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677 South Segoe Rd., Madison, WI 53711 USA

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Consequences of Rising Atmospheric Carbon Dioxide Levels for the Belowground Microbiota Associated with White Oak

David B. Ringelberg,* Julia O. Stair, Jonas Almeida, Richard J. Norby, Elizabeth G. O'Neill, and David C. White

ABSTRACT

The consequences for belowground microbiota under conditions of rising atmospheric CO_2 are largely unknown. In this research we examined the microbiota associated with white oak (*Quercus alba* L.). It was our hypothesis that an increase in CO_2 level would induce a change in the rhizosphere-associated microbial abundance and community composition. To provide an in situ estimation of microbial abundance and community composition, ester-linked polar lipid fatty acid (PLFA) technology was utilized. This technology, based on the quantitative measurement of membrane lipid fatty acids, has been utilized in the accurate identification and description of bacterial isolates and communities. Initial experiments demonstrated that a clear distinction in lipid patterns and microbial biomass existed between sterile roots and those of roots containing an associated viable microbiota. Statistical approaches were then used to determine what differences existed between individual PLFA and PLFA patterns obtained from white oak fine roots and bulk soils. An analysis of variance (ANOVA) showed significant differences to exist in the relative percentages of individual prokaryotic PLFA collected under ambient vs. elevated CO_2 and between those associated with fine roots and bulk soils. Multivariate statistics showed distinct differences in the patterns of prokaryotic PLFA detected in the rhizosphere vs. the surrounding bulk soil, but did not identify differences related to elevated CO_2 exposures. An artificial neural network recognized PLFA patterns unique to three different CO_2 exposures: ~35, ~50, and ~65 Pa. Results of the three statistical tests were viewed as supportive of the hypothesis describing significant differences in individual PLFA and patterns of PLFA as a result of elevated CO_2 exposure.

One of the prominent weaknesses in our understanding of how forest ecosystems will respond to increases in atmospheric CO_2 concentration is the limited knowledge of responses of roots and their associated rhizosphere microbial community. This ecosystem component has a potentially large effect on belowground C storage (O'Neill, 1994). Since host–pathogen relationships, defenses against physical stresses, the capacity to overcome resource shortages, and rhizodeposition are all related to rhizosphere organisms (Rogers et al., 1994), any change in microbial biomass, community composition, or metabolic status of the rhizosphere could directly or indirectly affect the process of soil C storage as well as aboveground processes.

One possible effect of CO_2 enrichment on the rhizosphere microbiota is via increased allocation of photosynthate to root systems, thus stimulating the exudation of C compounds into the rhizosphere and consequently stimulating the rhizosphere microflora (Zak et al., 1993; Norby et al., 1995a). Although a seedling study that performed at the same site as this study failed to produce clear evidence of increased exudation under conditions of elevated CO_2 (Norby et al., 1987), fine root growth and mycorrhization were shown to increase (O'Neill et al., 1987; O'Neill, 1994; Lewis et al., 1994). To address ecosystem-level questions using data from experiments with isolated plants, it is necessary to know how rhizosphere responses will scale with fine root dynamics. The possible responses include: (i) a compensatory or negative feedback effect, whereby increased fine root density is associated with a reduction in rhizosphere microbial mass per unit root, (ii) a constant microbial mass per unit root, or (iii) a specific stimulation of the rhizosphere microbial community. This study looked at the possibility of these scenarios by examining the rhizosphere microbiota for changes in biomass, community composition and/or physiological status through the analysis of membrane lipid fatty acids (PLFA).

In the past, responses of rhizosphere microbiota to environmental change have been difficult to quantify in terms of biomass, community composition, and physiological health. It has been repeatedly documented that classical techniques for the detection of microorganisms in soils and sediments, which are based on isolation from the soil matrix and subsequent culture to form visible colonies, are inadequate because the resulting counts may represent 0.1 to 10% of the extant community (Balkwill et al., 1988; Tunlid and White, 1991; White, 1983, 1986, 1988). Consequently, methods of assessing the total microbial community that do not require the isolation of the intact and viable organisms from the environmental matrices were developed. One of the most comprehensive and quantitative assays available for the assessment of environmental microbial communities is the analyses for signature lipid biomarkers.

Signature lipid biomarker (SLB) methods involve the assay of cellular components which have proven to be an excellent means by which to quantitatively define the viable microbial biomass, community composition, and metabolic status (Ringelberg et al., 1988; Tunlid and White, 1991; Vestal and White, 1989; White, 1983).

D.B. Ringelberg, Dyntel Corp., Waterways Experiment Station, 3909 Halls Ferry Road, Vicksburg, MS 39180-6199; J.O. Stair and J. Almeida, Center for Environ. Biotechnol., Univ. of Tennessee, 10515 Research Dr., Suite 300, Knoxville, TN 37932-2575; and R.J. Norby, E.G. O'Neill, and D.C. White, Environ. Sciences Div., Oak Ridge National Lab., Oak Ridge, TN 37831. Received 1 Mar. 1996. *Corresponding author (dringelbe@aol.com).

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Abbreviations: PLFA, polar lipid fatty acid; ANOVA, analysis of variance; SLB, signature lipid biomarker; PHB, poly-hydroxy butyric acid; ANN, artificial neural network.

