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Atmospheric CO_2 elevation has little effect on nitrifying and denitrifying enzyme activity in four European grasslands

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Abstract

The objective of this study was to determine what patterns, if any, existed in the response of nitrifying enzyme activity (NEA), denitrifying enzyme activity (DEA), soil microbial N and soil inorganic N to elevated CO_2 across a broad range of grassland environments. We studied the response of these N pools and microbial activities in four CO_2-enrichment sites of the MEGARICH project (Managing European Grasslands as a Sustainable Resource in a Changing Climate). CO_2 treatment was studied in factorial combination with a cutting frequency treatment at two sites and with a temperature treatment at one site. Our study showed that microbial biomass N, NEA, DEA and extractable soil [NH_4^+] and [NO_3^-] were generally not affected by elevated CO_2 in these grassland ecosystems after several years of treatment, nor by cutting frequency or temperature at the sites that included these treatments. Exceptions to this were that DEA and soil [NO_3^-] decreased by 22% and 45%, respectively, at the French site at elevated CO_2. We discuss the possible explanations for this lack of response.

Keywords: ammonium, global change, immobilization, MEGARICH project, nitrate

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Introduction

Microbially mediated processes of the N cycle are expected to be modified by rising CO_2 concentrations, because soil micro-organisms are sensitive to changes in soil environmental factors that are often modified by elevated CO_2, such as soil water content and the quality and quantity of organic material entering the soil (Hu *et al*., 1999; Hungate, 1999). Understanding the effects of elevated CO_2 on processes such as nitrification and denitrification is of considerable importance, as they play a key role in regulating soil inorganic N concentrations, nitrate (NO_3^-) leaching and the production of nitrous oxide (N_2O), a highly active greenhouse gas that also contributes to stratospheric ozone destruction (Smith, 1997). Likewise, understanding the effects of elevated CO_2 on microbial biomass N is essential because changes in N sequestered in microbial biomass could have strong negative or positive feedbacks on plant productivity in N limited ecosystems (Diaz *et al*., 1993; Zak *et al*., 1993). Although the drivers of nitrification and denitrification processes, soil inorganic N and microbial N immobilization are relatively well known (e.g. Prosser, 1989; Tiedje, 1994), the impact of elevated CO_2 on the complex interactions that exist in the N cycle are poorly understood, and the responses of these processes and N pools to elevated CO_2 show few clear patterns (Niklaus & Körner, 1996; Sadowsky & Schortemeyer, 1997; Hungate, 1999; Hungate *et al*., 1999; Robinson & Conroy, 1999).

In the present study, we have investigated the effects of elevated CO_2 on nitrifying enzyme activity (NEA),

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denitrifying enzyme activity (DEA), extractable inorganic nitrogen concentrations in the soil ($[NH_4^+]$ and $[NO_3^-]$), and microbial biomass N in four, long-term CO_2-enrichment sites of the MEGARICH project (Managing European Grasslands as a Sustainable Resource in a Changing Climate) in European grasslands. The CO_2 treatment was studied in factorial combination with a cutting frequency treatment at two of the four sites, and with a temperature treatment at one of the sites. The objective of this study was to determine the response of these N pools and microbial activities to elevated CO_2 across a broad range of environmental conditions.

Materials and methods

Study sites

This study was conducted at the elevated CO_2 facilities set up and managed by four partner sites of the European MEGARICH project. The characteristics of the different sites are summarized in Table 1. The main treatment at all sites is an elevation of atmospheric CO_2 concentration, with values of elevated CO_2 that are comparable between sites (i.e. between 600 and 660 $\mu mol\,mol^{-1}$ as compared with ambient values, close to 370 $\mu mol\,mol^{-1}$).

France (INRA Clermont-Ferrand)

