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Soil Biology & Biochemistry 38 (2006) 2448–2460

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Response of soil microbial biomass and enzyme activities to the transient elevation of carbon dioxide in a semi-arid grassland

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Received 2 December 2005; received in revised form 28 January 2006; accepted 28 February 2006
Available online 18 April 2006

Abstract

Although elevation of CO_2 has been reported to impact soil microbial functions, little information is available on the spatial and temporal variation of this effect. The objective of this study was to determine the microbial response in a northern Colorado shortgrass steppe to a 5-year elevation of atmospheric CO_2 as well as the reversibility of the microbial response during a period of several months after shutting off the CO_2 amendment. The experiment was comprised of nine experimental plots: three chambered plots maintained at ambient CO_2 levels of 360 μmol mol^{-1} (ambient treatment), three chambered plots maintained at 720 μmol mol^{-1} CO_2 (elevated treatment) and three unchambered plots of equal ground area used as controls to monitor the chamber effect.

Elevated CO_2 induced mainly an increase of enzyme activities (protease, xylanase, invertase, alkaline phosphatase, arylsulfatase) in the upper 5 cm of the soil and did not change microbial biomass in the soil profile. Since rhizodeposition and newly formed roots enlarged the pool of easily available substrates mainly in the upper soil layers, enzyme regulation (production and activity) rather than shifts in microbial abundance was the driving factor for higher enzyme activities in the upper soil. Repeated soil sampling during the third to fifth year of the experiment revealed an enhancement of enzyme activities which varied in the range of 20–80%. Discriminant analysis including all microbiological properties revealed that the enzyme pattern in 1999 and 2000 was dominated by the CO_2 and chamber effect, while in 2001 the influence of elevated CO_2 increased and the chamber effect decreased.

Although microbial biomass did not show any response to elevated CO_2 during the main experiment, a significant increase of soil microbial N was detected as a post-treatment effect probably due to lower nutrient (nitrogen) competition between microorganisms and plants in this N-limited ecosystem. Whereas most enzyme activities showed a significant post-CO_2 effect in spring 2002 (following the conclusion of CO_2 enrichment the previous autumn, 2001), selective depletion of substrates is speculated to be the cause for non-significant treatment effects of most enzyme activities later in summer and autumn, 2002. Therefore, additional belowground carbon input mainly entered the fast cycling carbon pool and contributed little to long-term carbon storage in the semi-arid grassland.

Keywords: Carbon dioxide; Climate change; Soil enzymes; Microbial biomass; Shortgrass steppe; Carbon cycling; Below ground processes

1. Introduction

Increased atmospheric CO_2 concentration often stimulates plant photosynthesis, enhances carbon allocation belowground, increases plant nutrient uptake, and improves the efficiency of plant water use (Hu et al., 1999). Results from 16 free-air CO_2 enrichment (FACE) sites representing four different vegetation types indicate that most herbaceous species had reduced leaf nitrogen content and modest enhancement of leaf CO_2 assimilation under elevated CO_2 (see review by Nowak et al., 2004).

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doi:10.1016/j.soilbio.2006.02.021

Belowground microbial processes are likely to be affected through altered plant inputs under elevated CO_2 (Diaz et al., 1993; Zak et al., 1993; Rice et al., 1994; Dhillion et al., 1996). A major component of plant input is derived from litter fall and root turnover. Inputs also derive from rhizodeposition which may account for up to 40% of assimilated C (Paterson et al., 1997; Norby and Luo, 2004). Additional belowground carbon input could enter either the fast cycling carbon pool or be sequestered in persistent organic materials, such as humus (Schlesinger and Lichter, 2001; Niklaus et al., 2003). Since many experiments under elevated CO_2 were performed in young developing ecosystems where microorganisms appear to be regulated by relatively large pools of soil organic matter (Jones et al., 1998), rather than by additional input of organic substrates under elevated CO_2, information on microbial response to CO_2-induced changes in natural ecosystems with nutrient limitations are rare. Only a few studies have found evidence that enzyme activities are directly and/or indirectly affected by elevated CO_2 (e.g. Körner and Arnone, 1992; Kandeler et al., 1998; Kampichler et al., 1998; Kang et al., 2001; Ebersberger et al., 2003, Henry et al., 2005). In addition, the long-term persistence of the effect of elevated CO_2 on soil microbial function has been poorly studied. With the exception of the paper of Mosier et al. (2003), we are not aware of any study estimating the reversibility of the CO_2 effect on microbial processes over a longer period. To study the resilience of an ecosystem after a pertubation is important, because we expect that the elevation of CO_2 will probably decrease or slow down due to political reasons.

The shortgrass steppe is a semi-arid grassland along the western edge of the Great Plains of the United States, stretching from south-eastern New Mexico and western Texas north to the Colorado–Wyoming border at 41°N (Lauenroth and Milchunas, 1991). Vegetation is dominated by warm-season, C_4 grasses (*Bouteloua* and *Buchloe* spp.), and contains also cool-season, C_3 grasses (e.g. *Pascopyrum* and *Stipa* spp.) as well as a variety of C_3 forbs and woody vegetation; C_4 forbs and woody vegetation are uncommon (Morgan et al., 2001). Using an open-top chamber (OTC) experiment on the shortgrass steppe in northern Colorado, Morgan et al. (2004) found consistent and strong CO_2-induced production responses mainly due to preferentially enhanced growth of only one of the dominant grasses, the C_3, *Stipa comata*. Transpiration use efficiency of plants was around $0.25\,g\,m^{-2}\,mm^{-1}$ on this native grassland, with 68–78% of annual precipitation used in transpiration (Ferretti et al., 2003). Elevated CO_2 reduced stomatal conductance and promoted soil moisture storage in the shortgrass steppe (Morgan et al., 2001; Pendall et al., 2003). Following the dynamics and demography of roots in this native, semi-arid steppe grassland over a period of 5 years, Milchunas et al. (2005a) revealed that elevated CO_2 induced greater growth, but also greater loss of roots. In addition, they calculated, from growth-to-loss ratio regressions, very long turnover times of 5.3–7.0 years.

We expected that soil microorganisms in the top soil layer would have the highest benefit of the additional carbon allocation due to the high root biomass and rhizodeposition in this layer. Therefore, the first aim of the present study was to test whether soil microorganisms of different soil layers made use of the additional substrates available under elevated CO_2 in the soil profile of the short grass steppe. Secondly, the OTC experiment gave us the opportunity to follow the long-term response of microbially mediated belowground processes over a period of 3 years during elevated atmospheric CO_2 as well as the post-effect during a period of 12 months after CO_2 amendment ended. We measured the activities of several different soil enzymes because these extracellular enzymes mediate microbial decomposition of organic substrates and because they reflect changes in the function of soil microdecomposer communities (Marx et al., 2001). We selected enzymes according to their use of either low molecular weight or high molecular weight substrates (invertase and xylanase) and according to their importance to release of N, P and S for plant nutrition (protease, urease, alkaline phosphatase and arylsulfatase). We hypothesised that elevated CO_2 may stimulate below ground microbial processes mainly in the top soil of the semi-arid grassland soil due to increased plant biomass production and that these effects might be only transient due to relatively fast turnover times of carbon in the top soil.