Some of the more descriptive cellular components are the ester-linked phospholipid fatty acids (PLFA). Phospholipids are rapidly turned over in the environment making them valuable markers for living cells (White et al., 1979). As a result, estimates of the total viable microbial biomass, which are comparable to other direct measurements, can be made. For example, a study of subsurface sediments showed estimates of microbial cell numbers based on PLFA to be equivalent to those determined by acridine orange direct counts (Balkwill et al., 1988). In addition to estimating microbial biomass, certain PLFA are discriminatory enough to allow various subsets of the microbial community to be identified (Vestal and White, 1989), thus providing insight into microbial community composition. Differences in the end products of fatty acid biosynthetic pathways provide insight into the origin of these various subsets. For example, the branched chain pathway, which is utilized by gram-positive bacteria, typically results in the formation of terminally methyl branched saturated fatty acids, whereas the anaerobic desaturase pathway utilized by gram-negative bacteria typically results in the formation of monounsaturated fatty acids. Although there are many variations of the above generalizations, the fact that basic differences do exist provides descriptive power when interpreting a PLFA profile. The quantification of certain PLFA ratios also provides insight into the metabolic status of specific components of the microbial community. For example, increases in the ratios of *trans* to *cis* PLFA and cyclopropyl to monoenoic precursor PLFA have been shown to indicate an increase in starvation and anaerobic respiration in cultures undergoing nutrient deprivation (Guckert et al., 1986). Another component of the SLB analysis is the quantification of poly-hydroxy alkanoates, in particular, poly-hydroxy butyric acid or PHB. This polyester functions as a storage lipid in a number of prokaryotes (Doi, 1990). In many instances, under conditions of nutrient limitation with sufficient utilizable C, PHB is accumulated within the cell (Findlay and White, 1983). By relating the concentration of PHB to that of PLFA, an indication of the growth characteristic of the organism or community can be obtained. For example, high values for PHB/PLFA indicates unbalanced growth in that C is being stored but cells are not dividing.

This study utilized the PLFA and PHB components of the SLB analysis to assess the viable biomass, community composition, and nutritional status of the rhizosphere and bulk soil associated microbiota of white oak (*Quercus alba* L.) saplings grown in open top chambers under 35, 50, and 65 Pa atmospheric CO_2 levels.

MATERIALS AND METHODS

The experimental facility for exposure of white oak seedlings to elevated CO_2 is located at the Oak Ridge National Environmental Research Park in Roane County, Tennessee (Wullschleger et al., 1992; Gunderson et al., 1993). An experimental array of open top chambers (Rogers et al., 1983) was established with seedlings originated from a half-sib collection of acorns that had previously been grown for 8 wk in growth cabinets containing 35, 50, or 65 Pa CO_2. During this period, seedlings were grown in a commercial soil-free mix (Promix), which had been inoculated with a vegetative culture of the ectomycorrhizal fungus, *Pisolithus tinctorius* (Pers, Coker, and Couch). After dormancy induction and a cold treatment, the dormant seedlings were planted in the field in May 1989, where they were grown for four full growing seasons under ambient conditions of light, temperature, precipitation, and soil. There were five seedlings in each of two replicate chambers per CO_2 concentration. The atmospheric CO_2 concentration in the chambers was maintained continuously (from April through November) at 35, 50, or 65 Pa. In November 1992, the experiment was terminated and plants harvested. Fine root density was measured in five soil cores (10 cm diam. by 15 cm deep) extracted from each chamber (Norby et al., 1995b)

Sections of root for rhizosphere analysis were collected from the array in November 1992. Roots with adhering soil were removed from within 1 m of the stem of each tree, placed in sterile packs, and then placed on ice for transportation. From the root sections, fine roots (<1 mm diam.) totaling approximately 0.5 g were collected for PLFA and poly-hydroxy butyric acid (PHB) analyses. In addition, a soil core from one chamber representing each CO_2 level was collected. The cores were divided into 0- to 5-, 5- to 10-, and 10- to 15-cm depths sections. A total of approximately 37 g of this soil was used to obtain PLFA and PHB profiles and concentrations.

Soils and roots were extracted in a single phase chloroform–methanol solvent system (Bligh and Dyer, 1959) modified to include a PO_4 buffer (White et al., 1979). The extract was separated into aqueous and organic (lipid-containing) phases by the addition of equal parts of chloroform and nanopure water. The organic phase was then fractionated into neutral-, glyco-, and polar-lipids on a column of silicic acid (Gehron and White, 1983). The polar lipid fractions were treated in a mild alkaline system to transesterify polar lipid fatty acids into fatty acid methyl esters for gas chromatographic analysis (White et al., 1979). Following strong acid ethanolysis of the glycolipid fraction, poly-hydroxy butyric acid (PHB) was analyzed using gas chromatography (Nickels et al., 1979). Structural verification of all lipid moieties was by gas chromatography–mass spectrometry as described in Ringelberg et al. (1994).

Polar lipid fatty acid are designated by the number of carbons followed by the number of double bonds, the position of the double bond from the aliphatic end (w) of the molecule and by the isomeric configuration of the double bond as either *cis* (c) or *trans* (t). For example: 16:1w7c contains 16 carbons with 1 double bond located 7 carbons from the methyl end of the molecule in the *cis* configuration. Terminal branching is indicated by i (iso) or a (anteiso), representing the location of a methyl branch 1 or 2 carbons in from the aliphatic end of the molecule (e.g., i15:0). Mid-chain branching is designated by the position of the methyl group from the acid end of the molecule (e.g., br19:1). Cyclopropyl fatty acids are indicated by the prefix cy (e.g., cy17:0).

Previous work demonstrated that PLFA analysis could discriminate between root and rhizosphere microbiota in the herbaceous rape plant (*Brassica napus* L.) (Tunlid et al., 1985). To determine whether the technique was valid for the woody plant white oak, fine root material with adhering rhizosphere soil were twice sonicated (5 min each time) in 20% sodium hypochlorite at 25°C. This oxidative treatment destroys microbial lipids through lysis and, in effect, *sterilizes* the roots. Polar lipid fatty acids are then dephosphorylated by exogenous and endogenous phospholipases into diglycerides, which elute as a neutral lipid on the silicic acid columns. Samples were processed as described above and compared with white oak rhizosphere and soil core lipid profiles to ascertain which

PLFA were attributable to microorganisms, i.e., those affected by the hypochlorite treatment.

