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Nine years of enriched CO_2 changes the function and structural diversity of soil microorganisms in a grassland

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Summary

To gain insight into microbial function following increased atmospheric CO_2 concentration, we investigated the influence of 9 years of enriched CO_2 (600 μl litre^{-1}) on the function and structural diversity of soil microorganisms in a grassland ecosystem under free air carbon dioxide enrichment (FACE), as affected by plant species (*Trifolium repens* L. and *Lolium perenne* L. in monocultures and mixed culture) and nitrogen (N) supply. We measured biomass and activities of enzymes covering cycles of the most important elements (C, N and P). The microbial community was profiled by molecular techniques of phospholipid fatty acid (PLFA) and denaturing gradient gel electrophoresis (DGGE) analysis. The enrichment in CO_2 increased soil microbial biomass (+48.1%) as well as activities of invertase (+36.2%), xylanase (+22.9%), urease (+23.8%), protease (+40.2%) and alkaline phosphomonoesterase (+54.1%) in spring 2002. In autumn, the stimulation of microbial biomass was 25% less and that of enzymes 3–12% less than in spring. Strong correlations between activities of invertase, protease, urease and alkaline phosphomonoesterase and microbial biomass were found. The stimulation of microbial activity in the enriched atmosphere was probably caused by changes in the quantity and kind of root litter and rhizodeposition. The response of soil microorganisms to enriched CO_2 was most pronounced under *Trifolium* monoculture and under greater N supply. The PLFA analysis revealed that total PLFA contents were greater by 24.7% on average, whereby the proportion of bioindicators representative of Gram-negative bacteria increased significantly in the enriched CO_2 under less N-fertilized *Lolium* culture. Discriminant analysis showed marked differences between the PLFA profiles of the three plant communities. Shannon diversity indices calculated from DGGE patterns were greater (+12.5%) in the enriched CO_2, indicating increased soil bacterial diversity. We conclude that greater microbial biomass and enzyme activity buffer the potential increase in C sequestration occurring from greater C addition in enriched CO_2 due to greater mineralization of soil organic matter.

Introduction

The increasing concentration of atmospheric carbon dioxide (CO_2) is now generally recognized as causing changes in the global climate. The increase from 280 μl litre^{-1} at the beginning of industrialization to the present 365 μl litre^{-1} is due mainly to burning of fossil fuel, deforestation and intensification of agriculture. At the current CO_2 concentration, photosynthesis of C_3 plants in most ecosystems is limited by lack of CO_2. Therefore, an increase in CO_2 concentration is likely to affect the amount of biomass produced by increasing the net assimilation of carbon (C). At least part of the extra C fixed is allocated below ground, resulting in more root biomass, increased root-to-shoot ratio and faster rhizodeposition (Rogers *et al*., 1994). The C/N ratio of plant tissues grown in an atmosphere enriched in CO_2 is known to widen (Jongen *et al*., 1995), and this can retard decomposition (Cotrufo *et al*., 1998). However, there are several studies reporting little or no change in the chemistry of plant litter (Franck *et al*., 1997; Hirschel *et al*., 1997; Gahrooee, 1998). It seems that retarded decomposition does not occur via CO_2-induced changes in the C/N ratio of plant material (Gorissen & Cotrufo, 2000). Enrichment in CO_2 also results in greater water-use efficiency of plants and larger soil water contents as a result of diminished transpiration (Körner, 2000).

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Increased concentrations of CO_2 *per se* in the atmosphere are unlikely to have direct effects on soil microorganisms. But as the microorganisms are constrained by limits of the available C, microbial reactions are probably indirectly influenced by changes in the chemistry of plant tissues and rhizodeposition.

On the one hand, the microorganisms are stimulated by increased availability of substrate from the increased biomass. On the other hand, soil water content plays an essential role in regulating microbial activity through osmosis and regulation of nutrient supply (Killham, 1994). Soil microorganisms play a key role in the decomposition of organic matter and mineralization of C and N, so the cyclings of these two elements are closely coupled. Changes in the inputs of C are likely to influence the dynamics of N, which in turn affects plant growth. Zak *et al.* (1993) suggested positive feedback reactions between increases in CO_2 and increases in root growth, microbial activity and availability of N in the rhizosphere. Daepp *et al.* (2000) showed that the change in the cycling of C and N is crucial in the responses of ecosystems to changes in the concentration of CO_2.

Grasslands occupy much of the land area worldwide. Therefore, potential changes in the ecological stability of them caused by changes in the function and structure of soil microorganisms are of great ecological and pastural importance. Although changes in the soil microbial biomass and activity caused by CO_2 have been reported (Zak *et al.*, 1993; Dhillion *et al.*, 1996), only a few people have investigated the effects of increased CO_2 on the diversity and composition of soil microbial communities (Marilley *et al.*, 1999; Rønn *et al.*, 2002).