The experimental design consisted of 12 mini-FACE rings (Miglietta *et al.*, 2001), each containing four grassland monoliths ($0.4 \times 0.4 \times 0.4\,m^3$) extracted from a semi-natural grassland and set up at the INRA (Institut National de la Recherche Agronomique) research site in Clermont-Ferrand, France. The height of the rings was adjusted to the height of the plants to ensure fumigation of the entire canopy. A computer maintained the target CO_2 concentration inside each ring, based on wind speed and air sample readings of CO_2 concentration at the center of each ring. A detailed description of the experimental setup and management in this site is given by Teyssonneyre *et al.* (2002). The soil texture was 67% sand, 17% silt and 16% clay. For the 0–20 cm layer, total soil C and N was 3.38% and 0.32%, respectively. The experiment was a randomized two-factor design (atmospheric CO_2 concentration and cutting frequency). From August 1998 onwards, six rings were CO_2-enriched (600 $\mu mol\,mol^{-1}$), and six were ambient (370 $mol\,\mu mol^{-1}$). Half of the rings in each CO_2 treatment (high-frequency cutting treatment) were cut (6 cm residual height) at monthly intervals during the growing season (May to November); the other half (low-frequency cutting treatment) was cut half as frequently. The swards were fertilized once a year with P, K and S (150 $kg\,P_2O_5\,ha^{-1}$, 300 $kg\,K_2O\,ha^{-1}$, and 130 $SO_3\,ha^{-1}\,yr^{-1}$ in July 1998, March 1999, 2000 and 2001). Two hundred and forty kg N-$NH_4NO_3\,ha^{-1}\,yr^{-1}$ were also added (July 1998 and then in split applications after each cut, total fertilization rate being the same in all treatments). Throughout the experiment, monoliths were watered regularly to avoid water stress.

Germany (GSF Neuherberg)

The experimental setup consisted of 24 mini-FACE rings (Miglietta *et al.*, 2001) set up in a semi-natural grassland in the GSF (Forschungszentrum für Umwelt

Table 1 Environmental conditions and experimental setup of the four sites

Site	France INRA Clermont-Ferrand	Germany GSF Neuherberg	Italy Ce.S.I.A. Rapolano Terme	UK CEH Bangor
Location	45°43′N, 3°1′E	48°13′N, 11°36′E	43°17′N, 11°35′E	53°17′N, 4°43′W
Climate	Semi-continental	Continental	Mediterranean	Oceanic
Soil type	Sandy loam	Sandy clay loam	Loamy clay	Sandy loam
Two dominant plant species	*Holcus lanatus*, *Lolium perenne*	*Bromus erectus*, *Festuca rubra*	*Cynodon dactylon*, *Avena barbata*	*Holcus lanatus*, *Anthoxanthum odoratum*
Setup	12 mini-FACE, monoliths	24 mini-FACE, *in situ*	12 mini-FACE, *in situ*	8 solar domes, monoliths
Treatment	2 CO_2 levels × 2 cutting frequencies	2 CO_2 levels × 2 cutting frequencies	2 CO_2 levels	2 CO_2 levels × 2 temperature levels
Management	Fertilization, irrigation	None	Summer irrigation, cutting	Irrigation, cutting
Fumigation start	August 1998	May 1999	December 1998	September 1998
Soil sampling date	June 2001	July 2001	August 2001	September 2002
CO_2 exposure (months)	34	26	20	48

und Gesundheit) research site in Neuherberg, Germany. The soil was a calcareous Regosol. The topsoil was a sandy clay loam (soil texture was 42% sand, 32% silt and 25% clay), almost decalcified, approximately 25 cm deep. The underlying soil layers consisted mainly of calcareous gravel with a layer of secondary lime to a 1 m depth. Total soil C and N was 4.37% and 0.38%, respectively. The experiment was a randomized two-factor design (atmospheric CO_2 concentration and cutting frequency). From May 1999 onwards, 12 rings were CO_2-enriched (660 $\mu mol\,mol^{-1}$) and 12 were ambient. Half of the rings in each CO_2 treatment (high-frequency cutting treatment) were cut (3 cm height) twice a year (July and October), the other half (low-frequency cutting treatment) once a year (July). No fertilizer or water was supplied to the plots.

Italy (Ce.S.I.A Rapolano Terme)

Twelve mini-FACE rings (Miglietta *et al*., 2001) were set up in Rapolano Terme, Siena, Italy, by the Ce.S.I.A. (Centro Studi per l'Applicazione dell'Informatica in Agricoltura). The study site was an unmanaged, naturally regenerating old field, which had not been fertilized for at least 5 years before the beginning of the experiment (di Toppi *et al*., 2002). Soil was a loamy clay. Organic C and total N was 2.13% and 0.22%, respectively. The experiment was a randomized one-factor design (atmospheric CO_2 concentration), the cutting frequency was the same in all rings (three cuts a year, 6 cm residual height). From December 1996 onwards, six rings were CO_2-enriched (600 $\mu mol\,mol^{-1}$) and six were ambient. The rings were irrigated daily in the summer with the same amount of water, no fertilizer was added to the plots.