Statistical analyses were selected to provide a comprehensive overview of the resulting data set. An analysis of variance using a Scheffe one-way ANOVA was performed on nontransformed data to determine if significant differences existed between the means of individual PLFA in relation to the CO_2 treatments (Manugistics, 1995). Significance was determined at 90, 95, and 99% confidence levels. Multivariate statistics in the form of a hierarchical cluster analysis was utilized to observe relationships between entire patterns of PLFA, as related to the different CO_2 levels. The cluster analysis was performed on arcsin transformed mole percentages using the complete linkage method based on euclidean distances (Everitt and Dum, 1991). Although this analysis relates samples based on profile similarities, there are no established criteria for indicating the presence of one or more communities and results are typically significant only within the given data set. Another analysis was then utilized whereby the ability of the test to recognize a pattern of PLFA descriptive of the varying CO_2 treatments was investigated. Feed forward multiperception artificial neural networks (ANN) were developed using log transformed mole percentages. Artificial neural networks are Artificial Intelligence techniques consisting of assemblies of processing units (nodes) based on the structure of a nervous system (Hinton, 1992). The networks' ability to represent complex nonlinear systems has been shown to be particularly suitable for bioprocess modeling and pattern recognition (Montague and Morris, 1994). The networks were trained with 90% of the data sets for rhizosphere (fine root material) and bulk soils independently. The accuracy of the networks were then cross-validated using the remaining, randomly selected 10%. For cross-validation, 10% of the PLFA patterns were randomly excluded from the data set and used for determination of a generalized recognition by the ANN. When the recognition error for both data sets started to diverge, the optimization process was stopped. A low cross-validation error coupled with a minimum number of hidden nodes determined the optimal ANN configuration. Statistics were run using the software packages Statgraphics Plus for Windows version 1.0 (Manugistics, Rockland, MD) for Scheffe ANOVA, Einsight version 3.0 (Infometrix, Seattle, WA) for hierarchical cluster analysis and Braincell 2.0 (Promised Land Tech, UT) for development of the artificial neural networks.

RESULTS AND DISCUSSION

Bleaching the fine root material resulted in the loss of specific PLFA known to be associated with prokaryotic cell membranes (Fig. 1A), but did not significantly affect those PLFA typically associated with eukaryotes (Fig. 1B) (Vestal and White, 1989). The PLFA that decreased in mole percentage as a result of the bleaching treatment were of 19 carbons or less with at most one degree of unsaturation. The only PLFA detected in the fine root material fitting this description that did not show a decrease in mole percentage were the saturates 14:0 through 18:0 and the monounsaturate 18:1w9c. This monounsaturate, as well as residing in the membranes of certain gram-negative bacteria (Ratledge and Wilkinson, 1988), is also a precursor in the formation of polyunsaturated fatty acids (via the aerobic desaturase biosynthetic pathway), which are typically associated with eukaryotic organisms (Harwood and Russell, 1984). Those PLFA not affected by the bleaching treatment were either of 20 carbons or greater or contained 2 or more degrees of unsaturation. Based on these findings, results and interpretations of the effects of elevated atmospheric CO_2 on belowground prokaryotic microbiota will be restricted to the PLFA listed in the top half of Table 1. These results are presented for the rhizosphere or fine root associated microbiota and the bulk soil or nonroot associated microbiota independently. The only exception to the above was in the development of the artificial neural networks for the rhizosphere and bulk soil data sets, which utilized a combination of the prokaryotic and eukaryotic PLFA described in Table 1 adjusted so that the total mole percentage per sample equaled 100%.

Biomass

A significant decrease in the rhizosphere associated microbial biomass (per gram weight of fine root material) occurred between the ambient and 50 Pa CO_2 exposures. No significant differences were measured between the means of 35 and 65 Pa rhizosphere CO_2 exposures or between any of the three CO_2 exposures in the bulk soils (Table 2). Prokaryotic biomass was estimated from the abundance of PLFA affected by the bleaching treatment and eukaryotic biomass from those PLFA not significantly affected by the treatment, as previously described. The degree to which bleaching affected PLFA of fungi (especially ectomycorrhizae) could not be determined from the PLFA analysis because of overlap with root PLFA and would have necessitated the analysis of sterols.

Above and belowground plant biomass in the highest CO_2 exposure (by measures other than PLFA) were found to be more than double those in the ambient exposure. The growth response occurred early in the experiment with no sustained effect of CO_2 on growth rate, even though photosynthesis remained higher (Norby et al., 1995b; Gunderson et al., 1993). There was little evidence of either a compensatory or negative feedback effect between rhizosphere microbial biomass and fine root density. Fine root density increased 140% at the highest CO_2 concentration (Norby et al., 1995b), but results of this study showed there to be a significant effect on microbial biomass per unit fine root only between the 35 and 50 Pa CO_2 treatments and not between the 35 and 65 Pa treatments. A linear relationship between rhizosphere microbial biomass and fine root density was identified, implying an increase in total microbial biomass in the system (Table 3), at least until a maximum fine root density is attained. There was little indication of a negative or compensatory feedback between fine root mass density and rhizosphere bacterial biomass, since microbial biomass was not found to significantly decrease per unit fine root between 35 and 65 Pa CO_2 exposures.