We investigated the effects of enriched CO_2 on the function and structure of soil microorganisms in a grassland. We used the Swiss Free Air Carbon Dioxide Enrichment (FACE) facility for this purpose. In the ninth year of this experiment, soil beneath *Trifolium repens* L. (legume), *Lolium perenne* L. (non-legume) and a mixed culture was sampled in spring and autumn. We measured enzymes related to the C, N and P cycling to assess functional diversity. Structural diversity was characterized by microbial biomass and 'fingerprinting' techniques (PLFA and DGGE patterns). Both functional and structural aspects were examined under several N-fertilization treatments. Additionally, the heterogeneity of the soil at the experimental site allowed us to investigate CO_2 effects under the influence of varied soil physico-chemical properties. We hypothesized that (i) the amount of microbial biomass and of enzyme activity increases as the concentration of CO_2 is increased, because of the increase in root litter and root exudates, and (ii) enzyme activities in enriched CO_2 depend on the kinds of plants (legumes and non-legumes). So we thought that the effects of enriched CO_2 and N concentrations on the microbial structure and function depend on plant species. We also tested the hypothesis that (iii) stimulation of enzyme activity under enriched CO_2 is greater under high-N treatment. Finally, we hypothesized (iv) that enriching the atmosphere in CO_2 changes the structural diversity of the microorganisms in the soil.

Materials and methods

Study site and experimental design

The study site is at Eschikon (47°27′N, 8°41′E, 550 m above sea level) near Zürich, Switzerland. The soil is a fertile clay loam soil (Eutric Cambisol) with pH (1:2.5 in water) between 6.9 and 7.6. It consists of 27% clay, 32% silt, 37% sand and 4% organic matter. The FACE experiment was installed in 1993 and set up in three fumigated (600 μl CO_2 litre^{-1}) and three control (350 μl CO_2 litre^{-1}) replicate rings, each with a diameter of 18 m. The distance between fumigated and control areas was at least 100 m. Each FACE plot contained subplots (1.9 × 2.8 m) in which monocultures and mixed cultures of *Trifolium repens* L. cv. Milkanowa and *Lolium perenne* L. cv. Bastion were examined. The atmosphere of the FACE plots was maintained with CO_2-enriched air during the daytime and throughout the growing season. The swards were exposed to two different N treatments (solid NH_4NO_3; low N, 14 g m^{-2} year^{-1}; high N, 56 g m^{-2} year^{-1}) and were cut five times a year at a height of approximately 5 cm above ground level. The harvested aboveground plant material was removed from the plots.

Soil sampling and storage

Soil samples were taken from *Trifolium*, *Lolium* and mixture plots (low and high N-fertilizer treatments) in all six rings in June and October 2002 (total $n = 72$). From each subplot within the rings, eight random soil cores (2.5 cm diameter) were taken to a depth of 10 cm and mixed to one sample. Soil samples were stored at –20°C, and before each analysis, samples were allowed to thaw at 4°C for 2 days and then sieved (to pass 2 mm).

Soil microbiological analyses

Microbial biomass and enzyme activities. Microbial biomass-N and enzyme activities were determined on samples taken in June and October. The PLFAs and DGGE patterns were determined only on samples from October. Microbial biomass-N was determined by the chloroform–fumigation–extraction (CFE) method (Amato & Ladd, 1988). Soil samples were fumigated with chloroform for 24 hours, and ninhydrin-reactive-N (NHR-N) was extracted from the soil with 2 M KCl and determined by colorimetry at 570 nm. Invertase activity (Schinner & von Mersi, 1990) was measured by incubation of the soil with 35 mM sucrose solution for 3 hours at 50°C. The released reducing sugars reduced potassium hexacyanoferrate (III) to potassium hexacyanoferrate (II), which was determined colorimetrically by the Prussian blue reaction. Xylanase activity was determined by the method of Schinner & von Mersi (1990). After incubation of soil samples with xylan solution for 24 hours at 50°C, the reducing sugars released were determined as described for invertase activity. Results of invertase

and xylanase activity were expressed as glucose equivalents. To measure protease activity (Ladd & Butler, 1972), we incubated soil samples with sodium caseinate solution at 50°C for 2 hours, extracted the aromatic amino-acids produced with trichloracetic acid and determined them colorimetrically at 700 nm after adding Folin-Ciocalteau reagent. We expressed the results as tyrosine equivalents. Urease activity was determined as described by Kandeler & Gerber (1988). After incubation of soil samples with an aqueous urea solution at 37°C for 2 hours, the NH_4^+ –N released was extracted with 1 M KCl and 0.01 M HCl and measured colorimetrically at 660 nm by a modified indophenol reaction. Alkaline phosphomonoesterase activity was assayed as described by Hoffmann (1968). Soil was incubated with disodium phenylphosphate solution for 3 hours at 37°C and the phenol released was determined by colorimetry at 614 nm.

