Dynamics of nitrifying activities, denitrifying activities and nitrogen in grassland mesocosms as altered by elevated CO\textsubscript{2}

Romain Barnard\textsuperscript{1}, Laure Barthes\textsuperscript{1}, Xavier Le Roux\textsuperscript{2} and Paul W. Leadley\textsuperscript{1}

\textsuperscript{1}Université Paris-Sud XI, Laboratoire d’Ecologie, Systématique et Evolution, UMR CNRS 8079, F-91405 Orsay Cedex, France; \textsuperscript{2}Ecologie Microbienne, UMR 5557 (CNRS – Université Lyon 1 – USC 1193 INRA), 43 Bd du 11 Novembre 1918, F-69622 Villeurbanne Cedex, France

Summary

- The objective of this study was to better identify the mechanisms by which elevated CO\textsubscript{2} (c. 665 µmol mol\textsuperscript{-1}) alters soil nitrifying and denitrifying enzyme activity (NEA and DEA), and the dynamics of plant and microbial N pools.
- We measured the effects of elevated CO\textsubscript{2} on plant biomass and N, soil microbial biomass N, soil ammonium and nitrate concentrations, NEA and DEA in monospecific grassland mesocosms (\textit{Holcus lanatus} and \textit{Festuca rubra}) grown for 15 months in reconstituted grassland soil.
- NEA strongly decreased at elevated CO\textsubscript{2} in the \textit{Holcus} mesocosms, but was not affected in \textit{Festuca} systems. DEA was less sensitive to elevated CO\textsubscript{2} than NEA. In \textit{Holcus} mesocosms, microbial N showed an initial growth phase that appeared to limit plant N acquisition, but was only transient.
- CO\textsubscript{2}-induced changes in NEA and DEA were best explained by factors that could reduce soil [O\textsubscript{2}] (increased soil water content and organic C). Elevated CO\textsubscript{2} may increase microbial immobilisation in highly disturbed systems, this effect weakening as the system gets closer to equilibrium.

Key words: ammonium, denitrification enzyme activity, elevated CO\textsubscript{2}, \textit{Festuca rubra}, \textit{Holcus lanatus}, immobilisation, nitrate, nitrification enzyme activity.

Introduction

The responses of many terrestrial ecosystems to elevated CO\textsubscript{2} will depend in large part on the interactions between elevated CO\textsubscript{2} and nitrogen transformations in the soil, including mineralisation, nitrification and denitrification (Hungate, 1999). Studies of the effects of elevated CO\textsubscript{2} on N transformations in the soil have primarily focused on microbial mineralisation and immobilisation (Zak \textit{et al.}, 2000). We have focused on nitrification and denitrification processes, which are responsible for the transformation of N into forms that are likely to be lost from ecosystems. Ammonium, which is tightly retained in ecosystems, can be transformed by nitrifying bacteria into nitrate (NO\textsubscript{3}\textsuperscript{-}) that can easily be lost through leaching. Denitrifying bacteria can transform nitrate into volatile gaseous nitrogen forms, of which N\textsubscript{2}O and NO are important air pollutants (Houghton \textit{et al.}, 2001). Several studies have measured increased gaseous N emissions from grassland systems at elevated CO\textsubscript{2} (Arnone & Bohlen, 1998; Ineson \textit{et al.}, 1998; Kammann, 2001; Baggs \textit{et al.}, 2003a; Baggs \textit{et al.}, 2003b). To provide insight into the mechanisms that underlie the response of N transformations to elevated CO\textsubscript{2} there is still a need for integrated studies in simplified systems, in addition to long-term, \textit{in situ} experiments. In the present study, we used reconstituted, monospecific grass mesocosms to better understand how the biological processes involved in the transformation of nitrogen from its ammonium form onwards are affected by elevated CO\textsubscript{2}.

Despite a large number of studies in pots, monoliths or field conditions, the response of the N cycle to elevated CO\textsubscript{2} remains unclear, due to the complexity of the interactions between plant, soil and soil microorganisms (Berntson &
Bazzaz, 1998; Hodge et al., 2000; Zak et al., 2000). It is thought that increased soil labile C, soil water content, and litter C:N ratio are the primary mechanisms by which elevated CO₂ alters N cycling (Hungate, 1999). These responses are indirect consequences of more direct effects of elevated CO₂ on plants such as increased photosynthesis and decreased stomatal conductance (Stitt & Krapp, 1999; Wand et al., 1999).