The PLFA analysis clearly demonstrated the existence of the *rhizosphere effect*, or the stimulation of microbial growth by matter released by the plant (Elliott et al., 1979) by showing prokaryotic concentrations in the rhizospheres to be at least three times that observed in the surrounding bulk soils (Table 1). Significant decreases in PLFA were observed with depth increase in the bulk soil sections (0–5 cm, 18 $\pm$ 6 μmol kg^{-1}; 5–10 cm, 14

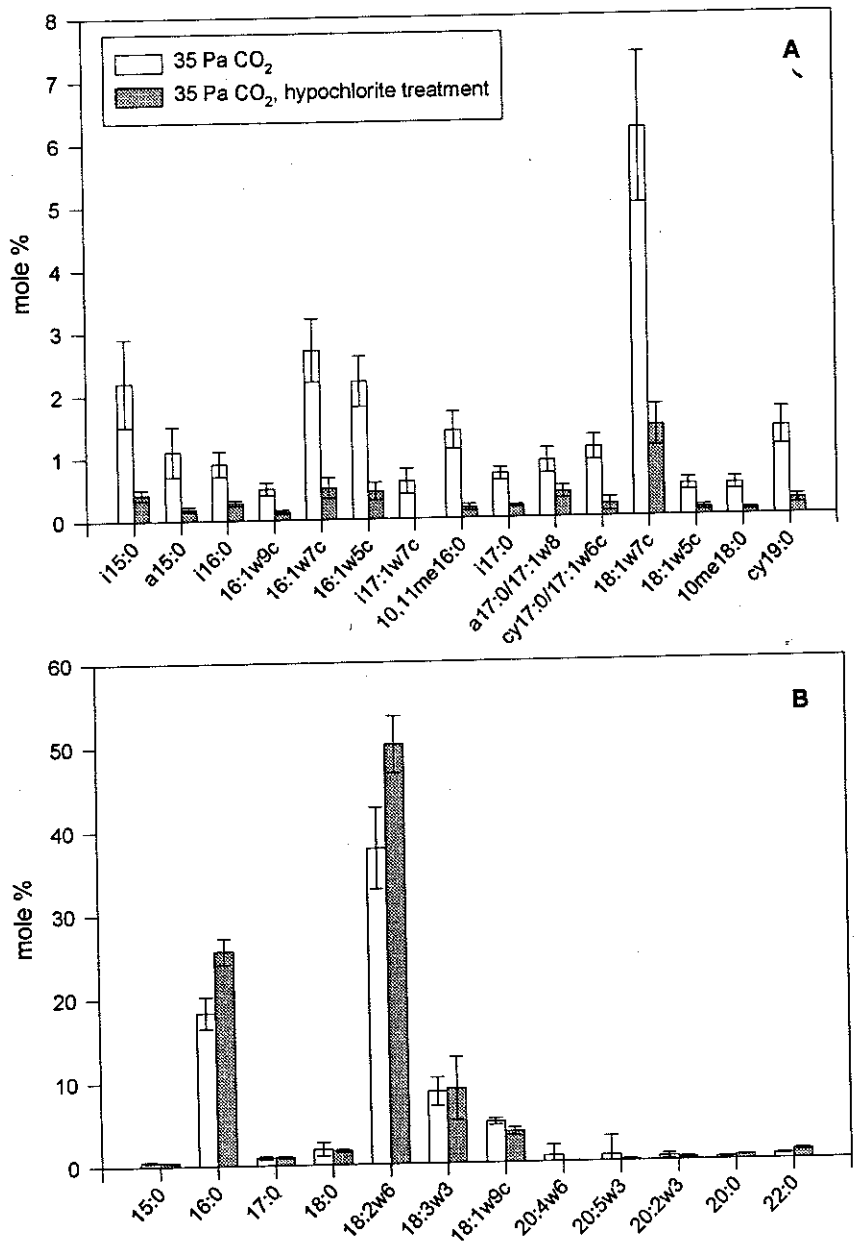

Fig. 1. Effect of hypochlorite treatment on white oak fine root material. (*A*) Prokaryotic PLFA decreased in mole percentage, while (*B*) eukaryotic PLFA showed no affect from the treatment. Error bars represent 1 SD, $n = 5$.

$\pm$ 4 μmol kg^{-1}; and 10–15 cm, 9 $\pm$ 2 μmol kg^{-1}; average $\pm$ standard deviation) but there were no significant differences in belowground microbial biomass with respect to the three CO_2 exposures. This latter result is similar to that observed by Zak et al. (1995). In their study, aspen (*Populus* sp.) was exposed to ambient and twice ambient atmospheric CO_2 concentrations in open top chambers. Neither the rhizosphere nor the nonrhizosphere associated soils showed a significant difference in the PLFA concentrations under ambient and elevated CO_2.

Community Structure

An analysis of variance (ANOVA) indicated that prokaryotic PLFA profiles in the rhizosphere and the bulk soils were affected by exposure to elevated atmospheric CO_2. However, the ANOVA showed significant differences to exist primarily between the 35 and 50 Pa exposures (Table 2) and not between the 35 and 65 Pa CO_2 treatments. The only PLFA to show a significant difference in mole percentage between the 35 and 65 Pa exposures was 10me18:0 or tuberculostearic acid. This acid has been described as a biomarker for the actinomycetes (Kroppenstadt, 1985). The significant differences between the lower and mid-CO_2 exposures correlates with the biomass observations. Although the decrease in biomass, as a result of the 50 Pa exposure, was associated with a subtle shift in prokaryotic community composition (Table 2) the limited number of PLFA, which were affected precludes an attempt at accurately

Table 1. Percent composition (mole %) of PLFA profiles recovered from white oak rhizospheres (fine root material).