Phospholipid fatty acid (PLFA) profiles. Total lipids were extracted from soil (2 g) with a chloroform–methanol–citrate buffer mixture (Bligh & Dyer, 1959). We fractionated them as described by Frostegård *et al*. (1991), using silicic acid columns (Varian), into neutral lipids, glycolipids and phospholipids, by elution with 5 ml of chloroform, 20 ml of acetone and 5 ml of methanol, respectively. After drying the phospholipids with N_2, we added 6.9 μg of nonadecanoate (fatty acid methyl ester 19:0) as an internal standard. Phospholipids were transformed into fatty acid methyl esters (FAMEs) by alkaline methanolysis, which were quantified by gas chromatography (GC/FID, AutoSystem XL Gas Chromatograph, Perkin-Elmer, Rodgau-Jügesheim, Germany) fitted with a 50 m capillary column and helium as carrier gas. The injector temperature was 260°C, the flame ionization detector temperature was 280°C, and the initial temperature was 70°C (for 2 minutes); it was increased to 160°C at 30°C minute^{-1} and then to 280°C at 3°C minute^{-1}. Calibration, sample sequencing, peak integration and naming were controlled by the Turbochrom Workstation software (Perkin-Elmer). In all 24 PLFAs were detected and identified. PLFA bioindicators were selected according to Federle (1986), Vestal & White (1989), Frostegård *et al*. (1993) and Zelles & Bai (1994). Those representing Gram-positive bacterial PLFAs were i15:0, a15:0, i16:0 and i17:0, and those representing Gram-negative bacterial PLFAs were 16:1ω7, cy17:0, 18:1ω7 and cy19:0. Signature PLFAs for fungi were 18:1ω9, 18:2ω6 and 18:3ω3. Contents of PLFA bioindicators were calculated and used to characterize the effect of increased CO_2 on microbial groups.

Denaturing gradient gel electrophoresis (DGGE) patterns. Total microbial community DNA was extracted from soil by the Fast DNA SPIN Kit for Soil (Qbiogen, Heidelberg) according to the manufacturer's protocol. We measured the extinction of DNA at 260 nm to assess the DNA yield and quality. Specific sequences in bacterial DNA were amplified by the bacterial primer pair F984-968 GC, containing a GC clamp and R1378-1401 as described by Heuer *et al*. (1997). These primers span the regions between nucleotides 968 and 1401 of 16S rRNA genes. Polymerase chain reactions (PCR) were carried out by addition of about 10 ng of extracted DNA as template to 25-μl-PCR reaction mixtures. These PCR mixtures contained each deoxynucleotide triphosphate at a concentration of 2 mM, 2.5 μl of 10-times concentrated reaction buffer, each primer at a concentration of 5 μM, 1 U of Taq DNA polymerase (Qbiogen) and sterile distilled water. The PCR programme included an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes. During the last cycle, the extension step lasted 12 minutes. The PCR products were then purified by the QIAquick PCR Purification Kit (Qiagen, Hitden, Germany) following the manufacturer's protocol. The PCR products were analysed by gel electrophoresis in 2% agarose gels stained with SYBR-Green. The 16S rRNA gene sequences were separated by the D Code System (Bio-Rad, München) on 6% polyacrylamide gels containing a linear gradient ranging from 35% to 55% denaturant solution, whereby the 100% denaturing solution contained 7 M urea and 40% by volume deionized formamide. After polymerization with tetramethylethylenediamine (TEMED) and ammonium persulphate and sample loading, DGGE was done at 90 V and 60°C in TAE buffer (40 mM tris-hydroxymethyl-aminomethane (Tris), 20 mM sodium acetate and 1 mM EDTA (pH 7.4)) for 16 hours. Gels were stained by the Silver Stain Kit (Bio-Rad) following the manufacturer's instructions. After scanning, gels were analysed by Geldoc Quantity One software (Bio-Rad) to identify the number, intensity and position of individual bands in the several lanes of the gels.

Soil water content. We determined soil water content gravimetrically by drying moist soil samples to constant mass at 105°C.

Statistical procedure. The experiment was analysed as a split-plot arrangement of treatments, whereby CO_2 concentration was the main-plot factor. Plant species composition and nitrogen fertilization were the subplot factors. To identify the inherent natural pattern of the investigated plots, hierarchical cluster analysis was applied. The resulting dendrogram revealed that the microbial data of the third replicate of the CO_2 treatment formed a separate cluster, regardless of the treatment imposed (results not shown). These results confirmed other research done at the Eschikon FACE site, which has shown that the third FACE ring had different soil characteristics from those of the other two, including lower pH, and smaller concentrations of organic C, extractable P and greater bulk density (Lüscher *et al*., 1998). These differences resulted in differences in plant growth, soil microbial biomass, soil enzyme activities and composition in the microbial community. Soil microbial properties are sensitive to changes in the physico-chemical properties of the soil resulting from site

disturbance. The entire experiment consisted of 72 experimental units (three blocks × two pCO_2 × three plant cultures × two nitrogen fertilization levels × two sampling dates). Chemical analyses of each sample (total n of 36 in each of spring and autumn) were carried out in duplicate, except for microbial biomass-N measurements, which were determined in triplicate. Results of soil enzyme measurements and PLFA analysis were calculated on a dry soil weight basis. The statistical analyses were carried out by the procedure Mixed of SAS 9.1 (SAS Institute Inc., Cary, NC, USA). The model was a split-plot with CO_2 as the main plot factor, and block × CO_2 were tested as random effects. Plant culture, nitrogen fertilization and their interaction were tested as fixed effects. For the autumn sampling, Pearson's product–moment correlation coefficient was calculated to assess the relationship between individual enzyme activities, microbial biomass (NHR-N and PLFA) and water content. The canonical discriminant analysis was applied to determine whether the plant cultures could be identified by their microbial PLFA composition pattern and what the discriminatory importance of each variable is. The groups were defined according to the plant culture. Independent variables were entered simultaneously. Bacterial diversity was determined by the Shannon index H, based on DGGE patterns, with H calculated from

$$H = -\sum_{i=1}^{S} \frac{n_i}{N} \ln \frac{n_i}{N}, \quad (1)$$

where n_i is the intensity of the ith band, N is the total intensity of all bands and S is the total number of bands. Statistical significance for all analyses was accepted at the $P = 0.05$ level of probability.