The microorganisms that control the major N transformation processes in the soil (mineralisation, nitrification, and denitrification) are likely to be sensitive to these CO₂-induced changes in soil water content, soil labile C, and litter quality. Increases in soil water content (Knapp et al., 1996; Hungate et al., 2002) and labile soil C (Cortufo & Gorissen, 1997; van Ginkel et al., 2000) that are often observed at elevated CO₂ will generally favour denitrification and limit nitrification (Tiedje, 1988; Paul & Clark, 1989). DEA and NEA should also be sensitive to changes in inorganic N substrate availability, but the response of soil NH₄⁺ and NO₃⁻ availability to CO₂ remains difficult to predict because they are determined by a large number of interacting processes (Paul & Clark, 1989; Hungate, 1999). Competing microbial and plant N uptake represent major sinks for soil NH₄⁺ and NO₃⁻. The outcome of this competition can be modified at elevated CO₂, with consequences that have led to conflicting hypotheses as to whether increased root exudation at elevated CO₂ would tend to stimulate microbial N immobilisation, leading to decreased N availability for plants (Diaz et al., 1993), or stimulate N mineralisation, resulting in increased N availability for plants (Zak et al., 1993).

We studied the impact of ambient and elevated CO₂ concentrations on several aspects of nitrogen cycling in grassland mesocosms of Holcus lanatus and Festuca rubra, grown as monocultures in reconstituted grassland soil. We measured the effects of elevated CO₂ on plant biomass and N, soil microbial biomass N, extractable inorganic nitrogen concentrations in the soil ([NH₄⁺] and [NO₃⁻]), nitrifying enzyme activity (NEA), denitrifying enzyme activity (DEA), and labile C in leachate. The objective of this study was to better identify the mechanisms by which elevated CO₂ may alter NEA, DEA and the dynamics of plant and microbial N pools. Our hypothesis was that elevated CO₂ should favour DEA and reduce NEA due to increased soil water content, but that this could be modified by changes in NH₄⁺ or NO₃⁻ availability.

Materials and Methods

Experimental setting and management

The experiment was conducted on two perennial C₃ grasses monocultures, Holcus lanatus L. (Yorkshire fog) and Festuca rubra L. (red fescue), grown in mesocosms in naturally lit, CO₂ controlled chambers. Deep PVC pots (14×19×50 cm) were filled with a reconstituted sandy loam soil. Two soil horizons (0–20 cm and 20–40 cm) were dug out of a seminatural grassland (St. Pierre-les-Nemours, France), and then sieved using a 1 cm mesh sieve. Holcus lanatus and Festuca rubra are common and sometimes dominant species in European temperate grasslands and are also present in several plant communities studied in a multisite experiment where we have measured the effects of elevated CO₂ on NEA and DEA (Barnard et al., 2004). These species also showed a differential response to elevated CO₂ in a previous mesocosm study (L. Barthes et al., unpublished data). The pots were filled with 9 cm of sand, then 19 cm of soil from the 20–40 cm horizon and 19 cm of soil from the 0–20 cm horizon. For the top horizon, the cation exchange capacity was 4.1 cmol kg⁻¹, SOM was 28.8 g kg⁻¹, total N 1.27 g kg⁻¹, total C 16.77 g kg⁻¹ and pH 5.3. The texture was 71% sand, 8% clay and 21% silt. For the second horizon, cation exchange capacity was 3.4 cmol kg⁻¹, SOM was 19.5 g kg⁻¹, total N 0.74 g kg⁻¹, total C 11.32 g kg⁻¹ and pH 5.6. The texture was 73% sand, 7% clay and 20% silt.

Holcus and Festuca seeds (obtained from Herbiseed, Twyford, UK) were set to germinate in 7 cm of soil from the first horizon on 4 March 2001, and the plantlets were transferred into the mesocosms on 14 March 2001. The experimental design consisted of 12 naturally lit chambers (wooden frame and clear plastic walls, 65 cm × 67 cm × 100 cm high) set up inside a large glasshouse. Each chamber contained nine mesocosms. Six chambers were ventilated with ambient air taken from outside the glasshouse (daytime [CO₂] = c. 365 µmol mol⁻¹), the six others with ambient air enriched with CO₂. CO₂ enrichment was started the 7 April 2001. Elevated CO₂ concentrations were maintained day and night by adding a small amount of pure CO₂ to the air blown through each chamber. This gave a relatively constant differential of 300 µmol mol⁻¹ above ambient in the elevated CO₂ treatment, resulting in daytime CO₂ concentrations of c. 665 µmol mol⁻¹. An infrared gas analyser (ADC-225-MK3, Analytical Development Co., Hoddesdon, UK) coupled to a sequential sampling manifold was used to monitor the CO₂ concentrations in the chambers throughout the experiment. The use of closed chambers, as opposed to open-top chambers, meant that CO₂ concentrations rarely deviated more than 50 µmol mol⁻¹ from their set point and that CO₂ concentrations were homogeneous within the chamber. The temperature in each chamber was also monitored, and there were no significant differences in temperature between the ambient and elevated chambers (data not shown). Mean daily minimum and maximum temperatures across all chambers were 9.0 and 17.7°C in January 2002 and 14.7 and 35.9°C in July 2002. To avoid position effects inside the chambers, a rotation was made after each harvest: each rotation consisted of randomly switching positions of the mesocosms within the chambers. The mesocosms were watered once to three times per week, each mesocosm receiving the same amount of water.