	Rhizosphere‡						Bulk soil§					
	35 Pa	SD	50 Pa	SD	65 Pa	SD	35 Pa	SD	50 Pa	SD	65 Pa	SD
Prokaryotic PLFA†												
i14:0	–	–	–	–	–	–	–	–	1.1	0.0	0.7	0.5
i15:1w7c	–	–	–	–	–	–	1.0	0.1	1.1	0.1	0.7	0.5
i15:0	9.1	2.1	7.4	2.6	8.1	1.9	9.6	1.3	9.5	0.4	9.3	1.5
a15:0	4.8	1.1	4.1	1.5	4.4	0.9	6.0	0.9	6.6	0.2	9.6	3.0
br15:0a	–	–	–	–	–	–	0.2	0.3	0.6	0.0	0.6	0.1
br16:1	–	–	–	–	–	–	0.7	0.1	0.8	0.2	0.8	0.1
i16:0	3.7	0.6	3.6	0.7	3.7	0.6	3.4	0.2	3.6	0.1	4.0	0.2
16:1w9c	2.0	0.3	1.7	0.3	1.8	0.2	2.4	0.0	2.2	0.1	2.6	0.5
16:1w7c	11.7	1.3	10.4	2.2	11.3	1.5	10.1	0.4	9.9	0.1	11.8	2.9
16:1w5c	9.5	1.1	9.1	1.1	9.2	0.9	8.7	1.0	8.0	0.2	9.2	2.4
i17:1w7c	2.7	0.8	3.2	0.4	3.1	0.6	4.8	0.3	4.3	0.0	5.3	0.3
i17:1	–	–	–	–	–	–	0.6	0.0	0.7	0.4	0.6	0.1
10,11me16:0	5.9	0.8	5.8	1.4	5.2	1.1	10.0	0.7	9.1	0.5	10.5	0.8
br16:0a	–	–	–	–	–	–	1.0	0.1	0.9	0.1	1.1	0.1
i17:0	3.0	0.3	2.6	1.1	3.0	0.3	2.9	0.1	2.7	0.1	3.2	0.2
a17:0/17:1w8	3.9	0.5	4.3	0.6	4.0	0.4	3.2	0.1	3.3	0.0	2.5	1.8
cy17:0/17:1w6c	4.6	0.7	5.2	0.9	4.9	0.6	4.2	0.2	4.9	0.3	4.9	0.8
br17:0a	0.9	0.2	1.2	0.4	1.0	0.3	1.3	0.3	1.4	0.1	1.0	0.4
br17:0b	–	–	–	–	–	–	0.5	0.1	0.6	0.1	0.3	0.2
10me17:0	–	–	–	–	–	–	0.8	0.2	1.1	0.1	1.2	0.3
18:1w7c	26.4	2.9	27.0	3.9	27.2	3.0	15.7	2.9	14.5	0.9	4.7	6.1
18:1w5c	2.3	0.5	2.7	0.8	2.3	0.3	1.9	0.1	2.1	0.2	2.3	0.2
br19:1a	1.6	0.3	1.6	0.3	1.7	0.2	1.2	0.2	1.2	0.0	0.9	0.5
10me18:0	2.3	0.6	3.0	0.6	2.9	0.7	2.3	0.6	3.1	0.2	2.9	1.7
12me18:0	–	–	–	–	–	–	–	–	0.2	0.3	0.1	0.1
cy19:0	5.7	0.8	6.9	1.8	6.3	1.2	7.7	0.3	6.4	0.2	9.1	2.0
Total	100.0	–	100.0	–	100.0	–	100.0	–	100.0	–	100.0	–
Total μmol kg^{-1}	69.0	15.5	56.2	20.0	67.0	12.2	18.0	6.0	22.0	4.2	16.2	1.8
Eukaryotic PLFA												
14:0	–	–	–	–	–	–	1.9	0.5	2.2	0.1	2.3	0.6
15:0	0.7	0.2	0.6	0.3	0.8	0.4	1.4	0.3	1.7	0.1	1.7	0.1
16:0	24.0	2.5	22.4	3.4	23.6	1.8	40.0	2.8	39.0	1.5	41.2	1.0
17:0	1.2	0.3	1.1	0.2	1.0	0.2	1.6	0.1	1.9	0.1	1.8	0.1
18:0	2.5	1.1	2.7	0.6	2.3	0.6	8.9	0.5	8.6	0.4	7.7	2.6
18:2w6	49.3	4.9	48.7	3.6	51.1	3.1	10.8	2.4	12.8	1.5	13.6	3.1
18:3w3	11.2	1.9	11.0	1.6	11.4	1.7	–	–	–	–	–	–
18:1w9c	6.4	0.8	7.5	1.4	6.5	1.7	26.0	0.3	25.1	1.7	25.7	1.0
20:4w6	1.0	1.8	1.2	1.3	0.5	0.2	2.9	0.4	3.6	1.2	2.2	0.7
20:5w3	1.0	3.1	0.7	0.8	0.2	0.1	2.1	0.7	2.1	0.8	1.1	0.8
20:2w3	0.7	0.5	1.1	0.7	0.6	0.2	–	–	–	–	–	–
20:1w9c	0.4	0.9	0.5	0.6	0.2	0.1	2.7	0.6	1.3	0.9	0.7	0.9
20:0	0.4	0.1	0.5	0.1	0.4	0.2	0.8	0.1	0.8	0.0	1.5	0.8
22:0	0.8	0.1	1.1	0.3	0.9	0.1	0.6	0.4	0.6	0.4	0.6	0.4
24:0	0.4	0.1	0.8	0.3	0.5	0.2	0.2	0.3	0.3	0.2	0.1	0.2
Total	100.0	–	100.0	–	100.0	–	100.0	–	100.0	–	100.0	–
Total μmol kg^{-1}	2327.1	696.8	1527.1	685.8	2246.2	827.3	6.8	2.0	8.6	1.7	7.2	2.0
Total PLFA μmol kg^{-1}	301.7	78.4	217.3	65.0	306.5	82.5	24.8	7.9	30.5	5.9	23.5	3.8

