



# A possible plant-mediated feedback between elevated CO<sub>2</sub>, denitrification and the enhanced greenhouse effect

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## Abstract

Natural abundances ( $\delta$ ) of <sup>15</sup>N were used to detect effects of elevated atmospheric CO<sub>2</sub> concentration ([CO<sub>2</sub>]) and soil wetness on soil N transformations in the presence or absence of plants. An elevated [CO<sub>2</sub>] of 1000 μl l<sup>-1</sup> reduced water use by the perennial C<sub>4</sub> grass *Panicum coloratum* and stimulated root and whole-plant growth. Soil remained wetter between infrequent irrigations than in soil supporting *P. coloratum* grown in an ambient [CO<sub>2</sub>] (350 μl l<sup>-1</sup>). The δ<sup>15</sup>N value of soil nitrate increased from -2.4 to +9.6‰ as nitrate was depleted from the soil, but remained unchanged in unplanted soil. The change in δ<sup>15</sup>N of soil nitrate was greatest in frequently watered soil regardless of [CO<sub>2</sub>], and in infrequently watered soil only in elevated [CO<sub>2</sub>]. It was least in the infrequently watered, ambient [CO<sub>2</sub>] treatment. Isotope mass balances and <sup>15</sup>N/<sup>14</sup>N fractionation theory identified denitrification as the most probable cause of this effect, through the effect of elevated [CO<sub>2</sub>] on soil wetness. Nitrification, nitrogen assimilation, leaching or ammonia volatilisation were unlikely causes. The data suggest a positive, plant-induced effect of elevated atmospheric [CO<sub>2</sub>] on denitrification. The possibility exists, therefore, for a positive feedback between elevated atmospheric [CO<sub>2</sub>], a greater soil-to-atmosphere N<sub>2</sub>O flux and an exacerbation of the enhanced greenhouse effect. © 1998 Elsevier Science Ltd. All rights reserved.

**Keywords:** Denitrification; Elevated CO<sub>2</sub>; *Panicum coloratum*; δ<sup>15</sup>N

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## 1. Introduction

The concentration of CO<sub>2</sub> in the atmosphere ([CO<sub>2</sub>]) can influence biological processes in soil. This influence is rarely direct, but is transmitted indirectly, via plants (Körner and Arnone, 1992; Hungate et al., 1997a,b,c).

Plants are often larger when grown in elevated [CO<sub>2</sub>], whether C<sub>4</sub> or C<sub>3</sub> species (Ghannoum et al., 1997). Much of the growth response to atmospheric [CO<sub>2</sub>] is below-ground (Van Vuuren et al., 1997), but this depends on, among other things, nutrient availability (Conroy, 1992). When there is a growth response to atmospheric [CO<sub>2</sub>], it is possible that, per plant, more C as exudates and detritus is transferred into the soil. If some of this C is used by heterotrophic microbes, there will probably be an effect on soil N transformations which can be traced back to the increased [CO<sub>2</sub>] (Hungate et al., 1997c).

Elevated [CO<sub>2</sub>] may influence soil in another way. O<sub>2</sub> consumption by microbes or roots may be increased by the provision of more C or by there being more living roots per unit soil volume under elevated [CO<sub>2</sub>] (see above). In well-aerated soil, O<sub>2</sub> consumption is balanced by diffusion from atmosphere, but this is impeded significantly by moisture. O<sub>2</sub> diffusivity in water is smaller by a factor of 10<sup>4</sup> compared with diffusion in air. Therefore, the wetter the soil, the more likely it is that the inward diffusion of O<sub>2</sub> will fail to compensate fully for its consumption. Localised hypoxia or anoxia then results.

Soil wetness is influenced by, among other things, transpiration, and this also depends on atmospheric [CO<sub>2</sub>]. Elevated [CO<sub>2</sub>] reduces stomatal conductances of C<sub>3</sub> and C<sub>4</sub> species (Ghannoum et al., 1997). Such plants consequently have slower transpiration rates and so remove less water from the soil per unit time (Jackson et al., 1994; Samarakoon and Gifford, 1996; Hungate et al., 1997a; Van Vuuren et al., 1997). The

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soil may then remain slightly wetter between rain events or irrigations. Soil wetness may then affect soil aeration as described above. If it does, N transformations may be subject to this indirect influence of atmospheric  $[\text{CO}_2]$ .

Clearly, the associations between  $[\text{CO}_2]$  and soil N transformations are complex. It is impracticable to measure all potentially important processes. But an alternative approach is available. It is sometimes possible to monitor the operation of a complex system by measuring variations in one or a few key features, e.g. particular substrates or products. Then, it is necessary to use that information to deduce which process(es) were most likely to have been responsible for those variations. This is one of the tasks to which natural abundances of stable isotopes are particularly well suited (e.g., Mariotti et al., 1981, 1988; Handley and Raven, 1992).

Stable isotope natural abundances are measured by mass spectrometry as ratios ( $R$ ) of heavy to light isotopes. These are usually reported as  $\delta$  values (‰) defined as:

$$\delta = \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \times 10^3 \quad (1)$$

$R_{\text{sample}}$  is the isotope ratio of a sample and  $R_{\text{standard}}$  that of the standard material with which the sample is compared. For  $^{15}\text{N}/^{14}\text{N}$ , the standard is atmospheric  $\text{N}_2$  and  $R_{\text{standard}} = 0.0036765$ .