We do not have measures of the quantity or quality of root litter inputs, but because we regularly removed above-ground
plant material, the above-ground litter inputs into our system were small. It should also be noted that this experiment did not involve legumes and that the mesocosms were never watered to the drip point. Biological fixation of $N_2$, leaching, and potential effects of a modification of litter quality were therefore not taken into account in this study.

Soil moisture and pH
Gravimetric soil water content was measured at each sampling date for each mesocosm by comparing the mass of $c.$ 5 g of soil before and after drying at 105°C. The effect of CO$_2$ on soil moisture was also measured in Holcus mesocosms during a 30 d dry-down period. The mesocosms were initially brought to field capacity ($c.$ 20% gravimetric soil water content). Between 31 May and 28 June 2002, volumetric soil moisture was measured at 5, 15 and 25 cm depths on 10 dates with a ThetaProbe ML1 (Delta-T Devices, Cambridge, UK). Soil pH was measured in September 2001 on 1 : 1 mixtures of soil and distilled water. In July 2002, pH was measured in soil leachate using the following method. A tube was inserted into the bottom of each pot before the start of the experiment to aid in collecting leachate. The mesocosms were slowly brought to field capacity with deionised water, and then one extra litre was added slowly. One litre of leachate was collected from the tube, then rapidly filtered using a syringe tip filter (0.45 µm mesh) before the pH measurement was performed.

Plant, soil and microbial nitrogen pools
Plants were harvested five times: on 10 May 2001, 4 September 2001, 21 January 2002, 17 April 2002 and 15 July 2002, respectively, 1, 5, 9, 12 and 15 months after fumigation started. At each harvest, measurements were made in at least one pot from each glasshouse, resulting in $n = 6$ for each treatment at each harvest. Below-ground biomass was measured in September 2001 and July 2002 for Holcus and in July 2002 for Festuca. Total N concentrations in above- and below-ground biomass were measured with an elemental analyser (NA-1500, Carlo Erba Instruments, Milano, Italy).

Nitrate and ammonium concentrations were measured in 0–10, 10–20 and 20–30 cm soil layers cores (one 2.5 cm diameter core per layer and per mesocosm) taken in September 2001 and July 2002 in Holcus and Festuca systems. Another measurement was made in Holcus in the 0–10 cm soil layer in January 2002 (two 1 cm diameter × 10 cm deep cores). Nitrate and ammonium were extracted in 40 ml of 2 M KCl from 5 g sieved (1 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, Netherlands) following cadmium reduction ($\lambda = 540$ 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.

Soil microbial N was measured in 0–10, 10–20 and 20–30 cm soil layers (one 2.5 cm diameter core per layer and per mesocosm) taken in September 2001 and July 2002 in Holcus systems and in July 2002 in Festuca systems. Another measurement was made in Holcus systems in the 0–10 cm soil layer in January 2002 (two 1 cm diameter × 10 cm deep cores). We used the chloroform fumigation-extraction method (Brookes et al., 1985): 5 g soil samples were sieved (1 mm mesh) and then fumigated for 24 h with chloroform vapour. Control samples were not fumigated. After extraction in 0.5 M K$_2$SO$_4$ 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 nonfumigated soil)]/0.54 (Brookes et al., 1985).

Nitrifying and denitrifying enzyme activities
Nitrifying and denitrifying enzyme activities (NEA and DEA, respectively) were measured in the 0–10 cm soil cores taken in September 2001, January 2002 and July 2002 in Holcus systems, and in July 2002 in Festuca systems.