† Ester-linked polar lipid fatty acid.
‡ Fine root material (<1 mm in diam.) with attached soil.
§ Soil core collected from a 0- to 5-cm depth interval away from the visible root zone of the plant.

describing the shift. Those PLFA that did shift are all consistent with a gram-negative bacterial population including the actinomycetes. This is indicated by the monounsaturates that comprise the bulk of the fatty acids in the cell membranes of this classification of bacteria (Ratledge and Wilkinson, 1988) and by tuberculostearic acid for the actinomycetes.

A hierarchical cluster analysis of the average prokaryotic PLFA profiles per sample type and treatment indicated that a clear distinction existed between bulk soil and rhizosphere PLFA profiles (Fig. 2). The bulk soils showed greater mole percentages (compared with the rhizosphere samples) of the terminally branched saturated PLFA i15:0 and a15:0. These PLFA are found in the cell membranes of many gram-positive bacteria, some actinomycetes, and some of the obligate anaerobes such as the sulfate reducing bacteria (Ratledge and Wilkinson, 1988). A classic example of a branched chain synthesizer is the gram-positive *Arthrobacter* sp., some of which contain as much as 80% of their membrane fatty acid as a15:0. The soils also showed increases in the mole percentages of i17:1w7c and 10me16:0, which are also found in the cell membranes of some actinomycetes and the sulfate reducing bacteria. Although the likelihood of anaerobic bacterial colonization in these soils is small, obligate anaerobes have been recovered from topsoils following depletion or exclusion of oxygen (Roger et al., 1992). In contrast with the soils, the rhizosphere samples showed greater percentages of two monounsaturated PLFA, 16:1w7c and 18:1w7c, both of which are terminal points in a biosynthetic pathway utilized by most gram-negative bacteria for fatty acid synthesis. A typical bacterial example is the *Pseudomonas* genus. A summary table describing PLFA in terms of primary origin is provided in Table 4.

The hierarchical analysis did not consistently relate

Table 2. A comparison of the individual PLFA recovered from white oak rhizospheres and bulk soils expressed as either a percentage (mole %) or concentration (μmol kg^{-1}).

	CO_2 exposures		
	35–50 Pa	50–65 Pa	35–65 Pa
Rhizosphere (fine root <1 mm diam.)			
PLFA (mole %)			
a17:0	*		
br17:0	*		
cy17:0	*		
cy19:0	*		
i17:1w7c	*		
10me18:0	***		**
16:1w7c	*		
16:1w9c	**		
18:1w5c	*		
Biomass (μmol kg^{-1})			
Prokaryotic	*		
Bulk soil (0- to 5-cm depth interval)			
PLFA (mole %)			
i14:0	**		
i16:0			**
i17:0		**	
i17:1w7c		**	
18:1w7c			*

*,**,*** Significance determined by ANOVA (Scheffe) at 90%, 95%, and 99% confidence intervals, respectively.

PLFA profiles recovered from the varying CO_2 exposures. Within each sample cluster there was no consistent pattern with respect to the hierarchical linkage of the 65 Pa treatment with the other two CO_2 exposures (data not shown). Since hierarchical analyses are based on positive correlations only, an artificial neural network (ANN) was applied to determine if a pattern of PLFA could be uniquely recognized for each CO_2 level. In this test the total PLFA profile from Table 1 was utilized allowing the ANN to attempt a recognition of CO_2 effects on both prokaryotic and eukaryotic components.

An ANN was developed for each sample type (rhizosphere or bulk soil) independently. The cross validation using randomly excluded data (see methods section) ensured that any recognition was based on general implicit rules instead of the reliance on the memory of experimental data points. The recognition of CO_2 exposure was digitized according to concentration. Consequently, a PLFA pattern of a rhizosphere or bulk soil exposed to 35 Pa CO_2 would be associated with the vector (1, 0, 0) for 50 Pa (0, 1, 0) and for 65 Pa (0, 0, 1) by three independent ANN, one for each coordinate. The ANN predictions are then represented in a three-dimensional plot where the existence of a recognizable association between PLFA pattern and CO_2 level can be visualized (Fig. 3 and 4). Although both ANN were able to recognize PLFA patterns representative of the three CO_2 exposures, the ANN for the bulk soils was able to do so with less recognition error (data not shown). Dynamics associated with root–microbe interactions likely complicated if not interfered with the ANN ability to recognize a PLFA pattern in the rhizosphere samples. This analysis did include PLFA attributable to both plant and microbe. Assuming there to be a *root effect*, the ANN analysis showed that in the presence of low organic soil content, a lower CO_2 concentration resulted in a clearer recognition. That is, the 35 and 50 Pa CO_2 exposures resulted in a PLFA pattern more clearly recognizable by the ANN in the bulk soils, whereas the most clearly recognizable PLFA patterns in the rhizosphere samples were associated with the two highest CO_2 exposures.

Table 3. An estimation of the belowground fine root associated prokaryotic biomass per square meter of soil at three CO_2 concentrations.