2. Materials and methods

2.1. Study site

The study site is located at the USDA-ARS Central Plains Experimental Range (CPER), 40°40′N, 104°45′W, in the shortgrass steppe region of northeastern Colorado, about 56 km northeast of Fort Collins, CO (Lauenroth and Milchunas, 1991). The soil at the experimental site is a Remmit fine sandy loam (Ustollic Camborthids according to the USDA classification system) containing 0.89% total carbon, 0.10% total nitrogen and a pH (H_2O) value of 7.2 in the upper 20 cm layer (Mosier et al., 2002). The soil holds 18% water at field capacity, and 4% at the permanent wilting point. The semi-arid grassland site is at 1650 m elevation and has a long-term mean annual precipitation of 320 mm, with the majority occurring in May, June and July. A variety of seasonal precipitation patterns typical for shortgrass steppe region occurred during the 5-year study (Milchunas et al., 2005b). The third year of the experiment (1999) had nearly twice the average annual precipitation due to both wet spring and autumn periods. Year four of the experiment (2000) was a little below annual average precipitation, but a very long drought occurred from spring until late summer, when a few very heavy storms result in what appeared as a spring flush of growth in autumn. Year five was only slightly above the annual precipitation, but was very different seasonally. A long drought during the entire

latter half of the growing season occurred in year five, but the very wet spring raised the annual amount received. Mean monthly air temperature ranges from 22 °C in July to below 0 °C in January. Frost-free days average 133 days.

The native grassland community, with a basal cover of 25–35% of the soil, is comprised of over 25 species of forbs and grasses, but dominated by three grass species: *Bouteloua gracilis* (H.B.K.) Lag. (a warm season, C_4 grass, accounts for approximately 42% of total aboveground biomass), *Pascopyrum smithii* (Rydb.) A. (C_3 grass, 26% of total aboveground biomass) and *Stipa comata* (C_3 grass, 26% of total aboveground biomass). Root biomass (including crowns) is responsible for around 70% of net primary production (NPP) in the ecosystem: belowground biomass NPP averages 223 g $m^{-2} yr^{-1}$, root crowns contribute 57 g $m^{-2} yr^{-1}$, while aboveground biomass NPP averages 109 g $m^{-2} yr^{-1}$ (Milchunas and Lauenroth, 2001). Nine experimental plots of similar botanical composition and similar chemical and physical soil properties were selected for the OTC experiment. The field had been grazed by cattle at a light to moderate intensity (about 30% annual forage removal) until the start of the experiment in March 1997.

2.2. Experimental design

From 1997 to 2001, open-top chambers (4.5 m diameter × 3 m height) were used to evaluate the effects of CO_2 on the shortgrass steppe ecosystem. The use of the experiment site was extended for a further year to investigate the resilience of the CO_2 effects on the soil ecosystem. A detailed description of the climate change experiment is given by Morgan et al. (2001). Briefly, the experiment was comprised of nine experimental plots, three chambered plots maintained at ambient CO_2 levels of 360 ± 20 μmol mol^{-1} (ambient treatment), three chambered plots maintained at 720 ± 20 μmol mol^{-1} CO_2 (elevated treatment), and three unchambered plots of equal ground area for evaluation of any chamber effect. The chambers, similar in design to Owensby et al. (1993), were installed in early spring and remained in place for the entire growing season until mid-October. Chambers were removed in the winter (from November to March) when vegetation is dormant.

Each chamber was sub-divided into a northern and southern half. The northern half of the chambers was reserved for all of the destructive sampling and most of the periodic trace gas exchange and soil water measurements. Ten steel cylinders (20 cm diameter × 60 cm length) were pushed into the soil in the northern half at the beginning of the experiment for destructive sampling.

2.3. Soil sampling

At the end of the 1999, 2000, and 2001 growing seasons, two intact soil cores (20 cm diameter × 60 cm deep) were removed from each experimental plot. The sampling dates in autumn were chosen to avoid high year-to-year variations in temperature or moisture conditions of the soil. The cores were transported to Fort Collins where they were stored at 4 °C until they were destructively sampled during the following 3 days. Sampling consisted of cutting the steel containment cylinder and sectioning the cylinder into 0–5, 5–10, 10–20, 20–30, 30–40 and 40–60 cm depth increments from the soil surface. Each soil section was sieved through a 1-cm screen to remove large roots. The sieved soil was mixed and subsampled to provide samples for the various analyses that were conducted on soil from the study.

Additional soil samples were taken from the 0–5 cm soil layers in March, June, and October 2002, 5, 8 and 12 months after the end of the CO_2 treatment, to test the reversibility of the microbial response to elevated CO_2. In general, soils for microbial analyses were immediately frozen after samples were weighed and were kept frozen until time of analyses. The frozen soils were express mailed to the University of Hohenheim under dry ice. For soil microbiological analyses, soils were allowed to thaw for 2 days at +4 °C. This procedure of storage and pretreatment preserves about 90% of microbial properties and is recommended for most soil microbial analyses (Schinner et al., 1996). Each sample was analyzed in duplicate.

2.4. Microbial biomass N, N-mineralisation and enzyme activities

Ninhydrin-reactive N (NHR-N) was measured according to a modified method of Amato and Ladd (1988). Briefly, 0.3–0.5 g of the moist soils were fumigated with 0.1 ml of chloroform for 24 h at 25 °C. Subsequently, the chloroform of the samples was removed. Samples and unfumigated controls were extracted with 5.0 ml of 2 M KCl solution for 60 min on an orbital shaker. After filtration, 2 ml of the filtrates were mixed with 0.5 ml of 0.4 M sodium citrate solution. Ninhydrin-reactive N was determined by a colorimetric procedure (Schinner et al., 1996).

N-mineralisation was measured by incubating soil samples (5.0 g) under waterlogged conditions in an enclosed tube at 40 °C for 7 days; the production of ammonium was measured by a colorimetric procedure (Kandeler and Gerber, 1988). Protease activity was determined using the method of Ladd and Butler (1972). The samples were incubated for 2 h in a buffered casein solution (pH 8.1) at 50 °C. The aromatic amino acids released were extracted with trichloroacetic acid (0.92 M) and measured colorimetrically after addition of the Folin–Ciocalteu reagent.

For the determination of urease activity, 0.3–0.4 g of the moist soils were incubated with 1.5 ml of a 79.9 mM urea solution for 2 h at 37 °C. Released ammonium was extracted with 13.5 ml of 2 M KCl solution and determined

colorimetrically by a modified Berthelot reaction (Kandeler and Gerber, 1988).

For the determination of xylanase activity, 0.5–1.0 g of the moist soils were incubated with 5.0 ml of a substrate solution (1.7% w v^{-1} xylan from oat spelts suspended in 2 M acetate buffer, pH 5.5) and 5.0 ml 2 M acetate buffer (pH 5.5) for 24 h at 50 °C. Before incubation, only the clay fractions were mixed for at least 1 min with 0.7 g of quartz to improve the dispersion of the suspension. Reducing sugars released during the incubation period reduced potassium hexacyanoferrate (III) in an alkaline solution. Potassium hexacyanoferrate (II) was measured colorimetrically according to the Prussian blue reaction (Schinner et al., 1996).

Alkaline phosphomonoesterase (alkaline phosphatase) activity was assayed using a modified disodium phenylphosphate method: 0.3–0.4 g of the soils were incubated in 2.0 ml of 0.2 M borate buffer (pH 10.0) and 1.0 ml buffered phenylphosphate solution at 37 °C for 3 h; released phenol was estimated by a colour reaction (Hoffmann, 1968).