UK (CEH Bangor)

The experiment was carried out at the Centre for Ecology and Hydrology Bangor (CEH Bangor) Solardome facility. Monoliths were extracted from a semi-natural grassland in North Wales, Valley, Anglesey, United Kingdom. The soil was a well-drained, slightly acidic (pH 5.4–5.7 at a depth of 0–30 cm), mesotrophic brown earth with a sandy loam texture on top of a sand layer found mainly below 40 cm. Soil C and N was 4.31% and 0.37%, respectively. Monoliths ($0.4 \times 0.4 \times 0.4\,m^3$) were extracted in August 1998 and transferred to the Climate Change Solardomes at CEH Bangor. A quantitative measure of the abundance of species was recorded for each monolith, using the Domin scale (Rodwell, 1992). Subsequently, representative monoliths were divided at random between eight solardomes.

Eight domes (4.4 m diameter) were used in a factorial design with two CO_2 concentrations (ambient and ambient + 235 $\mu mol\,mol^{-1}$), two temperatures (ambient and ambient + 3 °C) and two replicates for each CO_2 × temperature combination. A detailed description of the system is given by Rafarel *et al*. (1995). From September 1998 onwards the swards were exposed to ambient or elevated CO_2. All swards were cut to a residual height of 6 cm in October 1998. In 1999 and 2000, monoliths were cut six times a year (weeks 9, 17, 24, 31, 38 and 46) to a residual height of 6 cm. From March (week 9) to November (week 44), each sward was supplied weekly with a nutrient solution of NH_4NO_3, NH_4Cl and KH_2PO_4 resulting in a fertilizer rate of 100 $kg\,N\,ha^{-1}\,yr^{-1}$ ($NO_3:NH_4$ in ratio 2:3), 20 $kg\,P\,ha^{-1}\,yr^{-1}$, 20 $kg\,K\,ha^{-1}\,yr^{-1}$. Throughout the experiment, swards were kept close to field capacity (i.e. 40% soil water content) to minimize the confounding effects of soil water status.

Measurements

Soil cores (2.6 cm diameter × 10 cm deep) were sampled in each ring at the four sites: France on 10–13 June 2001, Germany on 5–6 July 2001, Italy on 2–3 August 2001, United Kingdom on 10 September 2002. For each site, the fresh soil was stored at 4 °C, and microbial biomass and activities were measured within a few days after sampling (which does not alter enzyme activities, e.g., Luo *et al*., 1996), as were soil inorganic nitrogen measurements. Gravimetric soil water content was calculated using fresh soil weights and oven-dry (105 °C) weights.

Root biomass and legume frequency

Root biomass in the monoliths (0–40 cm depth) was measured in the French site (C. Picon-Cochard, pers. commun.). For the Italian and German sites, root biomass in the 0–10 cm soil layer was measured in soil leftover after 2 mm mesh sieving.

The frequency of legumes in the French site was determined in June 2001 by Teyssonneyre *et al*. (2002) using the point quadrat method. The frequency of legumes was estimated in the Italian site according to the Westhoff and van der Maarel scale in October 2000 (A. Raschi, pers. commun.). The percentage of legumes in total aboveground biomass of harvest at the German site was measured in July 2001 by Winkler & Herbst (in press). The root biomass in the soil cores and the frequency of legumes was not determined for the British site; the frequency of legumes was very low in the UK swards (Harmens *et al*., unpublished data).

Microbial biomass N

Soil microbial N was measured using the chloroform fumigation-extraction method (Brookes *et al*., 1985). Soil samples (5 g) were sieved (2 mm mesh) and then fumigated 24 h with chloroform vapor. Control samples were not fumigated. After extraction in 0.5 M K_2SO_4 with vigorous shaking for 30 min, total N in the extracts was measured by dry combustion. Nitrogen in the soil microbial biomass was calculated as [(total N in fumigated soil)−(total N in non-fumigated soil)]/0.54 (Brookes *et al*., 1985).

Denitrifying and nitrifying enzyme activities

DEA (Smith & Tiedje, 1979) was measured over a short period (4 h) by making all the factors affecting the denitrification rate non-limiting. Ten grams equivalent dry soil were placed in a 150 mL plasma flask containing 1 mg C-glucose g^{-1} dry soil, 1 mg C-glutamic acid g^{-1} of dry soil and 0.1 mg N-NO_3^- g^{-1} dry soil. The atmosphere of each tube was replaced by a 90 : 10 He–C_2H_2 mixture to provide anaerobic conditions and inhibition of N_2O-reductase activity. The N_2O efflux was measured in this flask after four hours. N_2O concentrations were analysed on a gas chromatograph equipped with an electron capture detector (Varian Star 3400 CX, Varian Chromatography Group, CA, USA). DEA was expressed as μg N h^{-1} g^{-1} dry soil.