Results

Soil water content

The water content in the ambient CO_2 treatment was on average 21.6% in spring and 36.2% in autumn. The soil water content was reduced under high N-fertilization, and significantly affected by the kind of plant culture, whereby values were greatest under *Lolium* (Table 1a). Furthermore we found two-way interactions between the factors plant culture and CO_2 as well as between plant culture and N fertilization. We found moderate relations between water content and microbial biomass and enzyme activity. The correlation coefficients were $r = 0.54$ (NHR-N), $r = 0.71$ (PLFA) and $r = 0.64$–0.70 (invertase, protease, urease, alkaline phosphomonoesterase). No correlation was found for xylanase activity and Shannon index.

Microbial biomass

Microbial biomass (NHR-N) was somewhat greater in the enriched CO_2 treatment on both sampling dates, but the differences were not statistically significant (Table 1a). In contrast, high N-fertilization significantly reduced the amount of NHR-N in the soil compared with low N-fertilization in spring. The plant culture did not affect microbial biomass on either sampling date. In autumn, contents of NHR-N were positively correlated ($r = 0.80$) with contents of PLFA.

Enzyme activities

Enzyme activities were 22.9–54.1% larger under the enriched CO_2 in spring and 16.6–41.7% larger in autumn (Table 1a). However, these differences were not statistically significant. Nitrogen fertilization had significant effects on the activities of invertase, xylanase and protease. Differences in activity between soils of *Trifolium repens*, *Lolium perenne* and mixed culture were significant for xylanase and invertase in spring and autumn. Enzyme activities were largest in soil under *Lolium* except for xylanase and urease. There was no interaction between CO_2 treatment and plant culture. However, on both sampling dates, the stimulation of enzyme activities in enriched CO_2 was greatest under *Trifolium* (data not shown). In autumn, contents of NHR-N were positively correlated with activities of invertase ($r = 0.83$), protease ($r = 0.78$), urease ($r = 0.85$) and alkaline phosphomonoesterase ($r = 0.88$). Contents of PLFA showed positive correlations with activities of invertase ($r = 0.93$), protease ($r = 0.81$), urease ($r = 0.83$) and alkaline phosphomonoesterase ($r = 0.83$).

Phospholipid fatty acid (PLFA) profiles

The total content of PLFAs at ambient CO_2 was on average 57.38 nmol g^{-1} soil, and was increased by 24.7% in the enriched CO_2 (Table 1b). Both the NHR-N and PLFA analysis of microbial biomass indicated that enriched atmospheric CO_2 increased the soil microbial biomass. Most single PLFAs were also enhanced under enriched CO_2, whereby in the *Trifolium* culture the increase in single PLFA concentrations was greater in high N-fertilization treatments (Figure 1). Enriched CO_2 increased the content of 19 (*Trifolium*) and 17 (*Lolium*) out of 24 single PLFAs under low N-fertilization, while under the high-N treatment, contents of 18 and 20 PLFAs increased under *Trifolium* and *Lolium*, respectively. Similar PLFA profiles were observed for the mixed culture (data not shown). However, significant effects of CO_2 were detected only for i15:0 and cy17:0. N fertilization affected the unsaturated PLFAs 16:1ω7, 18:1ω7, 18:1ω9 and 18:3ω3, the contents of which decreased under high-N fertilizer. The subplot factor plant culture showed significant effects on Gram-negative bacterial PLFAs 18:1ω7 and cy19:0 as well as on fungal PLFAs 18:1ω9 and 18:2ω6, which were greatest under *Lolium*. With respect to the proportions of total PLFA bioindicators, we found a significant increase of PLFAs representative of Gram-negative bacteria in the soil under *Lolium* monoculture and low N-fertilizer treatment. Proportions of fungal PLFAs decreased under all cultures and N treatments (Table 2).