For the determination of arylsulfatase, 0.3–0.4 g of soils were incubated with 1 ml of *p*-nitrophenylsulfate and 4 ml of acetate buffer (0.05 M, pH 5.8) for 1 h at 37 °C (Tabatabai and Bremner, 1970). Released nitrophenol was determined photometrically at 420 nm.

2.5. Statistical analyses

Microbial biomass (NHR-N), N-mineralisation and enzyme activity (protease, urease, invertase, xylanase, phosphatase, arylsulfatase) were calculated on an oven-dry weight (105 °C) basis. Normal distribution of the data was tested by the Kolmogorov–Smirnov-Goodness-of-fit test, and homogeneity of the variance was tested by Levene's test. Differences of the means of the microbial measurements between ambient and elevated CO_2 treatment were tested by standard *T*-test for independent samples. The effect of CO_2 and experimental time on the soil microbiological variables was quantified using simple two-factorial analysis of variance (ANOVA; CO_2 and sampling year). Discriminant function analysis using Wilks' Lambda for the stepwise selection of the variables (N_{mic}, N-mineralisation, protease, urease, invertase, xylanase, phosphatase, arylsulfatase) was applied to assess the response of the microbial community to elevated CO_2. The groups were defined according to the CO_2 treatment (control, ambient, elevated). The discriminant scores of each discriminant function (DF 1, DF 2) were tested for significant differences between samples using univariate analysis of variance, followed by Student Newman Keuls test. The result of discriminant function analysis is a two-dimensional representation which shows every sample as one point. Relative changes in the functional diversity are visualised as the distances between the soils. Significance was accepted at the $P<0.05$ level of probability.

3. Results

3.1. Depth profiles of microbial biomass, N-mineralisation and soil enzyme activities

Ninhydrin-reactive nitrogen as a measure of microbial biomass N showed large spatial variability within each soil layer in 1999 (Fig. 1). CO_2 enhancement did not significantly change the contents of ninhydrin-reactive nitrogen within the soil profile. In addition, microbial biomass C and microbial biomass N measured by the chloroform fumigation method were similar in the 0–5 and 5–10 cm layers, but decreased in the deeper soil layers to less than 50% and 40% of their levels in the top soils (data not shown). In accordance to our results of the third year of the experiment, no significant treatment effect could be detected in the soil profile in the two subsequent years.

N-mineralisation and enzyme activities involved in C-, N-, P- and S-cycling decreased significantly with soil depth (Figs. 1 and 2). Data were in the range previously reported for grassland (Ebersberger et al., 2003). In general, highest process rates were detected in the upper 5 cm, whereas the degree of decline with soil depth up to 60 cm depended on the microbiological property investigated. Xylanase was mainly concentrated in the top soil layer (0–5 cm), and less than 50% of activity could even be found in the 5–10 cm layer. High N-mineralisation, protease, invertase, alkaline phosphatase and arylsulfatase activities in the upper 20 cm corresponded to the main rooting zone of the shortgrass steppe.

Elevated CO_2 induced an increase in xylanase, invertase, protease, alkaline phosphatase and arylsulfatase activities in the top 5 cm. N-mineralisation measured by an anaerobic incubation and urease activity were not significantly affected by elevated CO_2. Depth profiles showed clearly that the response of microbial processes to the elevation is restricted to the upper soil layer. Therefore, only the 0–5 cm layer was used for further studies during 2000–2002.

3.2. Temporal pattern of microbial biomass, N-mineralisation and soil enzyme activities during the experiment (1999–2001)

Repeated soil sampling in 2000 and 2001 supported our results of the first year (Table 1a). In general, elevated CO_2 significantly increased invertase, urease, protease and arylsulfatase activities of the 5 cm layer in the range of 20–80%, whereas xylanase activity showed only a trend for higher values under elevated CO_2 (Fig. 3). Simple two-factorial ANOVA revealed that both factors (time and CO_2 treatment) significantly influence ninhydrin reactive N and enzyme processes (Table 2a), but the factors did not interact with one another. Temporal variation of ninhydrin reactive N and protease activity were much higher than the variation of other soil microbiological properties probably

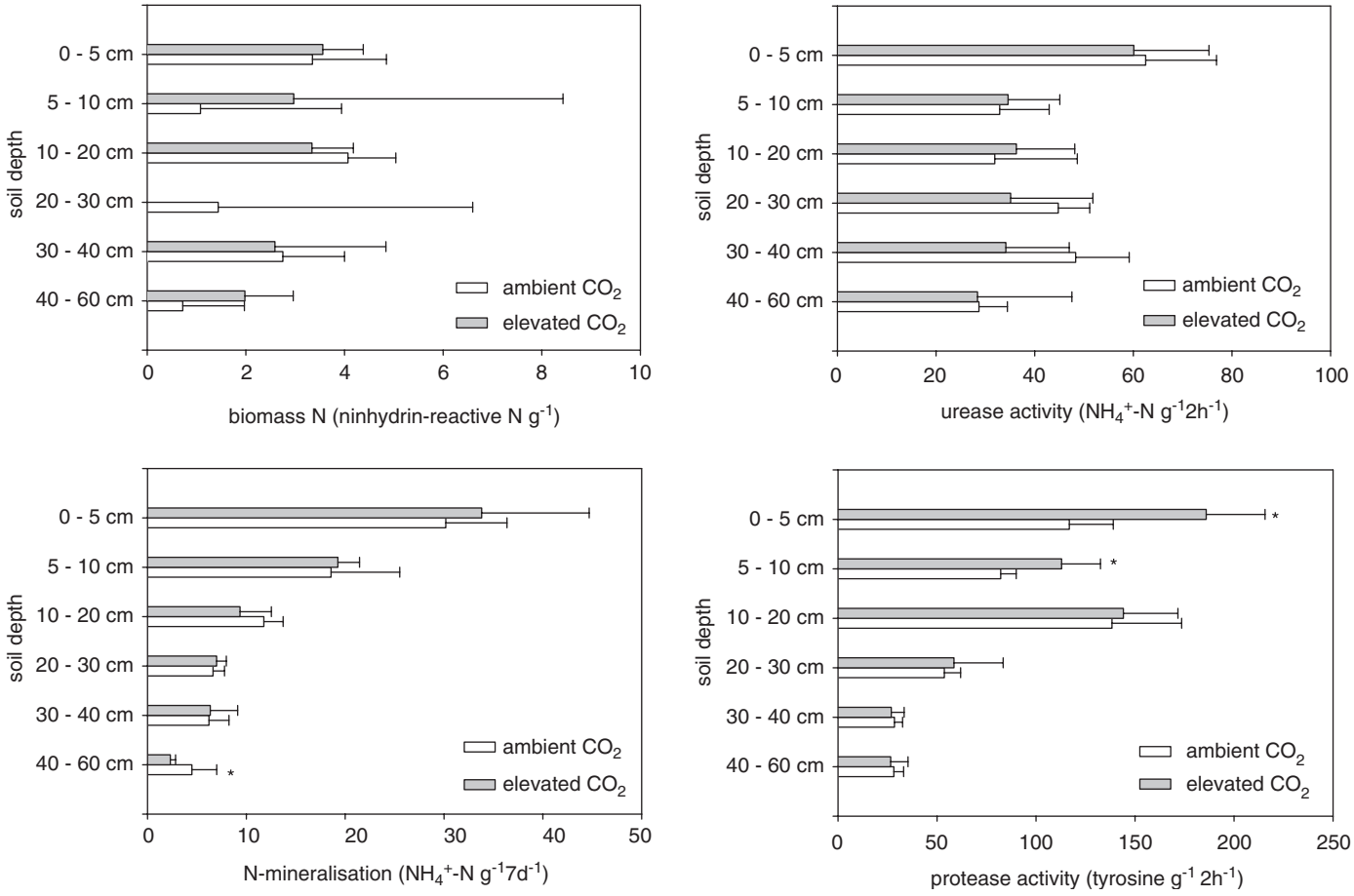

Fig. 1. The response of microbial biomass, N-mineralisation, protease activity and urease activities in the soil profile under the shortgrass steppe to elevated CO_2 (bars are means, whiskers indicate standard deviation of six soil replicates).

due to their sensitivity to differences in moisture content of soils.