DEA (Smith & Tiedje, 1979) was measured over a short period (4 h) by making all the factors affecting the denitrifying rate nonlimiting: 10 g 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$_2$H$_4$ mixture to provide anaerobic conditions and inhibit N$_2$O reductase activity. The N$_2$O efflux was measured in this flask after 4 h. N$_2$O 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 immediately sealed with a rubber stopper and its atmosphere replaced by a 90–10 He-C$_2$H$_4$ mixture to ensure anaerobic conditions and N$_2$O-reductase inhibition. Five millilitres of a suspension of a denitrifying bacteria (Pseudomonas fluorescens, O.D.$_{580} = 2$) in a solution containing 1 mg C-glucose g$^{-1}$ dry soil, 1 mg C-glutamic acid g$^{-1}$ dry soil and 0.1 mg N-NO$_3^{-}$ g$^{-1}$ dry soil. The flask was replaced by a 90–10 He-C$_2$H$_4$ mixture to ensure anaerobic conditions and N$_2$O-reductase inhibition. Five millilitres of a suspension of a denitrifying bacteria (Pseudomonas fluorescens, O.D.$_{580} = 2$) in a solution containing 1 mg C-glucose g$^{-1}$ dry soil, 1 mg C-glutamic acid g$^{-1}$ dry soil and 0.1 mg N-NO$_3^{-}$ g$^{-1}$ dry soil. The flask was
then sealed with parafilm® 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 *Pseudomonas fluorescens* suspension (O.D.<sub>580</sub> = 2) in a solution containing glucose and glutamic acid (same concentrations as above). Anaerobiosis and N<sub>2</sub>O-reductase inhibition were obtained in the flask as described above. N<sub>2</sub>O concentrations were analysed on a same gas chromatograph (Varian Star 3400 CX, Varian Chromatography Group, CA, USA). NEA was calculated by subtracting the N<sub>2</sub>O efflux for the first subsample from the second and expressed as µg N h<sup>−1</sup> g<sup>−1</sup> dry soil.

C in leachate

Carbon concentrations were measured in leachate in July 2002 in the *Holcus* and *Festuca* systems (see method for collecting leachate above). One litre of leachate was collected from pots and then quickly filtered with a syringe tip filter (0.45 µm mesh) and stored at −18°C. Total C in leachate was measured by dry combustion. Infra-red measurement of CO<sub>2</sub> emission of the sample injected in an acid media allowed us to measure inorganic C, then organic C was calculated as the difference between total C and inorganic C (Laboratoire d’Analyse des Sols, INRA Arras, France).

Statistical analysis

Analysis of variance was carried out using R 1.6.1 (The R Development Core Team) and JMP 3.5.1 (SAS Institute Inc., Cary, NC, USA). When necessary, variables were transformed to correct for nonnormality and unequal variances. A Wilcoxon nonparametric test was used when simple transformations could not correct the distribution and variance of the data. To analyse time responses, we used a repeated measurements multivariate analysis of variance (MANOVA) with a Pillai test. Results are given with mean ± SE (n = 6 for all measurements). The values per pot were converted in values per m<sup>2</sup> by multiplying them by 37.6 pot m<sup>−2</sup>.

Results

*Holcus mesocosms*

**Soil water content and organic C** Mesocosms in the elevated CO<sub>2</sub> treatment had higher soil water content in moderately dry to very dry soils in the drying experiment (Fig. 1). Gravimetric soil water content measurements at each harvest also showed a general increase in soil moisture at elevated CO<sub>2</sub>. In the 0–10 cm soil layer, water content was increased in the elevated CO<sub>2</sub> treatment in September 2001 and July 2002 (respectively +23%, P = 0.02 and +24%, P = 0.02), but was not modified in January 2001. In the 10–20 cm and 20–30 cm soil layers, it was also increased at elevated CO<sub>2</sub> in September 2001 (respectively +18%, P = 0.050 and +39%, P = 0.04) and in July 2002 (respectively +19%, P = 0.04 and +28%, P = 0.008). Gravimetric soil water contents at the harvests were between 6% and 11%, which corresponds to the range over which the drying experiment also showed a significant CO<sub>2</sub> effect on soil moisture.

We measured a significant increase (+30%, P = 0.008) of organic C in the leachate in July 2002, from 8.4 ± 0.5 mg C l<sup>−1</sup> in the ambient treatment to 10.9 ± 0.5 mg C l<sup>−1</sup> in the elevated
CO₂ treatment. We found no effect of elevated CO₂ on soil solution pH in September 2001 or July 2002.

**Plant biomass and N** Elevated CO₂ had a positive significant effect on *Holcus* above-ground biomass at all harvests except September 2001 (Fig. 2a). Root biomass was significantly increased in September 2001, but not in July 2002. Total plant biomass was significantly increased in July 2002 (+34%, *P* = 0.005) but not in September 2001. The percentage of N in above-ground and below-ground biomass tended to be lower
at all harvests, although not always significantly (Fig. 2b). N in above-ground biomass (Fig. 2c) decreased in the elevated CO2 treatment in September 2001, but tended to increase in July 2002. N in root biomass was increased in September 2001. Total plant N tended to be lower at elevated than ambient CO2 in September 2001 (−17%, P = 0.051) but to be higher in July 2002 (+25%, P = 0.056). We measured a significant time effect for above-ground and total biomass, %N and N content, and for root biomass and %N. We also found a significant time–CO2 treatment interaction for whole plant %N and N content.

