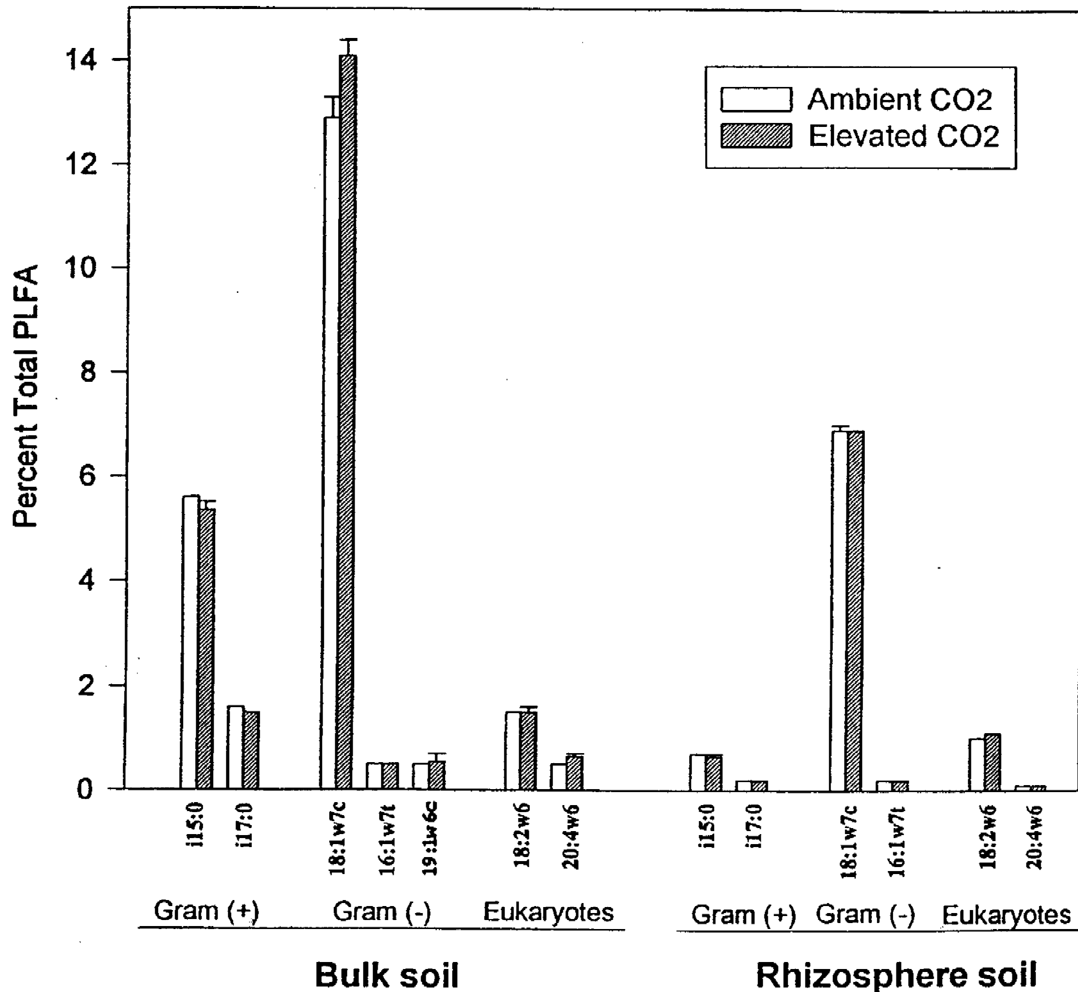

Figure 3. Selected phospholipid fatty acid (PLFA) profiles from micro-organisms found in rhizosphere and bulk soil of white clover grown in ambient (350 ppm) and elevated (600 ppm) atmospheric CO_2. Legend: □ ambient CO_2; ▨ elevated CO_2.

soil (Hodge, 1996; Hodge et al., 1998; Paterson et al., 1997; Sadowsky and Schortemeyer, 1997).

Rhizosphere microorganisms directly utilize plant-derived C (Merckx et al., 1987) and plants growing under CO_2 enrichment produce greater amounts of available C due to an increased amount of rhizodeposition (Diaz et al., 1993; Rogers et al., 1994; van Veen et al., 1991). Several studies have shown increased root exudation and enhanced translocation of assimilated C into the soil under elevated CO_2 (Cotrufo and Gorissen, 1997; Hodge et al., 1998; Paterson et al., 1996; Rattray et al., 1995). Schortemeyer et al. (2000), however, found no evidence of increased root exudation after two years of CO_2 enrichment in a study conducted in open-top chambers and reported that cumulative total organic C released in the root exudates was significantly higher under ambient CO_2. The contrasting results may be due to differences in plant species, methodologies, and the plant growth conditions used.