Discriminant analysis showed that the influence of the chamber effect decreased and the CO_2 treatment effect increased during the experimental run (Fig. 5). The discriminant scores of each discriminant function were tested for significant differences between treatments by analysis of variance. In 1999 and 2000 enzyme activity was controlled by both the chamber effect and the CO_2 enhancement: 52–67% of the variance were explained along axis 1, along which ambient (or control) and elevated CO_2 plots were grouped together, whereas along axis 2 (explaining 33–48% of the variance) the elevated CO_2 plots were significantly different from the ambient and control plots. In 2001 the effect of elevated CO_2 dominated over the chamber effect: the elevated CO_2 plots were significantly separated from the ambient (or control) plots along axis 1 explaining 85% of the variance. Axis 2 was of minor importance, contributing only 15% to the variance of the enzyme data set. Based on the canonical function coefficients, xylanase, invertase, protease, urease, phosphatase and microbial biomass were mainly responsible for the discrimination of samples according to their treatment (Table 3).

3.3. Reversibility of the carbon dioxide effect on soil microbiological properties after the end of CO_2 enhancement

Soil microbiological properties were studied in March, June, and October 2002 after the end of the CO_2 enhancement in autumn 2001 to investigate whether or not the abundance and function of soil microorganisms returned to levels of the ambient treatment within the following season (Fig. 4). Simple two-factorial ANOVA using time and treatment as factors showed that microbial biomass and enzyme activities were significantly affected after the end of the long-term elevated CO_2. Although microbial biomass was never significantly increased under elevated CO_2, microbial biomass showed a significant post-treatment effect during the following year (Table 1b, Fig. 5). The strong response of enzyme activities in March 2002 due to former elevation of CO_2 was reduced over the season and could not be detected any more in October 2002. We found only two exceptions from this general trend: alkaline phosphatase of the elevated CO_2 plot did not return to the level of the ambient treatment and xylanase did not show any significant difference between treatments in 2002 (Tables 1b and 2b).

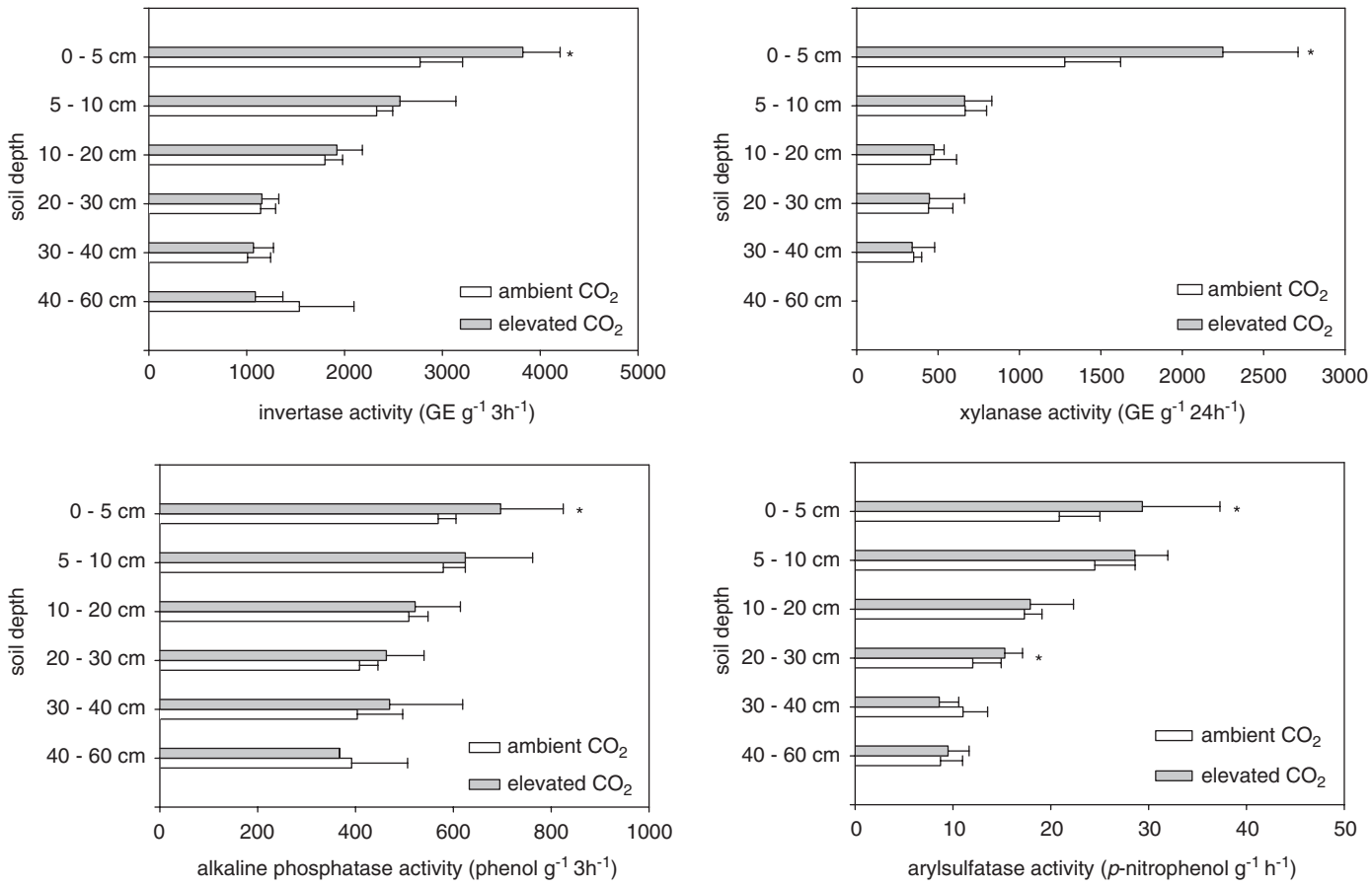

Fig. 2. The response of xylanase, invertase, alkaline phosphatase and arylsulfatase activities in the soil profile under the shortgrass steppe to elevated CO_2 (bars are means, whiskers indicate standard deviation of six soil replicates).