NEA was measured using the method described in Lensi *et al*. (1986). Two 10 g subsamples from each soil core were placed in 150 mL plasma flasks. One flask of each pair was immediatly sealed with a rubber stopper and its atmosphere replaced by a 90 : 10 He–C_2H_2 mixture to ensure anaerobic conditions and N_2O-reductase inhibition. In this flask, 5 mL of a suspension of a denitrifying bacteria (*Pseudomonas fluorescens*, $OD_{580} = 2$) in a solution containing 1 mg C-glucose g^{-1} dry soil, 1 mg C-glutamic acid g^{-1} dry soil were added. This subsample was set to denitrify for a few days to fully convert soil NO_3^- into N_2O, and served as a control for the second sample (i.e. to measure initial soil [NO_3^-]) in which we measured nitrifying activity. The second subsample was enriched with 1.4 mL of a $(NH_4)_2SO_4$ solution (final soil N content: 0.2 mg g^{-1} dry soil) in order to ensure a moisture content equivalent to 80% water holding capacity and no limitation by ammonium (high [NH_4^+] should also limit NO_3^- assimilation by micro-organisms). The flask was then sealed with parafilm© (American National Can, Chicago, IL, USA) and incubated at 25 °C for 48 h in a horizontal position to ensure good aeration of the soil. After this aerobic incubation, which allows nitrate to accumulate, the soil was enriched with 3.6 mL of a *P. fluorescens* suspension ($OD_{580} = 2$) in a solution containing glucose and glutamic acid (same concentrations as above). Anaerobiosis and N_2O-reductase inhibition were obtained in the flask as described above. N_2O concentrations were analysed on the same gas chromatograph. NEA was calculated by subtracting the N_2O efflux for the first subsample from the second and expressed as g N h^{-1} g^{-1} dry soil.

Soil inorganic nitrogen

Soil NH_4^+ and NO_3^- were extracted in 40 mL of 2 M KCl from 5 g sieved (2 mm mesh) soil samples which were shaken vigorously for 30 min. Extracts were filtered and the concentrations were measured using a continuous flow spectrophotometer (Skalar 5100, Breda, The Netherlands) following cadmium reduction (λ = 540 nm) for nitrate, and cation complexation ($\lambda = 660$ nm) for ammonium. This measure gives nitrate concentrations in soil solution plus the exchangeable nitrate adsorbed on the anion exchange complex. For ammonium, this measure represents the ions in the soil solution plus the exchangeable ammonium adsorbed on the cation exchange complex.

Statistical analysis

We analyzed the response of the variables to the different treatments by carrying out analyses of variance at each site with R 1.6.1. (The R Development Core Team). When necessary, variables were transformed to correct for non-normality and unequal variances. A Wilcoxon non-parametric test was used when we could not resolve problems of unequal variances with simple transformations. The number of replicates in each site is as follows: France: $n = 3$ for each CO_2 × cutting treatment combination, Germany: $n = 6$ for each CO_2 × cutting treatment combination, Italy: $n = 6$ for each CO_2 treatment, UK: $n = 2$ for each CO_2 treatment. Two rings that were consistently identified as extreme outliers were removed from the analysis in the German site. Treatment effect was calculated as follows: % effect = 100 × [treatment−control]/control.

Results

Soil water content, root biomass and legume frequency

Soil water content was significantly modified only in the German site at elevated CO_2, where it increased by 13% ($P = 0.027$), but not in any other site or treatment. Root biomass was significantly decreased by 30% in the French site at high cutting frequency ($P = 0.043$), and

was not significantly modified in any other site or treatment. The frequency of legumes in the aboveground biomass was significantly increased by 104% at elevated CO_2 at the French site (see Teyssonneyre *et al.*, 2002) and by 47% at the German site ($P = 0.014$). No significant interactions between treatments were measured at any of the sites where treatment combinations were applied.

Microbial biomass N, NEA and DEA

Microbial biomass N and NEA were not significantly modified at any site or treatment (Table 2, Fig. 1a and b for responses to CO_2 treatments), although microbial biomass N tended to decrease at elevated CO_2 in the UK site (−29% CO_2 effect) and increase at elevated temperature (+28%, $P = 0.061$). DEA decreased at elevated CO_2 in the French site (−22%), but was not significantly affected in any other site or treatment (Fig. 1c). At the UK site, the NEA values were close to our detection limit (Table 2) and, therefore, the effects of elevated CO_2 and temperature should be interpreted with caution. No significant interactions between treatments were measured at any of the sites where treatment combinations were applied.