**Microbial biomass N** We measured a significant CO2 increase in total microbial N in the whole pot in September 2001 (+26%, P = 0.015) but not in July 2002 (Fig. 3). Most of the CO2 effect on microbial biomass N in September 2001 came from changes in the 0–10 cm layer (+27%, P = 0.07). Between September 2001 and July 2002 the total microbial biomass N increased by 40.0 µg g−1 at ambient and 39.8 µg g−1 elevated CO2, resulting in a significant time effect in both treatments, but no difference between CO2 treatments. We also measured a significant time effect on microbial biomass N in the 20–30 cm layer, but not in the other layers. In both treatments, microbial biomass N in September 2001 was highest in the uppermost soil layer and lowest in the bottom soil layer, but it did not differ between the three soil layers in July 2002.

**Nitrifying and denitrifying enzyme activity** Elevated CO2 significantly decreased NEA at all three measurement dates and NEA significantly decreased over time in both treatments, but we measured no CO2–time interaction (Fig. 4a). At the final harvest, NEA in the elevated treatment was one third of that in the ambient treatment. DEA was not affected by time, and CO2 treatment had much smaller effects on DEA than on NEA: the former was significantly affected only in July 2002 (+14% CO2 effect, Fig. 4b).

**Soil [NO3−] and [NH4+]**

We measured no significant effect of elevated CO2 on extractable soil [NO3−] in the 0–10 cm soil layer (Fig. 5, top panels).

![Fig. 4](image)

(a) Nitrifying enzyme activity (NEA) and (b) denitrifying enzyme activity (DEA) in Holcus 0–10 cm soil in September 2001 and July 2002. Open and closed bars indicate mean ± SE at ambient and elevated CO2, respectively. Significance between treatments: *0.05 > P > 0.01; **0.01 > P > 0.001; ***0.001 > P.

![Fig. 3](image)

Microbial biomass N in the 0–10, 10–20 and 20–30 cm soil layers in Holcus soil (white, light gray and dark gray bars, respectively) in September 2001, January 2002 and July 2002. ‘A’ and ‘E’ are ambient and elevated CO2, respectively. Standard errors for the whole pot (0–30 cm) are indicated above the bars and standard errors for each layer are indicated within the bar for each layer. Significance between treatments: *0.05 > P > 0.01; **0.01 > P > 0.001; ***0.001 > P.
In September 2001, extractable soil \([\text{NO}_3^-]\) was significantly increased in the 10–20 cm layer (+38%, \(P = 0.032\)) and tended to increase in the 20–30 cm layer (+45%, \(P = 0.073\)) (Fig. 6a). It was not significantly modified in any soil layer in January 2001 (Fig. 5b) or July 2002 (Fig. 5c). Soil \([\text{NO}_3^-]\) significantly increased over time in all three layers.

Extractable soil \([\text{NH}_4^+]\) in the 0–10 cm soil layer was significantly increased at elevated \(\text{CO}_2\) in January 2002 (+71%, \(P < 0.001\); Fig. 5e) and July 2002 (+63%, \(P = 0.004\); Fig. 5f). It was not significantly modified by elevated \(\text{CO}_2\) in the other soil layers or sampling dates (Fig. 5, bottom panels).

**Festuca mesocosms**

**Soil water content and organic C** We measured no \(\text{CO}_2\) effect on soil water content in the 0–10 cm soil layer in September 2001, but did find a significant increase in July 2002 in the 0–10, 10–20 and 20–30 cm soil layers (respectively +41%, \(P = 0.005\); +23%, \(P = 0.008\); +31%, \(P = 0.002\)). Soil water contents at the harvests were between 4% and 9%.

We measured no significant effect of elevated \(\text{CO}_2\) on organic C in leachate in July 2002, values being, respectively, 7.4 ± 0.5 mg C l\(^{-1}\) and 8.4 ± 0.6 mg C l\(^{-1}\) in the ambient and elevated \(\text{CO}_2\) treatment.