Table 1
Amount of microbial biomass (N_{mic}) and enzyme activity of the 0–5 cm soil layer under ambient and elevated CO_2

	1999		2000		2001	
	Ambient CO_2	Elevated CO_2	Ambient CO_2	Elevated CO_2	Ambient CO_2	Elevated CO_2
(*a*)						
N_{mic} (μg NHR–N g^{-1})	3.4 (1.5)	3.6 (0.8)	2.7 (1.7)	5.9 (1.2)*	7.2 (1.8)	7.9 (2.3)
N-mineralisation (μg NH^{4+}–N g^{-1} $7d^{-1}$)	30.2 (6.2)	33.8 (10.9)	38.6 (16.8)	35.2 (8.5)	38.8 (11.5)	36.2 (8.1)
Protease (μg tyrosine g^{-1} $2h^{-1}$)	116.8 (22.2)	186.0 (29.7)*	91.6 (13.5)	147.1 (20.5)*	48.1 (4.8)	62.9 (13.8)*
Urease (μg NH^{4+}–N g^{-1} $2h^{-1}$)	62.5 (14.4)	60.1 (15.2)	63.9 (5.2)	76.0 (10.4)*	55.1 (8.7)	73.6 (16.5)*
Invertase (μg GE g^{-1} $3h^{-1}$)	2771.9 (433.5)	3822.0 (380.5)*	3670.7 (379.8)	5124.7 (1280.5)*	2525.5 (317.4)	3322.9 (467.7)*
Xylanase (μg GE g^{-1} 24^{-1})	1278.3 (343.1)	2249.8 (461.1)*	2083.0 (876.8)	2561.9 (793.1)	1085.0 (226.9)	1225.1 (408.1)
Phosphatase (μg phenol g^{-1} $3h^{-1}$)	569.1 (36.4)	696.6 (128.0)*	626.1 (28.6)	628.7 (143.5)	559.6 (95.3)	662.7 (38.0)*
Arylsulfatase (μg *p*-nitrophenol g^{-1} h^{-1})	20.9 (4.1)	29.4 (7.9)*	24.3 (6.7)	30.9 (14.5)	27.3 (8.1)	33.0 (5.4)
	March 2002		June 2002		October 2002	
	Ambient CO_2	Elevated CO_2	Ambient CO_2	Elevated CO_2	Ambient CO_2	Elevated CO_2
(*b*)						
N_{mic} (μg NHR–N g^{-1})	6.6 (1.7)	8.8 (1.1)*	6.5 (1.2)	8.6 (0.9)*	4.0 (1.4)	4.9 (1.3)
N-mineralisation (μg NH^{4+}–N g^{-1} $7d^{-1}$)	44.3 (7.4)	37.3 (25.9)	40.4 (10.8)	45.5 (8.7)	43.1 (11.2)	44.5 (14.7)
Protease (μg tyrosine g^{-1} $2h^{-1}$)	71.7 (32.9)	196.8 (58.7)*	119.6 (14.3)	233.1 (29.5)*	68.4 (24.3)	101.6 (33.9)
Urease (μg NH^{4+}–N g^{-1} $2h^{-1}$)	45.0 (13.2)	60.8 (9.9)*	48.7 (12.0)	63.0 (10.8)	63.3 (17.4)	63.9 (16.0)
Invertase (μg GE g^{-1} $3h^{-1}$)	3157.6 (424.0)	5773.3 (1076.8)*	4213.2 (374.3)	4556.9 (1005.5)	3317.4 (395.7)	4321.9 (1005.9)
Xylanase (μg GE g^{-1} 24^{-1})	2613.0 (1151.4)	3648.8 (1075.1)	5556.0 (3059.6)	5955.9 (1202.7)	5412.6 (1945.4)	4187.4 (846.4)
Phosphatase (μg phenol g^{-1} $3h^{-1}$)	530.9 (166.7)	820.0 (146.2)*	456.2 (224.5)	715.2 (110.8)*	442.0 (168.9)	759.4 (101.6)*
Arylsulfatase (μg *p*-nitrophenol g^{-1} h^{-1})	15.0 (5.1)	25.0 (7.6)*	17.1 (5.0)	23.1 (6.4)	19.5 (6.8)	19.3 (3.3)

Given are means and their standard deviation (sd). Asteriks indicate significant differences in elevated compared to ambient CO_2. GE, glucose equivalents.

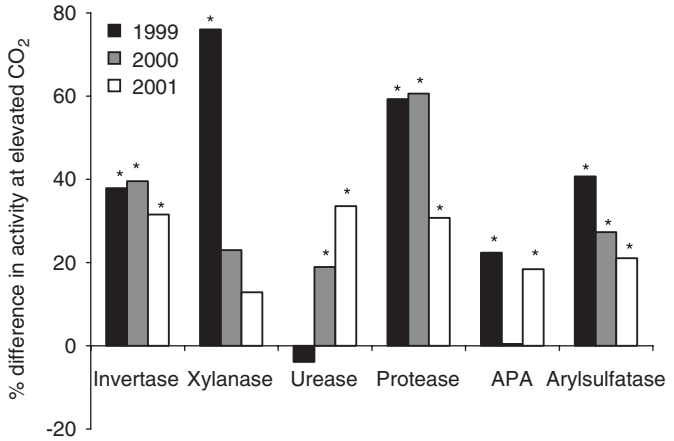

Fig. 3. Percentage difference in invertase, xylanase, urease, protease, alkaline phosphatase and arylsulfatase activities in the 0–5 cm soil layer after 3, 4, and 5 years of elevated CO_2, $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$.

Table 2
Significant differences between means of soil microbial components in the 0–5 cm layer estimated by simple two-factorial ANOVA

	Year (df = 2)	CO_2 (df = 2)	Year × CO_2 (df = 4)
(a) *Year of sampling and CO_2 treatment*			
N_{mic}	31.4^{***}	3.38^{*}	1.90
N-mineralisation	0.47	0.06	1.07
Protease	90.77^{***}	22.8^{***}	2.8^{*}
Urease	1.75	2.56	2.20
Invertase	19.8^{***}	20.3^{***}	0.81
Xylanase	13.24^{***}	5.87^{**}	1.07
Phosphatase	1.48	11.27^{***}	1.55
Arylsulfatase	0.83	5.8^{**}	1.27
	Month (df = 2)	CO_2 (df = 2)	Month × CO_2 (df = 4)
(b) *Month of sampling and CO_2 treatment*			
N_{mic}	17.70^{***}	9.22^{***}	1.87
N-mineralisation	0.51	0.44	0.81
Protease	33.36^{***}	35.40^{***}	3.77^{*}
Urease	3.53^{*}	16.22^{***}	1.07
Invertase	5.91^{**}	13.44^{***}	3.34^{*}
Xylanase	10.15^{***}	7.08^{**}	1.79
Phosphatase	0.24	21.47^{***}	1.37
Arylsulfatase	0.21	4.66^{*}	1.46

Given are the *F*-values and level of significance for the experimental run 1999–2001. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Discriminant analysis including all microbiological data revealed that there was a significant post-effect of the CO_2 enhancement on enzyme activity (Table 3). Fig. 6 shows that all treatments could be clearly distinguished according to their activity pattern. Univariate analysis of variance revealed significant differences of the discriminant scores along discriminant axis 1 (data not shown). While the size of this effect varied between March and June (82–84% of the variance was explained by this model), it largely decreased in October (67% explained variance).

4. Discussion

4.1. Depth profiles of microbial biomass, N-mineralisation and soil enzyme activities

The distribution of soil microorganisms as well as their extracellular enzymes followed their substrate availability within the soil profile. Investigations of root distribution in the shortgrass steppe of the same experiment have shown that approximately 60% of total root biomass occurs in the top 20 cm (LeCain et al., 2006). The depth profile of the xylanase activities reflected the distribution of the above-ground litter, because xylanase is mainly produced by fungi and is mainly bound to the particulate organic matter (Stemmer et al., 1999; Kandeler et al., 2000). The high variation of ninhydrin reactive N within each soil layer was evidence that soil microorganisms were unevenly distributed in the semi-arid grassland. The variable spacing between plants and distribution of fine roots were the most probable causes for the patchy distribution of soil microorganisms in this ecosystem (Ettema and Wardle, 2002).