CO_2 exposure	Fine root mass density	Prokaryotic PLFA	Total prokaryotic abundance
Pa	g of root m^{-2} ground	μmol PLFA kg^{-1}	μmol PLFA m^{-2} ground
35	85	69	6
50	157	56	9
65	186	67	12

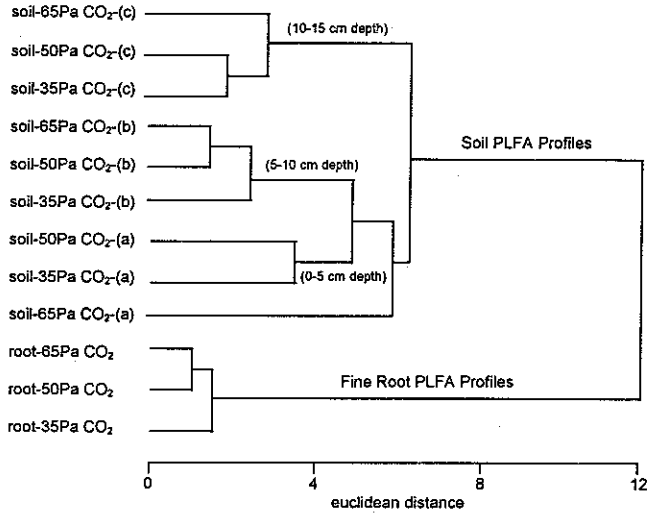

Fig. 2. A dendrogram representing the results of a hierarchical cluster analysis (complete linkage method) of averaged prokaryotic PLFA mole percentages (arcsin transformed) for rhizosphere (*Root*), and bulk soil (*Soil*) samples at three different CO_2 exposures: 35, 50, and 65 Pa. The bulk soil subsections are designated by the depth interval from which they were collected; (*a*) 0 to 5 cm, (*b*) 5 to 10 cm, (*c*) 10 to 15 cm. The analysis resulted in the formation of cluster groups representing the type of sample analyzed (root or soil), and the depth from which the soil samples were recovered. Since the PLFA profiles (prokaryotic community composition) of the individual samples show a greater similarity within a cluster group (linked by a lower euclidean distance) than they do between cluster groups (linked by a higher euclidean distance), these results indicate that the greatest difference occurs between fine root and soil samples and that, to a lesser extent, the soil samples exhibit different communities at different depths.

It is still unclear as to what the nature of the PLFA patterns are in terms of microbial community composition. Although an analysis of recognition sensitivity for individual PLFA is possible with an ANN, the highly nonlinear nature of the test makes it more appropriate to use other means (such as community level gene probes, isolations, etc.) for determining community composition. The above described results provide evidence of microbial community shifts as a result of elevated atmospheric CO_2 exposure. These findings, though not conclusive, are viewed as supportive of the hypothesis of this study in that significant changes in both PLFA abundance and composition were detected as a result of elevated CO_2 exposure. These changes are directly related to the be-

Table 4. A summary of PLFA moities in terms of primary origin. Adapted from Vestal and White, 1989.

PLFA	Functional group	Examples
i15:0, a15:0, i17:0, a17:0	Gram-positive	*Arthrobacter, Bacillus*
16:1ω7c, 18:1ω7c, cy17:0, cy19:0	Gram-negative	*Pseudomonas, Acinetobacter*
10me18:0	Actinomycetes	*Streptomyces*
10me16:0, i17:1ω7c	Sulfate-reducing bacteria	*Desulfobacter* (10me16:0), *Desulfovibrio* (i17:1w7c)
16:1ω8c, 18:1ω8c	Methane oxidizers	*Methylomonas* (16:1w8c), *Methylosinus* (18:1w8c)
18.2ω6, 18:3ω6, 18:3ω3	Fungi	*Pisolithus*
20:3ω6, 20:4ω6	Protozoa	*Naegleria*
18:1ω9c, 18:1ω11c, 18:3ω3, 20:5ω3	Higher plants	

lowground viable microbial biomass and community composition.

Nutritional Status

Specific ratios of PLFA associated with prokaryotic physiological status were not found to differ significantly as a result of elevated atmospheric CO_2 exposure Table 5). The ratios of cyclopropyl/monoenoic precursor and PHB/PLFA were both below levels previously observed in samples undergoing some form of nutritional imbalance (Ringelberg et al., 1988; Guckert et al., 1986; Table 4). Increases in the cyclopropyl/monoenoic precursor ratio have been observed in stationary phase cultures and in cultures showing increased anaerobic respiration (Guckert et al., 1986). The PHB/PLFA ratio reflects the proportion of C being stored to the number of viable cells present (Findlay and White, 1983). Increases in the ratio indicate unbalanced growth, in that cells accumulate C but are unable to undergo cell division due to nutrient limitations. Although the scope of these ratios is limited when considering the number of processes involved in microbial metabolism, there were no detectable changes in any of the three ratios as a result of CO_2 exposure. From the value of the ratios we can infer that the extant microbial community was not undergoing any form of undue environmental stress. As a result, activities related to C utilization and/or respiration were assumed to be unchanged under the elevated atmospheric CO_2 levels. The lack of detection of any shift in prokaryotic physiological status with increasing CO_2 concentrations suggests that the extant rhizosphere microbiota contained the ability to absorb any effects the elevated CO_2 levels may have elicited, such as nutrient limitations resulting from changes in rhizodeposition.