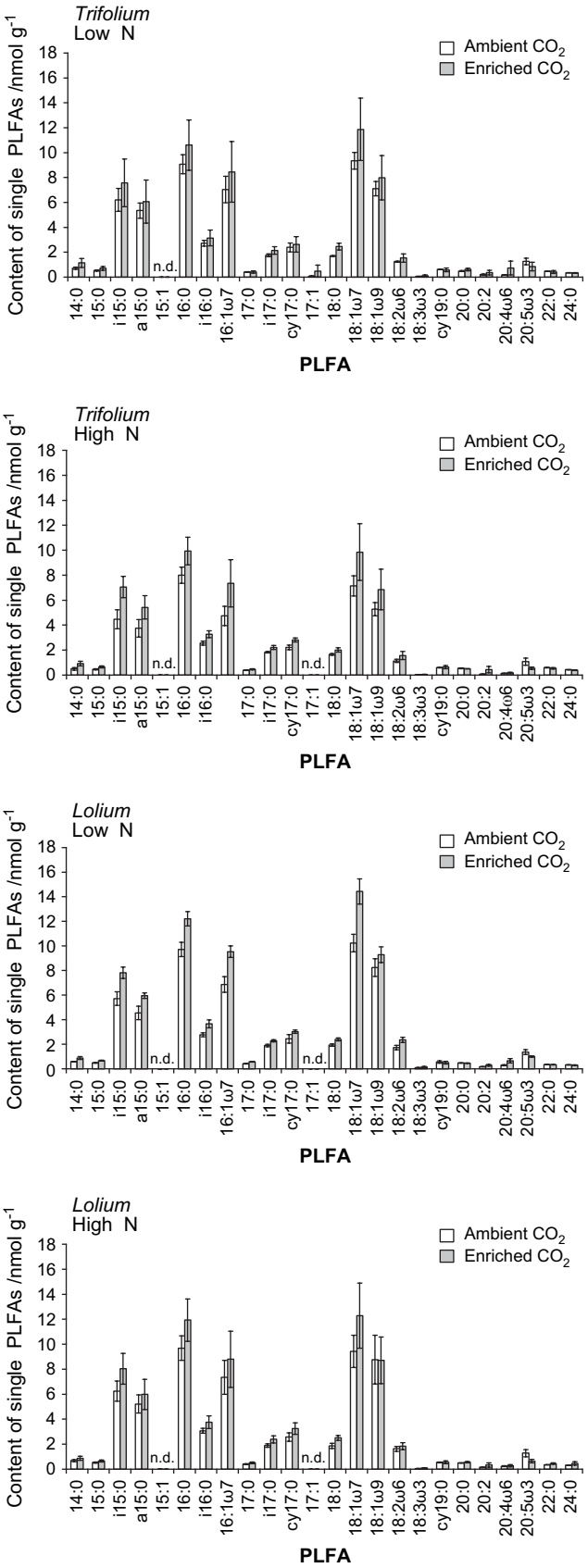
Table 1a Marginal means and standard errors (SE) of water content (%), microbial biomass-N (μg NHR-N g^{-1} soil), activities of invertase (mg GE g^{-1} soil 3 $hours^{-1}$), xylanase (mg GE g^{-1} soil 24 $hours^{-1}$), protease (mg TE g^{-1} soil 2 $hours^{-1}$), urease (mg NH_4^+-N g^{-1} soil 2 $hours^{-1}$) and alkaline phosphomonoesterase (mg phenol g^{-1} soil 3 $hours^{-1}$) for the effects of CO_2 treatment, N fertilization and plant culture. Marginal means for two-way interactions are not shown because they were not significant except for water content and Shannon index. GE, glucose equivalents; TE, tyrosine equivalents; NHR-N, ninhydrin-reactive-N

	CO_2 concentration / $\mu 11^{-1}$			N fertilization / g m^{-2} $years^{-2}$			Plant culture			
	350	600	SE	14	56	SE	*Trifolium*	*Lolium*	Mixture	SE
Spring										
Water content	21.61	23.54	0.93	24.27	20.88	0.75	20.61	24.49	22.63	0.83
Biomass-N	7.52	11.14	1.68	10.81	7.85	1.29	8.48	11.03	8.46	1.38
Invertase	6.10	8.31	0.67	7.88	6.54	0.55	6.16	8.30	7.16	0.62
Xylanase	1.98	2.43	0.30	1.81	2.59	0.25	1.22	2.45	2.94	0.28
Protease	0.44	0.61	0.06	0.55	0.49	0.05	0.48	0.56	0.53	0.05
Urease	0.09	0.11	0.01	0.10	0.09	0.01	0.10	0.09	0.09	0.01
Phosphatase	1.05	1.62	0.39	1.40	1.26	0.29	1.29	1.45	1.25	0.30
Autumn										
Water content	36.20	38.47	1.39	37.75	36.92	1.01	36.65	38.42	36.93	1.04
Biomass-N	21.04	26.00	4.35	25.62	21.41	3.30	20.42	25.11	25.03	3.51
Invertase	6.81	9.09	0.76	8.65	7.25	0.59	7.12	9.20	7.53	0.64
Xylanase	2.36	2.75	0.35	1.99	3.12	0.28	1.51	2.90	3.25	0.32
Protease	0.48	0.63	0.07	0.61	0.51	0.05	0.54	0.57	0.57	0.05
Urease	0.09	0.11	0.01	0.11	0.10	0.01	0.11	0.10	0.10	0.01
Phosphatase	1.18	1.67	0.40	1.52	1.33	0.29	1.37	1.55	1.37	0.30

The sensitivity of the PLFA profiles to the plant type was assessed by canonical discriminant analysis, which yielded two canonical variates for the three types. Canonical variate 1 discriminated strongly between *Lolium* and *Trifolium* monoculture (Figure 2), with the mixed culture lying between the two monocultures. It explained 86.4% of the total variance among the types. The correlation coefficients between the original variates and the canonical variates revealed that PLFAs 18:1ω9, 18:2ω6 and 22:0 contributed most to the discrimination (Table 3). Along canonical variate 2, which explained 13.6% of the variance and was dominated by PLFA 20:5ω3, the mixed culture was separated from the monocultures of *Lolium*