Elevated CO_2 induced mainly an increase of enzyme activities (protease, xylanase, invertase, alkaline phosphatase, arylsulfatase) in the upper 5 cm of the soil and did not change enzyme activities in deeper layers. Because the roots of most shortgrass steppe species are relatively long-lived (5–7 years based on results of Milchunas and Lauenroth (2001) and Milchunas et al. (2005a)), differences in total root biomass could not account for the observed differences in enzyme activities. Total root biomass as well as root C:N ratio were not significantly different under elevated CO_2 in the third year of the experiment. The minirhizotron data indicated that the increase in root production was primarily in the upper soil profile and that root decomposition also increased with elevated CO_2 (Milchunas et al., 2005a,b).

A further source of microbial substrates for enzymatic decomposition could be rhizodeposition derived from turnover of fine roots, root hairs and mycorrhizal gunfi; secretion of soluble root exudates; and turnover of rhizosphere-associated microbial biomass (Milchunas et al., 2005a). Rhizodeposition was roughly doubled in elevated compared with the ambient CO_2 treatment over the last 4 years of the experiment (Pendall et al., 2004). Since rhizodeposition and newly formed roots enlarged the pool of easily available substrates mainly in the upper soil layers, increased enzyme production from a rather stable pool of microbial biomass might be the main cause for higher enzyme activities in this layer. The hypothesis that enzyme regulation (production and activity) rather than shifts in microbial abundance is the driving factor for higher enzyme activities in the upper soil layer is supported by results of a Swiss CO_2 enrichment experiment (Ebersberger et al., 2003, 2004) as well as by results of the present study using different fingerprinting techniques (PLFA pattern, DGGE pattern using eubacterial primers, T-RFLP

Table 3
Results of discriminant analyses of the microbial variables (N_{mic}, xylanase, invertase, protease, urease, N-mineralisation, alkaline phosphatase and arylsulfatase activity) with 3 treatments (control, ambient, elevated) in the 0–5 cm soil layer (degrees of freedom for DF 1 = 16, DF 2 = 7)

	CO_2 run						Post CO_2 run					
	1999		2000		2001		March 2002		June 2002		October 2002	
	DF 1	DF 2	DF 1	DF 2	DF 1	DF 2	DF 1	DF 2	DF 1	DF 2	DF 1	DF 2
Wilks' lambda	0.13	0.37	0.04	0.27	0.14	0.62	0.06	0.44	0.03	0.36	0.11	0.42
Eigenvalue	1.84	1.68	5.52	2.77	3.49	0.61	6.00	1.26	9.38	1.77	2.77	1.36
Cumulative variance %	52	48	67	33	85	15	83	17	84	16	67	33
Canonical Correlation Coefficient[a]	0.81	0.79	0.92	0.86	0.88	0.62	0.93	0.75	0.95	0.80	0.86	0.76
Canonical function coefficient												
Xylanase	1.13	0.53	−0.27	0.90	−1.39	−0.86	−0.70	0.80	0.15	−1.13	0.45	0.47
Invertase	−0.48	0.63	−0.27	−0.17	0.77	−0.31	−1.26	−0.32	−0.45	−0.88	0.21	−0.49
Protease	−0.95	0.44	0.57	−1.13	1.26	0.02	−0.35	0.04	0.96	1.52	−0.93	−0.33
Urease	0.42	−0.41	0.86	0.31	0.74	−0.25	−0.55	0.45	0.60	−0.50	−0.60	1.51
N-mineralisation	0.30	0.12	−0.20	0.02	−0.23	1.11	−0.68	−0.04	0.31	0.21	1.01	−1.04
Phosphatase	0.49	−0.39	−0.81	−0.47	−0.24	0.15	0.01	−0.34	0.81	−0.73	−0.47	−0.50
Arylsulfatase	0.81	−0.39	−0.03	0.23	−0.45	0.26	0.97	−0.42	−0.92	0.23	0.33	−0.31
N_{mic}	0.12	0.12	1.00	0.44	−0.15	0.09	0.58	−0.59	0.12	−0.05	−0.23	0.47

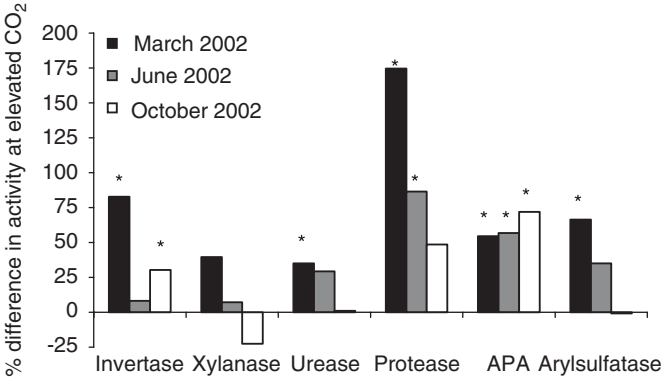

Fig. 4. Percentage difference in invertase, xylanase, urease, protease, alkaline phosphatase (APA) and arylsulfatase activities 5, 8, and 12 months after the end of CO_2 fumigation, $^{*}p \leqslant 0.05$, $^{**}p \leqslant 0.01$.

pattern using *Pseudomonas*-specific primers). These molecular techniques revealed that elevated CO_2 did not change the abundance and structure of the bacterial community (Kandeler et al., unpublished data; Bruce et al., 2000). Similar results were also obtained by Grayston et al. (1998) who found that bacterial metabolic activity (C utilisation rates measured by BIOLOG) and not population size (counts) were stimulated under elevated CO_2. Our results are also consistent with results of Hodge et al. (1998) who found no changes in microbial community diversity under elevated CO_2. Nevertheless, we have to consider that a lack of CO_2 effect on microbial biomass N does not necessarily mean that there is no effect on soil microorganisms: changes in microbial community can occur without changes in microbial N, and microbial turnover can also be increased due to increased microbial grazing (Lussenhop et al., 1998; Allen et al., 2000; Barnard et al., 2004).

N-mineralisation as well as urease activity did not change under elevated CO_2, although protease activity was significantly increased in the upper two layers (0–5 and 5–10 cm). This apparent discrepancy between the different measurements can be explained on the basis of increasing competition between microorganisms and plants for nitrogen under elevated CO_2 (King et al., 2004). Amino acids that were released by proteases due to higher substrate availability under elevated CO_2 may have been immediately assimilated by soil microorganisms and might not be taken up by plants. This hypothesis is supported by a wider C:N ratio of the shoot biomass under elevated CO_2 within the same ecosystem (King et al., 2004) and fits the competition hypothesis described by Cheng (1999).