**Plant biomass and N** Elevated \(\text{CO}_2\) had a positive significant effect on above-ground biomass at all harvests except May 2001 and January 2002 (Fig. 6a). In July 2002, we measured a large significant \(\text{CO}_2\) effect on root biomass. Total plant biomass was significantly increased in July 2002 (+67%, \(P < 0.001\)). The percentage of N in above-ground biomass was significantly lower at elevated \(\text{CO}_2\) throughout the experiment (Fig. 6b). Elevated \(\text{CO}_2\) decreased the percentage of N in root biomass and in total plant biomass (−27%, \(P < 0.001\)) in July 2002. N in above-ground biomass (Fig. 6c) was significantly decreased in the elevated \(\text{CO}_2\) treatment in September 2001 and July 2002. Elevated \(\text{CO}_2\) increased N in root biomass and total plant biomass (+21%, \(P = 0.03\)) in July 2002.

**Microbial biomass N** We measured no effect of elevated \(\text{CO}_2\) on microbial biomass N in any soil layer in July 2002. Values were 16.8 ± 3.4 and 17.0 ± 3.4 µg C g\(^{-1}\) dry soil at ambient and elevated \(\text{CO}_2\) in the 0–10 cm soil layer, 19.4 ± 2.7 and 20.3 ± 5.2 µg C g\(^{-1}\) dry soil at ambient and elevated \(\text{CO}_2\) in the 10–20 cm soil layer, and 14.9 ± 2.6 and 17.2 ± 2.8 µg N g\(^{-1}\) dry soil at ambient and elevated \(\text{CO}_2\) in the 20–30 cm soil layer.

**Nitrifying and denitrifying enzyme activity** In July 2002, elevated \(\text{CO}_2\) showed no significant effect on NEA (respectively 0.18 ± 0.01 and 0.16 ± 0.02 µg N h\(^{-1}\) g\(^{-1}\) dry soil at ambient and elevated \(\text{CO}_2\)) or DEA (respectively 0.16 ± 0.004 and 0.15 ± 0.01 µg N h\(^{-1}\) g\(^{-1}\) dry soil at ambient and elevated \(\text{CO}_2\)).

**Soil \([\text{NO}_3^-]\) and \([\text{NH}_4^+]\)** Elevated \(\text{CO}_2\) had no significant effect on extractable soil \([\text{NO}_3^-]\) in the 0–10 cm soil layer in September 2001 (Fig. 7a). In July 2002, we measured a marginally significant decrease of extractable soil \([\text{NO}_3^-]\) in the 0–10 cm soil layer, but no effect in the 10–20 and 20–30 cm soil layers. Extractable soil \([\text{NH}_4^+]\) was significantly decreased at elevated \(\text{CO}_2\) in the 0–10 cm soil layer in September 2001 (Fig. 7c). In July 2002, it increased significantly in the 0–10 cm soil layer (+9%, \(P = 0.017\)), but we measured no \(\text{CO}_2\) effect in the other soil layers (Fig. 7d).

**Discussion**

The drivers of NEA and DEA (soil water content, soluble carbon, and inorganic N) were affected by \(\text{CO}_2\) in different ways in *Holcus* and *Festuca* mesocosms and this may explain, in part, the differential responses of NEA and DEA in the mesocosms of these two species.
Soil water content, soluble carbon and inorganic nitrogen

Soil water content has generally been found to increase at elevated CO₂ due to reduced stomatal conductance (Knapp et al., 1996; Hungate et al., 1997; Arnone & Bohlen, 1998; Lutze & Gifford, 1998; Niklaus et al., 1998b; Hungate et al., 2002), and occurs even in some situations where leaf area index is increased at elevated CO₂ (Li et al., 2003). Our measurements of soil water content are consistent with these previous findings.

**Fig. 6** Festuca above-ground and below-ground biomass (a), N concentration (b) and N content (c) at the five harvest dates. Below-ground biomass and N were only measured in July 2002. Open and closed bars indicate mean ± SE at ambient and elevated CO₂, respectively. Significance between treatments: *0.05 > P > 0.01; **0.01 > P > 0.001; ***0.001 > P.
studies, since soil water content was generally increased at elevated CO₂ for both Holcus and Festuca mesocosms.

Increased rhizodeposits of labile C into soil have often been observed at elevated CO₂ (Cotrufo & Gorissen, 1997; van Ginkel et al., 2000). This has been found to arise from increased carbon availability in plants, together with greater root growth and carbon allocation (Bassirirad et al., 1996; Cotrufo & Gorissen, 1997; Niklaus et al., 2001a). Our measurements of organic C in leachate are only rough indicators of rhizodeposition, but they suggest that rhizodeposition increased in Holcus systems or was unchanged in Festuca systems at elevated CO₂.