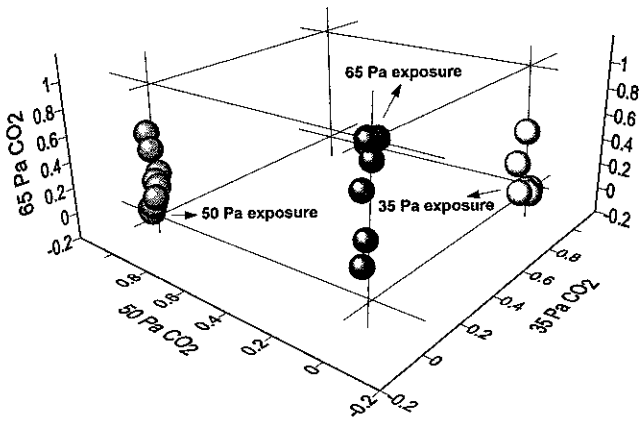

Fig. 3. Results of a trained artificial neural network's (ANN) ability to recognize a bulk soil (0–5 cm depth) PLFA pattern related to CO_2 exposure. The CO_2 exposures recognized were: 35 Pa CO_2 (clear sphere), 50 Pa CO_2 (grey sphere), and 65 Pa CO_2 (dark sphere). The three dimensional plot illustrates the digitized outcome of the ANN recognition process. For example, a complete recognition of a PLFA pattern related to the 65 Pa CO_2 exposure would result in the coordinates (0, 0, 1; *x*, *y*, *z*), for a 50-Pa CO_2 exposure (0; 1; 0; *x*, *y*, *z*) and for a 35-Pa CO_2 exposure (1, 0, 0, *x*, *y*, *z*). The tighter the cluster, the smaller the recognition error.

CONCLUSION

Our analyses indicated that exposure of white oak to elevated atmospheric CO_2 resulted in significant changes in microbial abundance and community composition. These observed differences are indirectly supportive of the hypothesis that elevated CO_2 exposures result in a change in the quantity and quality of root exudates. The results obtained in this study are more supportive of a specific stimulation of the belowground root associated microbiota than they are of a negative or compensatory feedback response. The nature of the specific stimulation is still unclear, yet the study resulted in consistent evidence of a stimulation of actinomycete (or related) type organisms.

Differences between the rhizosphere associated microbiota of white oak and the microbiota of surrounding nonrhizosphere soils were readily detected by analysis of the PLFA profiles. The assay was also capable of identifying shifts in belowground root associated biomass and community composition under the different CO_2

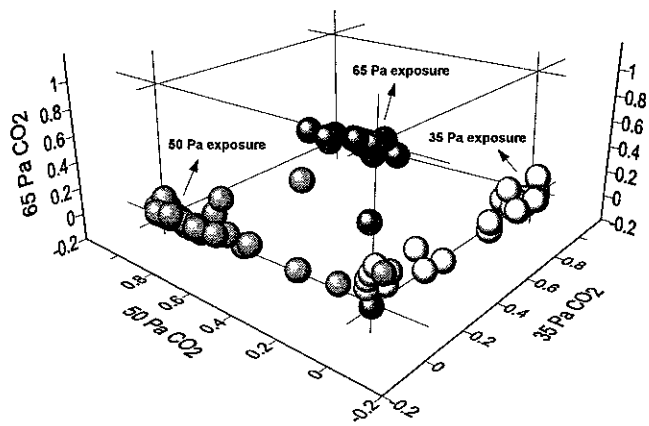

Fig. 4. Results of a trained artificial neural network's (ANN) ability to recognize a rhizosphere PLFA pattern related to CO_2 exposure. The CO_2 exposures recognized were: 35 Pa CO_2 (clear sphere), 50 Pa CO_2 (grey sphere), and 65 Pa CO_2 (dark sphere). The three dimensional plot illustrates the digitized outcome of the ANN recognition process as described in the legend for Fig. 3.

Table 5. An estimation of the prokaryotic physiological status by examination of specific PLFA ratios.

	cyclopropyl/monounsaturated precursor		nutritional imbalance
CO_2 exposure	cy17:0/16:1ω7c	cy19:0/18:1ω7c	PHB/PLFA
35 Pa	0.39 ± 0.06†	0.22 ± 0.03	0.01 ± 0.01
50 Pa	0.51 ± 0.18	0.25 ± 0.05	0.02 ± 0.02
65 Pa	0.43 ± 0.09	0.24 ± 0.05	0.04 ± 0.06

† Values represent the average of 18 replicates ± the standard deviation.

exposures. It is still unknown whether the magnitude of these shifts are substantial enough to affect ecosystem processes such as C allocation or utilization. Measured effects of elevated CO_2 on white oak were shown to be an increase in root dry weight and a shift in root to shoot allometry (Norby et al., 1995b). Therefore, it is known that elevated atmospheric CO_2 exposures do result in differences in the allocation of C within the plant. The observed belowground prokaryotic community shifts are likely directly related to this phenomenon or to that of the yet unmeasured but hypothesized change in root exudate quality and quantity. Measurements of general and specific microbial activities (^{14}C–acetate incorporation) would provide data addressing the magnitude of any belowground impact on ecosystem processes.

A limitation of the signature lipid biomarker technique when addressing community composition is in the resolution of the assay. The resolution is not fine enough to quantify the number of particular species or genera present when a mixed community is analyzed. The assay is, however, capable of identifying the presence of specific genera and/or classifications of bacteria (i.e., gram-negative). The assay is also, in specific instances, capable of quantifying the number of individuals of a single species in an environmental matrix (Ringelberg et al., 1994).

A concern not addressed in this study is seasonal variability and its effect on a plant's response to elevated atmospheric CO_2. Since the analyses were performed on samples collected at one time during the year, seasonal variability was not addressed. Variations in plant lipid composition occur during different stages of the plant's life cycle. As a result, differences in microbial colonization of the rhizosphere are also likely to occur. Whether effects of elevated CO_2 on the extant microbiota will be manifest in different forms during different stages of a plant's life cycle is currently being researched.

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