Soil [NH_4^+] and [NO_3^-]

Extractable [NO_3^-] decreased by 45% in the elevated CO_2 treatment at the French site (Fig. 2a). Soil [NH_4^+] and [NO_3^-] were not significantly modified at any other site or treatment (Fig. 2), although in the UK site, soil [NO_3^-] tended to decrease at elevated CO_2 (−96%). No significant interactions between treatments were measured at any of the sites where treatment combinations were applied.

Discussion

CO_2 effects on soil microbial N

Elevated CO_2 can have a positive effect (Diaz *et al.*, 1993; Zak *et al.*, 1993; Niklaus, 1998) or no effect (Berntson & Bazzaz, 1998; Niklaus, 1998; Zak *et al.*, 2000) on soil microbial biomass N (see also reviews by Paterson *et al.*, 1997; Sadowsky & Shortemeyer, 1997; Hu *et al.*, 1999). We found no significant microbial biomass N response to elevated CO_2 in any of the four sites. There are several possible explanations for this lack of responsiveness. Soil nutrient status may be a key factor in the microbial response to elevated CO_2 (Hungate, 1999). Niklaus & Körner (1996) suggested that most changes in microbial biomass occur in short-term experiments, disturbed soils or with fertilizer addition, whereas microbial biomass may be largely insensitive to CO_2 in intact, unfertilized soils. While nutrient limitations might explain the lack of responsiveness in the German and Italian sites, substantial amounts of fertilizer were added at the French and British sites. In addition, we had expected that increased legume frequency at the French and German

Table 2 Microbial biomass N, nitrifying enzyme activity (NEA), denitrifying enzyme activity (DEA), extractable soil [NH_4^+] and extractable soil [NO_3^-] in all treatment combinations at each site.

Sites	Treatments		Microbial biomass N ($\mu g\,N\,g^{-1}$ dry soil)	NEA ($\mu g\,N\,h^{-1}\,g^{-1}$ dry soil)	DEA ($\mu g\,N\,h^{-1}\,g^{-1}$ dry soil)	[NH_4^+] ($\mu g\,NH_4^+$-N g^{-1} dry soil)	[NO_3^-] ($\mu g\,NO_3^-$-N g^{-1} dry soil)
France	CO_2−	Cut−	113.7 ± 13.9	0.25 ± 0.07	1.37 ± 0.05	9.3 ± 0.5	1.4 ± 0.4
	CO_2−	Cut+	127.6 ± 2.0	0.20 ± 0.03	1.41 ± 0.13	8.5 ± 0.6	1.6 ± 0.1
	CO_2+	Cut−	126.6 ± 13.3	0.14 ± 0.04	1.12 ± 0.03	9.7 ± 1.2	0.9 ± 0.2
	CO_2+	Cut+	124.8 ± 8.0	0.19 ± 0.05	1.05 ± 0.06	9.7 ± 1.0	0.8 ± 0.1
Germany	CO_2−	Cut−	203.7 ± 4.6	0.66 ± 0.11	1.12 ± 0.05	2.1 ± 0.1	0.7 ± 0.1
	CO_2−	Cut+	197.2 ± 11.4	0.75 ± 0.05	1.08 ± 0.09	2.1 ± 0.1	0.8 ± 0.2
	CO_2+	Cut−	200.4 ± 14.2	0.68 ± 0.05	1.12 ± 0.09	2.2 ± 0.1	0.6 ± 0.1
	CO_2+	Cut+	190.8 ± 12.3	0.75 ± 0.06	1.12 ± 0.06	2.2 ± 0.1	0.9 ± 0.2
Italy	CO_2−		179.0 ± 19.5	0.92 ± 0.09	0.90 ± 0.07	2.1 ± 0.2	11.7 ± 1.5
	CO_2+		175.6 ± 6.4	0.85 ± 0.08	0.93 ± 0.07	2.0 ± 0.2	11.1 ± 1.0
UK	CO_2−	T−	129.1 ± 7.4	$0.83 \times 10^{-3} \pm 0.63 \times 10^{-3}$	0.55 ± 0.04	22.9 ± 15.6	0.6 ± 0.4
	CO_2−	T+	151.0 ± 15.0	0.21×10^{-3} ± NA	0.57 ± 0.05	6.3 ± 1.5	0.3 ± 0.2
	CO_2+	T−	91.6 ± 16.8	$0.21 \times 10^{-3} \pm 0$	0.51 ± 0.004	30.6 ± 25.4	0.02 ± NA
	CO_2+	T+	130.8 ± 1.0	$0.42 \times 10^{-3} \pm 0.21 \times 10^{-3}$	0.56 ± 0.05	9.1 ± 1.4	0.05 ± 0.0004

Different treatments are atmospheric CO_2 concentration (CO_2−: ambient, CO_2+: elevated), cutting frequency (Cut−: low frequency, Cut+: high frequency), and temperature (T−: ambient, T+: elevated). Results are shown as mean ± SE. NA indicates that no SE is available ($n = 1$ because only one sample had values above detection limits).