Table 1b Marginal means and standard errors (SE) of the diversity of 16S rDNA (Shannon index), total PLFA, Gram-negative bacteria, Gram-positive bacteria and fungal PLFA (all nmol g^{-1} soil) for the effects of CO_2 treatment, N fertilization and plant culture. Marginal means for two-way interactions are not shown because they were not significant except for water content and Shannon index

	CO_2 concentration / $\mu 11^{-1}$			N fertilization / g m^{-2} $year^{-1}$			Plant culture			
	350	600	SE	14	56	SE	*Trifolium*	*Lolium*	Mixture	SE
Shannon index	2.56	2.88	0.05	2.76	2.69	0.04	2.75	2.79	2.63	0.04
PLFA	57.38	71.56	2.91	68.13	60.81	2.59	60.50	69.42	63.48	3.02
Gram-negative bacteria										
16:1ω7	6.32	8.38	0.54	7.89	6.81	0.46	6.90	8.13	7.02	0.52
cy17:0	2.37	2.86	0.08	2.61	2.62	0.08	2.51	2.82	2.51	0.10
18:1ω7	9.13	11.84	0.60	11.54	9.43	0.53	9.55	11.59	10.31	0.62
cy19:0	0.59	0.57	0.08	0.58	0.58	0.06	0.61	0.54	0.59	0.06
Gram-positive bacteria										
i15:0	5.75	7.75	0.31	7.07	6.42	0.29	6.32	6.95	6.96	0.34
a15:0	4.64	6.00	0.44	5.60	5.03	0.34	5.15	5.42	5.39	0.37
i16:0	2.81	3.45	0.13	3.15	3.11	0.12	2.92	3.31	3.16	0.14
i17:0	1.88	2.25	0.09	2.09	2.03	0.08	1.99	2.11	2.09	0.08
Fungi										
18:1ω9	7.20	8.14	0.40	8.20	7.14	0.38	6.81	8.75	7.45	0.45
18:2ω6	1.43	1.73	0.09	1.66	1.50	0.08	1.37	1.89	1.47	0.10
18:3ω3	0.06	0.11	0.02	0.11	0.05	0.02	0.06	0.09	0.09	0.02



and *Trifolium*. Because of the dominance of canonical variate 1, the fatty acids 18:1ω9, 18:2ω6 and 22:0 seemed to be the most important variables in discriminating according to the plant type.

Bacterial community structure

The DGGE patterns showed that bacterial community structure changed as a result of enriched CO_2. The Shannon diversity indices calculated from DGGE patterns revealed that the enrichment of CO_2 increased bacterial diversity by 12.5% on average (Table 1b, Figure 3). Sub-plot factors plant culture and N fertilization showed significant influences on the Shannon indices, which were less when the soil was fertilized with large amounts of N or when cultivated with the mixed culture. In addition, the interaction between plant culture and CO_2 as well as between plant culture and N fertilization significantly affected Shannon indices, whereby the stimulation of bacterial diversity was greatest under *Trifolium* and high N-fertilization.

Discussion

Microbial processes in the soil were stimulated by exposure to increased CO_2 for 9 years. Invertase, xylanase, protease, urease and alkaline phosphomonoesterase responded with greater activities. Microbial biomass measured as NHR-N and total PLFA was stimulated as well. Other investigators (Dhillion *et al*., 1996; Kandeler *et al*., 2006) have also reported such increases of microbial activity caused by CO_2 enrichment. In general, the additional carbon input from roots and rhizodeposition boosts microbial processes (Rogers *et al*., 1994; Hodge *et al*., 1998). In our study, it seems that microbial biomass and enzyme activity were most likely stimulated by increase in roots under enriched CO_2.

Greater enzyme activities could be directly related to greater production of enzymes by the increased microbial biomass. Positive correlations between them support this view. The contribution of root-derived enzymes is expected to be small because enzymes showed similar reactions to alkaline phosphomonoesterase, which cannot be produced by plant roots.

Our study confirms previous results, for example those of Ross *et al*. (1995), who found that invertase activity significantly increased under enriched CO_2. Dhillion *et al*. (1996) observed that activities of xylanase and dehydrogenase were significantly greater by 61% and 13%, respectively, after enrichment of CO_2 (700 μl litre^{-1}) for 220 days. In contrast to the above, our results are based on a long-term CO_2 enrichment

Figure 1 Contents of single PLFAs in soil under *Trifolium* and *Lolium* monoculture at ambient (350 μl litre^{-1}) and in enriched (600 μl litre^{-1}) CO_2 concentrations and different N supply. Means of three field replicates and standard errors are shown. n.d., not detectable.