Carbon substrate availability in the bulk soil is much lower than that in the rhizosphere, and may limit microbial respiration. However, at this time we cannot discount that C availability increases in bulk soil under elevated CO_2, compared to ambient CO_2, resulting in the increase of total and metabolically active bacteria that we observed with soils near white clover. Increased substrate supply to the bulk soil may arrive via mycorrhizal hyphae. Others have found increased mycorrhizal growth under elevated atmospheric CO_2 levels (Rillig and Allen, 1998; Rouhier and Read, 1999). These hyphae may improve C supply

to microorganisms distant from the root. Alternatively, rapid root turnover in N-rich legumes (Dubach and Russelle, 1994; Goins and Russelle, 1996) may provide C for microbial growth in soil that is not next to roots present at sampling. Presence of adequate N to support plant growth is a critical component in plant response to elevated CO_2 (Luscher et al., 2000). Further studies will be necessary to determine how microbial populations in the bulk soil are stimulated by elevated atmospheric CO_2.

Several studies have suggested that elevated atmospheric CO_2 concentrations could alter the composition of soil microbial communities due to changes in the amount and/or composition of plant material input into the soil (O'Neill et al., 1987; Rogers et al., 1994). Plant-related changes in bacterial populations under elevated CO_2 have been reported. Schortemeyer et al. (1996) reported that the population of *R. leguminosarum* bv. *trifolii* in the rhizosphere of white clover plants subjected to elevated atmospheric CO_2 concentration increased two-fold in the Swiss FACE experiment, whereas no change was observed in the rhizosphere of ryegrass. The authors suggested that this change was due, in part, to plant-derived signal molecules (root exudates) which were selectively enhanced under elevated CO_2, resulting in an increase in the number of *R. leguminosarum* bv. *trifolii* in the rhizosphere. Similarly, Montealegre et al. (2000) showed a shift in the community composition of *R. leguminosarum* bv. *trifolii* in the Swiss FACE as a result of CO_2 enrichment. More recently, Marilley et al. (1999) reported that *Pseudomonas* sp. strains increased their dominance in the rhizosphere of ryegrass, while pseudomonad dominance decreased in the rhizosphere of white clover. In addition, studies done in a more artificial system, an Ecotron, have also shown that the composition of soil fungal species changes as a result of rising atmospheric CO_2 (Jones et al., 1998). While these studies indicate that specific microbial populations are altered in the rhizosphere of plants subjected to elevated atmospheric CO_2, the mechanism(s) by which this occurs is presently unknown.

In this present study, we used PLFA analysis to examine microbial community composition in rhizosphere soil from white clover plants grown under ambient and elevated CO_2. We chose only to examine clover plants because our previous results indicated that this plant system has substantive changes in microbial populations (Montealegre et al., 2000; Schortemeyer et al., 1996). Results of our studies indicated that while the PLFAs corresponding to prokaryotes were predominant in both rhizosphere and bulk soil (representing more than 90% of the total PLFAs extracted), clear differences between microbial communities in rhizosphere and bulk soil of white clover and their responses to the level of CO_2 were evident. PFLA analyses done by Zak et al. (1996) and Ringelberg et al. (1997) similarly showed differences between the microbial community composition of rhizosphere and non-rhizosphere soil of *Populus grandidentata* and *Quercus alba*, respectively. However, some studies have shown no alteration of microbial community composition due to elevated atmospheric CO_2 concentrations (Zak et al., 1996; 2000). Griffiths et al. (1998), using broad-scale DNA techniques, showed that the rhizosphere microbial communities of ryegrass and wheat (*Triticum aestivum* L.) were 86% similar under ambient and elevated CO_2, with indistinguishable mole% g + C profiles and Ringelberg et al. (1997) reported that elevated CO_2 caused only a subtle change in gram negative bacteria and actinomycetes.

Results from PLFA analyses in our study indicated that the proportion of eukaryotes in the rhizosphere increased under elevated CO_2, whereas the proportion of prokaryotes was not affected. However, phospholipid fatty acids indicative of gram negative bacteria increased in the bulk soil of plants exposed to CO_2 enrichment in accordance with the increase in microbial activity. We also observed that the PLFA profile $20{:}4\omega6$, a biomarker for protozoa, increased in the bulk soil of white clover grown under CO_2 enrichment. We hypothesize that this increase in protozoa is in response to the increase in the abundance of metabolically active bacteria observed when plants are grown under elevated CO_2 conditions.

In our study the effect of CO_2 level on particular PLFA profiles, although statistically significant ($P<0.05$ and $P<0.1$), were subtle and we cannot necessarily infer differences in metabolic activity (e.g. greater predation by protozoa) from these 'standing state' measurements done in the short term. Pulse-chase and stable isotope tracer studies may help elucidate differences in microbial activity in the rhizosphere and bulk soil that occur in response to elevated atmospheric CO_2. Moreover, since it is likely that alterations in microbial community composition might occur at a very slow rate or on the microscale, long term studies are required to determine if changes observed in this study will continue over time and will become more pronounced.

Belowground microbial processes play an essential role in nutrient cycling and organic matter turnover, and influence the growth of the plants by competing for nutrients. Therefore, changes in microbial activity and community composition occurring under elevated atmospheric CO_2 may have the potential to influence the cycling and storage of C and N within terrestrial ecosystems. Further studies are required to determine the mechanisms responsible for the alterations in microbial numbers, activity, and community composition observed in this study, but as we have shown in this research, both spatial (rhizosphere and bulk soil) and ecosystem-level analyses are needed.

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