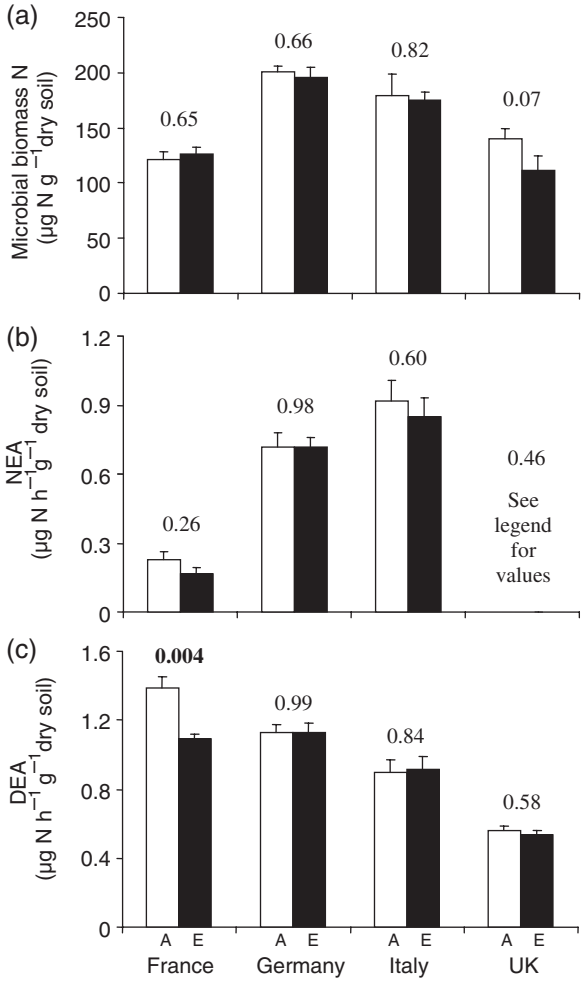

Fig. 1 Microbial biomass N (A); nitrifying enzyme activity, NEA (B); and denitrifying enzyme activity, DEA (C) at the four sites. UK values for NEA: $0.63 \times 10^{-3} \pm 0.42 \times 10^{-3}\,\mu g\,N\,h^{-1}\,g^{-1}$ dry soil in the ambient treatment and $0.31 \times 10^{-3} \pm 0.10 \times 10^{-4}\,\mu g\,N\,h^{-1}\,g^{-1}$ dry soil in the elevated CO_2 treatment. 'A' and 'E', respectively, stand for 'ambient' and 'elevated' [CO_2]. Values represent the mean and standard error of ambient and elevated CO_2 treatments at each site. At the sites that had CO_2 treatment in factorial combination with another treatment (France, Germany and Italy), the latter had no significant effect and no significant interaction between treatments was found. *P*-values for the CO_2 treatment main effect are indicated for each site above the bars and values of $P < 0.05$ are in bold.