4.2. Temporal pattern of microbial biomass, N-mineralisation and soil enzyme activities during the experiment (1999–2001)

Repeated soil sampling in autumn during the third to fifth year of the experiment allowed observation of the response of soil microorganisms to the duration of the CO_2 elevation. We expected that the response of specific enzyme activities would increase over time, because the ecosystem developed under conditions of higher plant biomass production and under slightly higher moisture conditions (LeCain et al., 2003). However we did not detect any year × CO_2 interaction by simple two-factorial ANOVA (Table 2a) and consequently no temporal trend of the CO_2 response of soil microorganisms. Similar results were found for root growth in the mini-rhizotron by Milchunas et al. (2005a). The extent of enhancement of enzyme activities, which varied in the range of 20–80%, might be caused by different amounts and distribution of precipitation over the years and/or by differences in substrate availability. The year with the highest precipitation (third) produced neither more roots nor more soluble exudates than the following years (Milchunas et al., 2005a,b). Therefore, large temporal

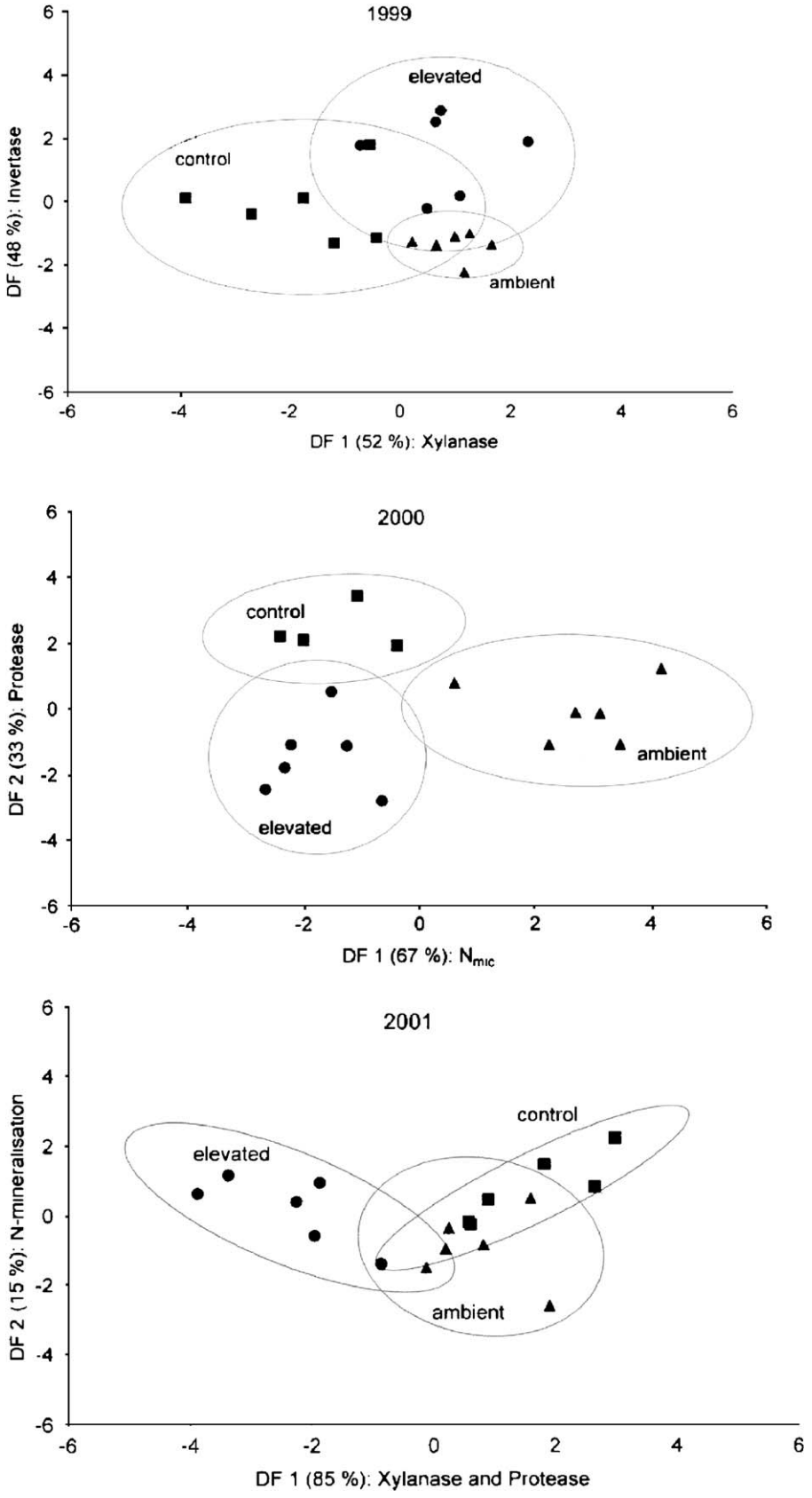

Fig. 5. Discriminant analyses of the soil enzymes in the 0–5 cm soil layer after 3, 4, and 5 years of elevated CO_2.

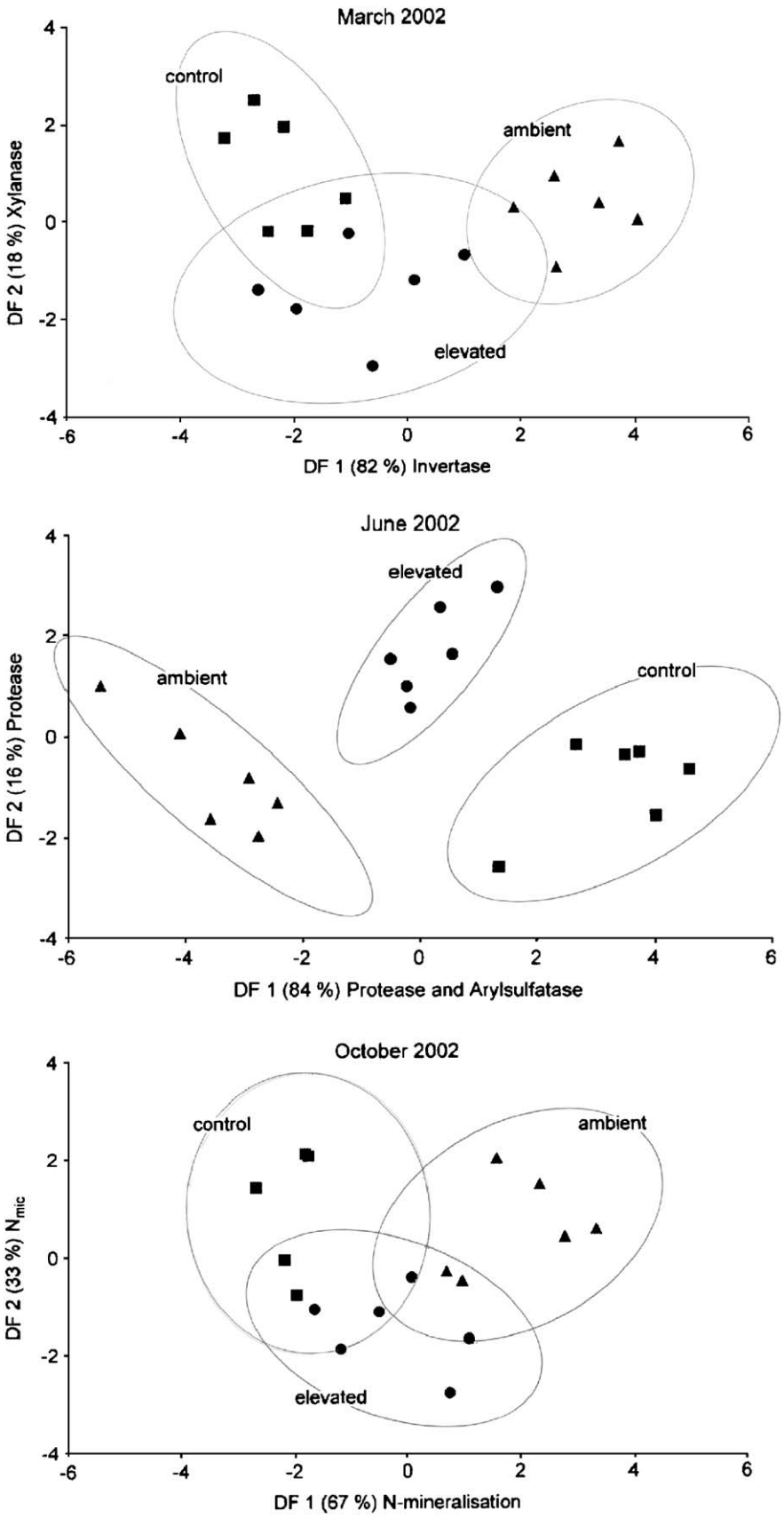

Fig. 6. Discriminant analyses of the soil enzymes in the 0–5 cm soil layer 5, 8, and 12 months after the end of CO_2 fumigation.