Soil [NH₄⁺] is generally unchanged at elevated CO₂ (Arnone & Bohlen, 1998; Niklaus et al., 1998a; Johnson et al., 2001; Niklaus et al., 2001b), or decreased (Berntson & Bazzaz, 1998; Matamala & Drake, 1999). Similarly, the response of soil [NO₃⁻] at elevated CO₂ is either no change (Arnone & Bohlen, 1998), or a decrease (Niklaus et al., 1998a; Johnson et al., 2001; Niklaus et al., 2001b). The lack of significant CO₂ effects on soil [NO₃⁻] in 2002 in Holcus and Festuca systems is consistent with these previous studies, but the increase in soil [NH₄⁺] at some dates is not. The increased soil [NH₄⁺] we measured in the Holcus mesocosms at elevated CO₂ may have arisen from several mechanisms including increased N mineralisation (which we did not measure) or decreased nitrification.

Nitrifying enzyme activity

NEA is generally favoured in well-aerated soils, at high NH₄⁺ availability and at moderate pH (Linn & Doran, 1984; Proser, 1989; Grundmann et al., 1995). In Holcus mesocosms, soil water content generally increased, soil [NH₄⁺] was unchanged or increased, and soil pH was unchanged at elevated CO₂. Thus, a reasonable explanation for the decrease in NEA at elevated CO₂ in Holcus systems throughout the experiment is the decrease in soil aeration due to increased soil water content. However, the CO₂ effects on soil water content were largest in moderately dry to very dry soils and these are not typically the soil moisture levels at which soil water content is likely to inhibit nitrification in the type of soil used in our experiment (Grundmann et al., 1995). In addition, the absence of CO₂ effects on NEA in Festuca mesocosms in July 2002, where soil water content also increased at elevated CO₂, is not fully consistent with this hypothesis.

Another possible explanation for decreased NEA in Holcus mesocosms is a decrease in soil oxygenation due to a stimulation of respiration in the soil. Indeed, heterotrophic respiratory activity in the soil is the major factor that removes oxygen from the soil environment (Tiedje, 1988). For example, Grundmann et al. (1995) found that increased soil respiration at elevated temperature reduced soil [O₂], thereby inhibiting NEA. Increased labile organic C also often reduces soil [O₂] through a stimulation of soil microbial activity (Tiedje, 1988) and this may explain the commonly observed reduction in nitrification when labile sources of carbon are added to soils (Paul & Clark, 1989). The observed increase in organic C in leachate is consistent with this mechanism. Increased root respiration might also have reduced soil [O₂] at elevated CO₂, since root biomass in Holcus systems was higher at elevated CO₂ at the September 2001 harvest. Grundmann et al. (1995) have shown that increased soil water content and soil respiration
Denitrifying enzyme activity

DEA is favoured when soils are more anaerobic, soil [NO$_3^-$] increases, labile C availability increases and as soil pH becomes more neutral (Tiedje, 1988; Paul & Clark, 1989; Merrill & Zak, 1992; Weier et al., 1993; Simek & Cooper, 2002; Strong & Fillery, 2002). DEA was relatively insensitive to elevated CO$_2$ in both Festuca and Holcus systems. In addition, DEA changed little over time in the Holcus systems and differed little between the two species. The small increase in DEA in Holcus mesocosms in July 2002 might have arisen from an increase in soil water content or soil C availability (no changes in soil [NO$_3^-$] or pH were detected).

Based on measurements of N$_2$O fluxes, several authors have suggested that denitrification should be favored at elevated CO$_2$ due to increased soil water content (Arnone & Bohlen, 1998; Baggs et al., 2003b). Using $^{15}$N natural abundance in soil NO$_3^-$, Robinson & Conroy (1999) suggested that CO$_2$-induced changes in denitrification were best explained by changes in soil wetness and not by changes in root derived C or in NO$_3^-$ production in their mesocosms. One possible explanation for the general unresponsiveness of DEA to elevated CO$_2$ in our study is that CO$_2$-induced changes in soil [O$_2$] via soil water status were not sufficient to reduce soil [O$_2$] to the point where it affected the synthesis of denitrifying enzymes (note that the soil moisture thresholds for inhibiting nitrification are much higher than the thresholds for inducing denitrification – Paul & Clark, 1989). The soils used in our experiment had very little development of soil structure (even in the field) due to high sand and low clay contents, which reduce the possibility to develop anaerobic microsites in soil aggregates (Tiedje, 1988; Sierra & Renault, 1996). A second possible explanation for the nonresponsiveness of DEA to CO$_2$ treatment in our experiment may be that NO$_3^-$ availability was the limiting factor.