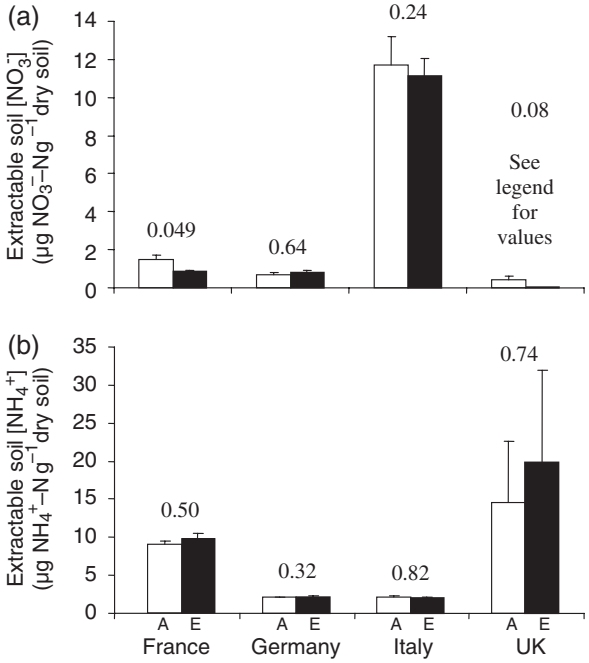

Fig. 2 Extractable soil [NO_3^-] (A) and [NH_4^+] (B) at the four sites. UK values for [NO_3^-]: $0.4 \pm 0.2\,\mu g\,NO_3^-$-N g^{-1} dry soil in the ambient treatment and $0.04 \pm 0.01\,\mu g\,NO_3^-$-N g^{-1} dry soil in the elevated CO_2 treatment. 'A' and 'E', respectively, stand for 'ambient' and 'elevated' [CO_2]. Values represent the mean and standard error of ambient and elevated CO_2 treatments at each site. At the sites that had CO_2 treatment in factorial combination with another treatment (France, Germany and Italy), the latter had no significant effect and no significant interaction between treatments was found. *P*-values for the CO_2 treatment main effect are indicated for each site above the bars and values of $P < 0.05$ are in bold.

sites would increase N inputs into these systems, and stimulate N sequestration in soil micro-organisms. Another possible explanation for the lack of responsiveness of microbial N to elevated CO_2 is that the duration of the experiments (between 20 and 34 months) may have been too short to induce measurable changes in microbial biomass N in our study. In a 3-year field study of calcareous grassland ecosystems, Niklaus (1998) measured a significant effect of elevated CO_2 on microbial biomass N only in the very last year of the study.

A lack of CO_2 effect on microbial biomass N does not necessarily mean that there are no effects on soil micro-organisms: changes in microbial community and activity can occur without changes in microbial biomass N (Hungate *et al*., 2000), changes in microbial biomass C can also occur, and microbial turnover can also be increased due to increased microbial grazing (Lussenhop *et al*., 1998).

CO_2 effects on [NH_4^+] and [NO_3^-]

We measured no significant effect of elevated CO_2 on exchangeable [NH_4^+] at any site. Soil [NH_4^+] is frequently unresponsive to elevated CO_2 (Arnone & Bohlen, 1998; Niklaus *et al*., 1998, 2001; Johnson *et al*., 2001), although soil [NH_4^+] decreased in some studies (Berntson & Bazzaz, 1998; Matamala & Drake, 1999).

We measured a large significant decrease of soil [NO_3^-] at the French site and a tendency for soil [NO_3^-] to decrease at the British site, but no significant effects at the other two sites. This is consistent with previous studies in which the response of soil [NO_3^-] to elevated CO_2 is either a decrease (Niklaus *et al.*, 1998, 2001; Johnson *et al.*, 2001) or no change (Arnone & Bohlen, 1998).

CO_2 effects on nitrifying and denitrifying enzyme activities

NEA is generally favoured in well-aerated soils, at moderate temperatures, and high NH_4^+ availability (Linn & Doran, 1984; Prosser, 1989; Grundmann *et al.*, 1995). DEA tends to be favoured at low soil aeration, high NO_3^- availability, high labile C substrate availability, moderate temperatures and modified by changes in soil pH (Linn & Doran, 1984; Tiedje, 1988; Merrill & Zak, 1992; Weier *et al.*, 1993; Simek *et al.*, 2002). Because elevated CO_2 is expected to affect most of these environmental variables (Hu *et al.*, 1999; Hungate, 1999), CO_2-induced changes in NEA and DEA can be expected. In medium- or long-term experiments in undisturbed systems, other studies found no effect of elevated CO_2 on DEA (Kammann, 2001), a tendency for DEA to decrease (Matamala & Drake, 1999), or contrasting responses depending on sampling date, which the authors attributed to differences in soil moisture (Billings *et al.*, 2002).

Our measurements of NEA and DEA suggest that elevated CO_2 may have limited effects on the amount of active nitrifying and denitrifying enzymes present in European grassland soils. The only significant effect of elevated CO_2 was a decrease in DEA at the French site. Given that soil water content was not affected by CO_2 elevation at the French site and that increased carbon inputs to the soil have often been measured at elevated CO_2 (Cheng & Johnson, 1998; Hungate, 1999; van Ginkel *et al.*, 1997, 2000), decreased substrate availability (i.e. [NO_3^-]) would seem to be the most plausible explanation for decreased DEA at this site.