pulses of root initiation and termination rates of entire individuals due to variation of precipitation may be the main cause for our results (Milchunas et al., 2005a). Tracing C movement through individual components of the shortgrass steppe in the third year revealed that elevated CO_2 stimulated not only plant productivity but also rhizodeposition and decomposition (Pendall et al., 2004; Milchunas et al., 2005a). Modeling this result demonstrated that substrate availability was at least as important as soil moisture in driving CO_2 treatment differences in soil organic matter decomposition rates (Pendall et al., 2003).

Additional information on microorganism responses to elevated CO_2 was obtained by comparing the level of activity of single enzymes across different years. Due to low stability of proteases under drought environments, the level of protease activity was much higher in the relatively moist third year of the experiment than in the fourth year with the extended spring drought and the fifth year with a long drought during the entire latter half of the growing season (Fig. 1, Table 1). The dry weather conditions in autumn before soil sampling might have also caused low xylanase activity as well as non-significant CO_2 effect on this enzyme in the fifth year. Because fungi are more sensitive to dry soil conditions than bacteria, lower fungal enzyme production of xylanase might explain our results. An alternative explanation is the low availability of the corresponding substrate: Milchunas et al. (2005b) estimated the lowest cellulose and hemicellulose concentrations in root tissue in the fifth year with the autumn drought.

Besides variation of substrate availability due to different moisture conditions or plant growth, the change in species composition of plants under elevated CO_2 probably also contributed to the different responses of individual enzymes. The CO_2 atmosphere enhanced the growth of *Stipa comata*, while having no effect on growth of the two perennial forage grasses, *Pascopyrum smithii* and *Bouteloua gracilis* (Morgan et al., 2004). The changing plant community composition, driven by elevated CO_2, might change the quality of rhizodeposition. This is evidenced by an 11% increase in the soluble fraction of new roots under elevated CO_2 (Milchunas et al., 2004). Therefore, additional low molecular weight substrates released by root exudates or other forms of rhizodeposition under elevated CO_2 may have been responsible for higher invertase activities (Fig. 3).

Changes in the functional diversity of the soil microbial community were tested by discriminant analyses (DA) including all microbiological properties followed by univariate analysis of variance of the discriminant scores (Fig. 4). A significant chamber effect detected in the third and fourth years may have been due to stimulated microbial turnover in ambient plots. These plots showed differences in microclimatic conditions, because canopy air and soil temperatures averaged 2.6 and 1.25 °C warmer inside than outside the chambers, respectively (Morgan et al., 2004). In general, the CO_2 effect increased and the chamber effect decreased over the experimental run. These results provide strong evidence that the functional diversity of the microbial community followed different patterns in the ambient and elevated CO_2-treatments. Since different microbial processes were responsible for the separation of the treatments by DA in the different years (see Table 3), soil microorganisms made use of the additional substrates by excreting enzymes depending on the quality of the available materials. Enzymes released by microorganisms might not be stabilised for a longer time in this soil, because high sand content (74%) as well as low organic matter content (0.89% total C) offer fewer sites for enzyme protection than other soils with a lower sand content and a higher organic matter content (Kandeler et al., 2005). Estimating turnover times of soil enzymes is a rather difficult task in soil microbiology, but should be used in the future to validate this hypothesis.

4.3. Reversibility of the carbon dioxide effect after the end of CO_2 enhancement (Post-CO_2 effect)

Continuing soil sampling after switching off the CO_2 elevation and measuring soil microbiological properties allowed us to investigate the possible post-effect of the CO_2 elevation. We expected that CO_2-induced variation in soil water that was detected during the main experiment (LeCain et al., 2003; Nelson et al., 2004) would no longer be present. Although microbial biomass did not show any response to elevated CO_2 during the main experiment, a signifcant increase of soil microbial N was detected as a post effect (Table 1b). Microorganisms might compete more successfully for nutrients after shutting down the CO_2 elevation than in the previous years, because plant growth was not stimulated any more by elevated CO_2. Enzyme data showed that soil microorganisms made selective use of the previous surplus of substrates. This hypothesis is based on studies showing that enzyme activities are closely linked to their substrate availability (Poll et al., 2006). Whereas most enzyme activities showed a significant post-CO_2 effect in spring 2002, selective depletion of substrates might be the cause for non-significant treatment effects of some enzyme activities in June and October 2002. The CO_2-induced variation in xylanase and invertase could no longer be detected in June 2002, whereas phosphatase and protease activities were still higher than the ambient treatment. Similar results are well known from short-term incubation experiments providing complex substrates as additional resources for microbial growth (Danneberg et al., 1988). Therefore, we suggest that the surplus of low molecular weight substrates, cellulose, and hemicellulose were first depleted, and then organic compounds containing nitrogen and phosphorus were utilised. The stepwise change of enzyme activities towards the ambient level can also be seen by discriminant analyses of the enzyme data (Fig. 6, Table 3). The post-effect decreased over 1 year, but was still evident in October 2002. Consequently, additional substrates that were accumulated under elevation of CO_2

over a period of 5 years were not completely diminished within the following year. Future investigations should clarify whether fast cycling organic pools and also more stable pools, characterised by turnover times of more than 10 years, will either sustain CO_2 elevation or will return to their original level within a short time period.

In conclusion, enzymes involved in C-, N-, P- and S-cycling (xylanase, invertase, phosphatase and arylsulfatase) showed higher activities from the third to the fifth year of the OTC experiment in northern Colorado. Slightly different reactions of individual enzymes were attributed to differences in substrate availability in different soil layers, to sequential use of substrates by soil microorganisms, as well as to differences in the amount and distribution of precipitation within the years. From our post CO_2 addition studies over a period of one year after shutting off the CO_2 amendment in the OTC experiment we have some evidence that at least the upper soil layer of the shortgrass steppe did not store the additional carbon inputs from the increased aboveground and belowground productivity under elevated CO_2. Therefore, additional belowground carbon inputs mainly entered the fast cycling carbon pool and did not contribute very much to the long-term storage of carbon. Since carbon did not accumulate in deeper mineral layers after a period of 5 years of CO_2 elevation, long-term net carbon sequestration in the semi-arid grassland is unlikely.

Acknowledgements

Field and laboratory assistance was provided by Mary Smith, Susan Crookall, David Jensen, Stacey Poland, and Larry Tisue. Funds for the study were provided by the Terrestrial Ecology and Climate Change Iniative, National Science Foundation IBN-9524068 and US Department of Agriculture NRICGP-98-134, with base support from US Department of Agriculture/Agricultural Research Service, Soil-Plant-Nutrient Research Unit and the Rangeland Resources Research Unit in Fort Collins, Colorado, and additional support from the Shortgrass Steppe Long-Term Ecological Research program (National Science Foundation DEB-0217631).

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