Table 2 Proportions (mol %) of total selected PLFA bioindicators specific for Gram-positive bacteria, Gram-negative bacteria and fungi at ambient (350 μl l^{-1}) and in enriched (600 μl l^{-1}) concentrations of CO_2, depending on different plant cultures (T, *Trifolium* monoculture; L, *Lolium* monoculture; M, mixed culture) and N supply (low N, high N). Calculation based on means produced by three field replicates per treatment

			CO_2 concentration/ μl l^{-1}	
Variable	Group	Treatment	350	600
Proportion of total PLFA bioindicators/ mol %	Gram-positive bacteria	T low N	36.6	36.3
		L low N	33.1	33.4
		M low N	35.3	38.0
		T high N	37.3	38.1
		L high N	35.1	36.2
		M high N	37.9	38.4
	Gram-negative bacteria	T low N	44.2	45.2
		L low N	44.6	46.6
		M low N	44.1	43.6
		T high N	43.6	43.9
		L high N	42.5	44.7
		M high N	42.8	43.0
	Fungi	T low N	19.2	18.5
		L low N	22.3	20.0
		M low N	20.6	18.3
		T high N	19.1	18.0
		L high N	22.3	19.1
		M high N	19.3	18.6

Table 3 Results of the discriminant analysis of the PLFA profiles. The groups were defined according to the plant culture. Independent variables were entered simultaneously

	Canonical variate	
	1	2
Wilks' lambda	<0.01	0.15
Eigenvalue	34.81	5.48
Degree of freedom	46	22
% of variance	86.4	13.6
Canonical correlation coefficient	0.99	0.92
Correlation coefficient[a]		
14:0	0.09	−0.02
15:0	0.01	0.04
i15:0	−0.13	−0.11
a15:0	−0.07	−0.04
16:0	−0.29	−0.03
i16:0	−0.25	−0.07
16:1ω7	−0.21	0.07
17:0	−0.29	−0.05
i17:0	−0.14	−0.88
cy17:0	−0.22	0.10
17:1	0.22	0.18
18:0	−0.21	−0.11
18:1ω7	−0.28	0.01
18:1ω9	**−0.37**	0.03
18:2ω6	**−0.53**	0.13
18:3ω3	−0.17	−0.17
cy19:0	0.20	−0.02
20:0	0.26	0.06
20:2	0.05	0.18
20:4ω6	−0.10	0.19
20:5ω3	−0.19	**0.43**
22:0	**0.57**	0.10
24:0	0.19	<0.01

[a]denotes correlation between the original variates and the canonical variates. Correlation is significant at the $P \leq 0.05$ level (values marked in bold).

experiment (9 years). Stimulation of enzyme activities in the enriched CO_2 indicated enhanced C, N and P cycling and greater availabilities of nutrients for microbial and plant growth. In fact, the Swiss FACE experiment showed that C mineralization and total C in adhering soil from *Lolium perenne* roots were stimulated under enriched CO_2 (S. Bazot, personal communication). Additionally, Schneider *et al.* (2004), who investigated the mobilization of N from soil under *Lolium*, showed that the proportion of N derived from soil organic matter in the above-ground biomass was significantly greater in the enriched CO_2. This indicated increased mobilization of N from soil organic matter in the enriched CO_2. In summary, our results support the hypothesis of positive feedback proposed by Zak *et al.* (1993), who stated that additional C stimulates microbial decomposition and thus leads to more available N under enriched CO_2.

With regard to our second hypothesis, that the effect of enriched CO_2 on enzyme activities depends on plant cultures, the data indicate that the CO_2 effect was more pronounced under *Trifolium* monoculture and mixed culture than under *Lolium* monoculture, though this interaction was not statistically significant. These results concur with the findings of Lüscher *et al.* (1998), who reported that legumes showed the largest and grasses the smallest increases in yield in the enriched CO_2 concentration. The stronger growth response of *Trifolium repens* and greater microbial activity under *Trifolium* monoculture are supported by the CO_2-induced increased symbiotic N_2-fixation of *Trifolium repens* (Zanetti & Hartwig, 1997). These reactions, balancing the increased N demand and leading to an additional N input into the system, are also responsible for a greater proportion of *Trifolium repens* in mixed cultures in the enriched CO_2 (Hebeisen *et al.*, 1997). This confirms the idea that the microbial activities of the mixed culture were more similar to those of *Trifolium* monoculture than those of *Lolium* monoculture. However, the different nitrogen acquisition strategies of legumes and non-legumes are probably not the only reason for the species-dependent CO_2 response. Less pronounced increases of

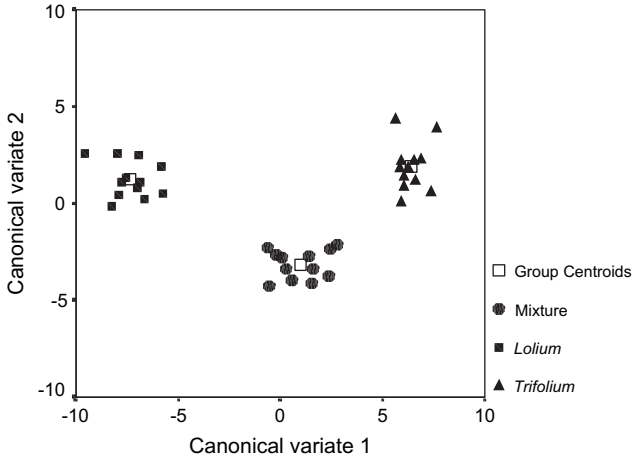


Figure 2 Canonical variates of the PLFA profiles for *Trifolium* and *Lolium* monoculture and mixed culture. Number of cases, $n = 36$.

enzyme activities under *Lolium* monoculture might have two causes. First, activities under the ambient CO_2 treatment were in general greater under *Lolium* than under *Trifolium* and the mixed culture. Comparison of these differences with those among CO_2 treatments shows that plant effects can be as large as CO_2 effects. Second, a larger C/N ratio of *Lolium* root material grown in the enriched CO_2 could have weakened the stimulation of enzymes. Indeed, Jongen *et al.* (1995) showed for the Swiss FACE experiment that C/N ratios of *Lolium* roots increased significantly as a result of decreases in the concentration of N in tissue, whereas for *Trifolium* it did not.