Why were CO_2 effects on NEA and DEA relatively small? Despite the large potential effects of elevated CO_2 on NEA and DEA, this study shows that the overall CO_2 effect was generally small in four grassland systems. We have focused on three possible explanations for this lack of response: (i) lack of statistical power, (ii) absence of CO_2 effects on drivers of NEA and DEA or compensation between drivers, and (iii) insensitivity of NEA and DEA to changes in their drivers.

First, statistical power was relatively high in most of the experiments (1,18 df in Germany; 1,10 df in Italy; and 1,8 df in France for main effects tests for CO_2). In addition, many of the CO_2 effects on NEA and DEA were quite small, far from significant, and the variance to mean ratio was relatively low (Table 2, Fig. 1b and c). However, lack of statistical power may have been a problem in the case of NEA at the French and UK sites since the CO_2 effects were large, but not significant.

Second, we do not have sufficient measurements of drivers of NEA and DEA, for example, soil water content or labile C concentrations, to draw conclusions about the effects of CO_2 on these drivers. It is interesting to note, however, that NEA and DEA showed very little response to CO_2 treatment in the German site, which is the only site that was never irrigated and which had significant CO_2 effects on soil water content for the date where the soil processes where sampled. It is possible that irrigation in the remaining sites reduced to various degrees the potential of CO_2 to alter NEA and DEA through changes in soil water content (note that the irrigation treatment at the UK site was intentionally designed to eliminate this effect). It is also possible that contrasting effects of elevated CO_2 on a number of drivers led to little overall effect.

Third, NEA and DEA may have been insensitive to CO_2-induced changes in their drivers. This could have occurred because the duration of CO_2 exposure before sampling was too short to detect changes in NEA and DEA. Laboratory and field experiments show, however, that NEA and DEA can respond rapidly to changes in soil conditions such as soil water content or temperature (Smith & Tiedje, 1979; Grundmann *et al.*, 1995), so the explanation for the lack of responsiveness of these variables may lie elsewhere. For example, NEA has a relatively broad soil moisture optimum (Grundmann *et al.*, 1995), so that slight CO_2-induced changes in soil water content may not have been sufficient to alter NEA.

Cutting frequency and temperature treatments

More frequent cutting (French and German sites) over nearly 3 years had no significant effect on microbial biomass N in our study. Defoliation is expected to reduce root biomass and belowground C allocation (Miller & Rose, 1992; Johansson, 1993; Holland *et al.*, 1996; Mackie-Dawson, 1999; but see Milchunas & Lauenroth, 1993). This could reduce C flow into the soil and, therefore, limit microbial C and N. However, the response of soil microbes to defoliation is inconsistent: an increase (Guitian & Bardgett, 2000), a decrease (Mikola *et al.*, 2001), or no change (Kuzyakov *et al.*, 2002) in microbial biomass have been found. The lack of response at the French site occurred despite a

significant 30% decrease in root biomass at high cutting frequency (C. Picon-Cochard, pers. commun.). At the two sites where the effect of cutting intensity was studied, no significant effect was observed either on DEA or NEA. This result is consistent with the small effect of cutting frequency or grazing intensity on DEA generally observed in grasslands (Groffman *et al*., 1993; Le Roux *et al*., 2003; but see Frank *et al*., 2000). In contrast, NEA is often enhanced by high cutting or grazing intensity (Groffman *et al*., 1993; Frank *et al*., 2000; Le Roux *et al*., 2003). However, the cutting frequencies studied here (monthly vs. every 2 months at the French site; once vs. twice a year at the German site) perhaps did not differ enough to induce marked changes in NEA.

We measured a tendency for increased microbial biomass N at elevated temperature at the UK site, but we do not know to what extent this might be representative of microbial responses to temperature, since most studies of temperature effects on soil microbial processes have focused on microbial activity and not on microbial N or C. Kandeler *et al.* (1998) and Tscherko *et al*. (2001) found no effect of increased temperature on microbial biomass N but did measure an increase of the micro-organism metabolic quotient (respiration to biomass ratio) and of rates of substrate use. In a review across 17 studies, Rustad *et al.* (2001) found that warming significantly increased soil respiration by 20%. The lack of response of NEA to temperature at the British site may be explained by increased soil respiration at elevated temperature, which reduces soil [O_2] (Grundmann *et al*., 1995). In contrast, DEA does not seem to be sensitive to increased temperature. The extremely low rates of NEA that we measured indicate that the potential for generating nitrate is very low, thereby limiting substrate availability for denitrification.

Conclusion

It has often been suggested that elevated CO_2 could substantially alter soil microbial N and nitrification and denitrification processes in natural and semi-natural ecosystems. We find little evidence of this in four grassland sites in Europe.

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