The small increase in DEA at elevated CO$_2$ in Holcus at the end of our experiment might be explained by the positive effect that increased soil soluble C often has on denitrification (Paul & Clark, 1989). Soil soluble C is thought to act primarily on denitrification through changes in soil [O$_2$] (Tiedje, 1988), but may also act through changes in C substrate availability for denitrifiers. Phillips et al. (2001) used laboratory manipulations of soil C availability to show that, together with NO$_3^-$ availability and soil water content, increased C inputs might explain increased DEA in forest soils at elevated CO$_2$. In contrast to Phillips et al. (2001) and our study, DEA has also been observed to be reduced at elevated CO$_2$ (Matamala & Drake, 1999; Kammann, 2001; Tscherko et al., 2001). Tscherko et al. (2001) found that DEA decreased at elevated CO$_2$ despite a large increase in dissolved soil organic C. Matamala & Drake (1999) suggest that reduced N availability may explain decreased DEA in their elevated CO$_2$ study.

The general response of DEA in herbaceous ecosystems (little change or reduction) contrasts with measurements of actual N$_2$O flux in herbaceous systems in glasshouse or field CO$_2$ experiments, which show substantial variability but generally increase (Arnone & Bohlen, 1998; Ineson et al., 1998; Kammann, 2001; Baggs et al., 2003b) or are unchanged (Mosier et al., 2002; Baggs et al., 2003b) at elevated CO$_2$. DEA and N$_2$O flux are linked to the same process, but measure very different components of denitrification because first N$_2$O flux is only a portion of the gaseous N flux produced by denitrification, and this portion is highly variable (Baggs et al., 2003a), second a large fraction of N$_2$O flux may come from nitrifiers (Phillips et al., 2001; Baggs et al., 2003a), and third DEA is a measure of the amount of functionally active denitrifying enzymes in the soil and not a measure of N flux (Smith & Tiedje, 1979). Measurements of the flux of N through denitrification are very reactive to modifications of soil environmental conditions, such as rain events that can trigger large N$_2$O emission peaks (Ineson et al., 1998). By contrast, enzyme activities are generally less variable than flux measurements, since they integrate the environmental constraints on microorganisms over a longer period of time (Simek et al., 2000). Therefore, N$_2$O flux measurements will be most useful in determining how elevated CO$_2$ modifies the amount of N$_2$O emitted to the atmosphere and DEA measurements will be most useful for understanding how CO$_2$ alters the environmental constraints on denitrifiers.

Plant and microbial N

It is thought that elevated CO$_2$ can favour immobilisation of N by soil microorganisms in fertile soils, rendering N less available for plants (Diaz et al., 1993; Hungate, 1999). Several results lead us to think that Holcus may have been N-limited in September 2001 due to microbial immobilisation, but not
at other sampling dates. First, elevated CO₂ had a positive effect on Holcus above-ground biomass at all harvests but September 2001. The September 2001 harvest is the only time when N content in the above-ground biomass was significantly lower in the elevated CO₂ treatment than in the ambient treatment. Second, total plant N tended to decrease at elevated CO₂ in September 2001, whereas above-ground N increased in all subsequent harvests and total plant N tended to increase at final July 2002 harvest. Plants in the elevated CO₂ treatment had also become visibly chlorotic before the September 2001 harvest. Third, elevated CO₂ had a significant positive effect on microbial biomass N in September 2001. Assuming that microbial biomass N was equivalent in both treatments at the beginning of the experiment, this means that more N was sequestered in microbial biomass between the beginning of the experiment and September 2001 at elevated CO₂.

Why was plant N limitation released after September 2001 in Holcus mesocosms? Hodge et al. (2000) suggest that microorganisms are better short-term competitors than plants, but are more liable to lose captured N through high turnover rates, whereas plants are able to better retain the captured N than microorganisms. Based on this logic, one would then expect strong microbial N sequestration in the early growth phase of microbial populations following an increase in substrate availability, but that the competitive advantage of soil microbes would diminish over the long-term. Based on an analysis of several CO₂ studies, Niklaus & Körner (1996) suggested that microbial biomass in near-equilibrium systems showed little response to elevated CO₂ compared with short-term experiments in disturbed soils. This pattern of response is consistent with our observations that plants seemed N-limited and microorganisms sequestered more N during earlier stages of development of the mesocosms, while plant N was unchanged or even increased at elevated CO₂ after this period.

Conclusions

Our results, together with other studies, suggest that elevated CO₂ may alter nitrification, denitrification, and N competition between plants and soil microorganisms in herbaceous ecosystems. The mechanisms that underlie the response of NEA and DEA to elevated CO₂ appear to involve complex interactions in the plant-soil system, in particular regarding factors that affect soil N availability, such as sequestration of N in plants and microorganisms, and factors that are likely to modify soil [O₃], such as changes in soil water content and heterotrophic respiration in the soil.

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