In agreement with the third hypothesis, the stimulation of enzyme activities under enriched CO_2 was in general more pronounced under the larger application of N. This was confirmed only for *Trifolium* and the mixed culture, except for protease activity, which showed greater activity under *Lolium*. In fact, plants show a greater response to enriched CO_2 when they have ample N and can therefore utilize the increased amount of C, which should result in more C for soil microorganisms. The availability of C and N is therefore likely to stimulate microbial growth and activity.

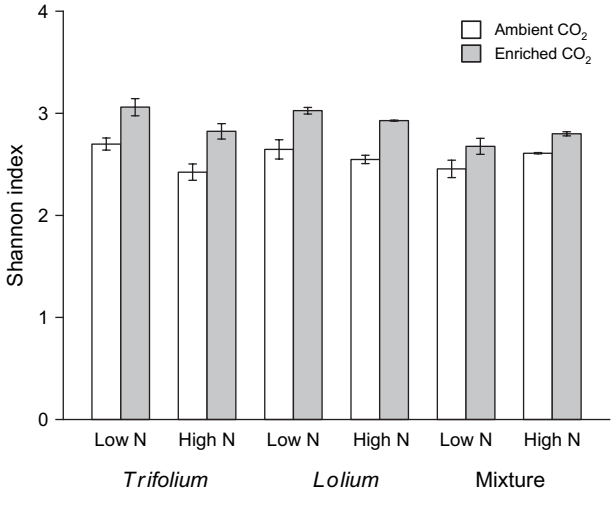


Figure 3 Shannon indices of soils from *Trifolium* monoculture, *Lolium* monoculture and mixed culture at ambient (350 μl litre^{-1}) and in enriched (600 μl litre^{-1}) CO_2 concentrations and different N supply. Means of three field replicates and standard errors are shown.

With regard to our fourth hypothesis (the structural diversity of soil microorganisms changes in the enriched CO_2), 9 years of enriched CO_2 concentration caused changes in the microbial community composition in the soil of all three cultures. The DGGE-based Shannon index suggested greater bacterial diversity in the enriched CO_2 concentration, indicating that stimulated microbial processes, in particular the mineralization of C and N, are directly correlated with adaptive changes in the structural diversity of soil microorganisms. In particular, the proportion of Gram-negative bacteria significantly increased in the enriched CO_2 concentration under *Lolium* and low N-supply. Montealegre *et al.* (2002) also detected an increase in PLFAs indicative of Gram-negative bacteria in the enriched CO_2. This enhances our conclusion that easily available, low-molecular-weight C substrates from rhizodeposition specifically favour Gram-negative bacteria. Several studies have shown increased root exudation and translocation of assimilated C into the soil under conditions of enriched CO_2 (see Hodge *et al.*, 1998). As CO_2-induced stimulation of soil microbial diversity in all culture plots was more pronounced under the larger application of N, in addition to carbon, N might have been limiting for microbial growth. Moreover, slightly greater soil water contents could have also affected the composition of microbial communities. The close relationship between microbial biomass (NHR-N, PLFA) and soil water content supports this hypothesis. According to Rillig *et al.* (1999), the fungal food chain under enriched CO_2 concentrations is more strongly stimulated than that of bacteria, as a result of the greater substrate efficiency of fungi. However, this hypothesis was not supported by the results of our study. In a similar study, Niklaus *et al.* (2003) found that no change in the ratio of fungi to bacteria was detected under nutrient-poor grassland exposed to 6 years of CO_2 enrichment. These different results show that soil microbial responses to enriched CO_2 depend strongly on several factors: effects of plant species, e.g. quantity and kind of litter, root biomass, turnover, exudation and plants' demand for N as well as the supply of N, and the physico-chemical properties of the soil. Activities of invertase, protease, urease and alkaline phosphomonoesterase, as well as NHR-N and total PLFA of one of the three replicates showed smaller values in correlation with less organic C, pH and C/N-values as well as less clay, and greater silt contents, respectively (H. Blum, unpublished data). Therefore, physico-chemical properties of the soil probably play an important role in mediating the effects of environmental changes on soil microbiota.

Conclusion

In conclusion, we found that the soil microbial biomass is increased and its activity is stimulated under conditions of increased atmospheric CO_2 concentrations. This is accompanied by a change in the structural diversity of the soil microorganisms. The driving mechanisms are probably greater root litter production and faster rhizodeposition. The extent of changes in the function and composition of soil microbial communities in the enriched CO_2 is likely to vary with the plant species and supply of N. As microbial processes play a key role in the decomposition of soil organic matter, changes in their functional and structural diversity are likely to affect nutrient cycling and plant growth. We conclude that greater mineralization of organic matter due to greater microbial activity buffers the potential increase in C sequestration in the soil under enriched CO_2. This study fosters our understanding of how soil microorganisms respond to enriched CO_2 in grassland ecosystems under current global environmental changes.

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