Changes  $\delta$  values may reflect the operation of processes which cause isotope fractionations, e.g. reactions of substrates containing heavy isotopes are often slower than those containing light isotopes. This can yield products with  $\delta$  values different from those of the substrates. The size of such a fractionation may be characteristic of the reaction. Or, a change in  $\delta$  may reflect the mixing of two or more pools with different isotopic compositions (see, e.g., Mariotti et al., 1981). Such processes may leave an isotopic 'legacy' or 'footprint' in substances in which they have occurred. Variations in  $\delta$  values can provide clues to the process(es) causing those variations after the processes have happened, provided that further isotopic changes are minimal. Such clues provide circumstantial — rather than definitive — evidence for the occurrence of specific processes (e.g., Koba et al., 1997). Nevertheless, this approach has at least three distinct advantages over others: unlike the addition of isotopically enriched tracers, it does not disturb the normal chemistry of a system; it can report on many processes which may be operating simultaneously, or have operated, in a system; and  $\delta$  values can be tested against rules which constrain them within predictable limits (see e.g., Mariotti et al., 1981).

Here we describe the use of  $\delta^{15}\text{N}$  to explore the possibility that elevated  $[\text{CO}_2]$  can, via plants, influence soil N transformations. We grew plants exposed to different  $[\text{CO}_2]$  and availabilities of soil water, in a soil fertilised with only one major N source,  $\text{NO}_3^-$ . We aimed to detect effects of plants on soil N by measuring the  $\delta^{15}\text{N}$  values of residual  $\text{NO}_3^-$  in planted soil, and comparing the latter with the  $\delta^{15}\text{N}$  values of  $\text{NO}_3^-$  in unplanted soil. Then, by comparing experimentally-determined  $\delta^{15}\text{N}$  values with known  $^{15}\text{N}/^{14}\text{N}$  effects for specific processes (e.g., N assimilation, denitrification, nitrification), applying  $^{15}\text{N}/^{14}\text{N}$  fractionation theory and calculating isotope mass balances, we aimed to identify likely processes responsible for observed  $\delta^{15}\text{N}$  patterns in relation to the different environmental variables.

## 2. Materials and methods

### 2.1. Soil and plants

Soil, an acid clay loam weathered from basalt, was collected from bushland at Mt. Tomah, NSW, Australia. Its pH (in water) was 4.7. The C-to-N mass ratio of the soil was  $17.9 \pm 1.85$ . The soil's total N had a mean  $\delta^{15}\text{N}$  value of  $+6.7 \pm 0.35\%$ , as determined by continuous-flow isotope ratio mass spectrometry (CF-IRMS; see below).

Fertiliser and lime were mixed with sieved soil (5 mm mesh) in the following amounts ( $\text{g kg}^{-1}$  dry soil):  $\text{Ca}(\text{NO}_3)_2$ , 2.64 (=0.45 g N,  $\delta^{15}\text{N} = -1.6 \pm 0.1\%$ );  $\text{KNO}_3$ , 0.82 (=0.11 g N,  $\delta^{15}\text{N} = +0.8 \pm 0.2\%$ );  $\text{CaHPO}_4$ , 1.86;  $\text{K}_2\text{SO}_4$ , 0.90;  $\text{MgCO}_3$ , 3.02;  $\text{CaCO}_3$ , 4.18;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.03;  $\text{H}_3\text{BO}_3$ , 0.04. The soil's pH increased to 6.5 as a result. Each pot received 3.1 g  $\text{NO}_3^-$ -N whose net  $\delta^{15}\text{N}$  value was  $-1.1 \pm 0.1\%$ .

Seven kg of moist, fertilised soil ( $\equiv 5.5$  kg dry soil at a mass wetness of  $0.28 \text{ g g}^{-1}$  dry soil) were packed into each of 96 cylindrical pots, 15 cm dia, 40 cm tall. After packing, water was added to the soil in each pot to give a mass wetness of  $0.4 \text{ g g}^{-1}$ , which was close to its field capacity. The base of each pot was perforated, but no significant drainage occurred. Each pot was weighed and allocated randomly to one of two controlled environment chambers (see below) and kept uncovered for three weeks before sowing. The mean concentration of  $\text{NO}_3^-$  in the soil solution ( $[\text{NO}_3^-]$ ) immediately before sowing was  $42 \pm 7 \text{ mM}$  ( $\equiv 1.2 \text{ g NO}_3^-$ -N per pot) and this had a  $\delta^{15}\text{N}$  value of  $-2.4 \pm 0.5\%$ . The concentration of extractable  $\text{NH}_4^+$  was always  $< 0.1 \text{ mM}$ .  $\text{NO}_3^-$  was, therefore, the major form of plant-available N in this soil.

Approximately 25 caryopses of the fast-growing perennial C<sub>4</sub> grass *Panicum coloratum* L. cv. Bambatsi

(Makarikari grass: Lloyd, 1981) were sown directly onto the soil in 40 pots in each of the two chambers. The remaining eight pots in each chamber were left unplanted. Sowing date was designated as day 0 of the experiment. Seedlings emerged 3 d later. Five days after emergence (day 8), seedlings were thinned to 6 per pot.

## 2.2. Treatments

Two environmental factors were varied: atmospheric  $[\text{CO}_2]$  and soil wetness.  $[\text{CO}_2]$  was maintained at  $350 \mu\text{l l}^{-1}$  ('ambient') in one chamber and at  $1000 \mu\text{l l}^{-1}$  ('elevated') in the other, as described previously (Ghannoum et al., 1997). Soil wetness was controlled by supplying water daily ('frequent' watering) to half the pots in each chamber (20 planted and 4 unplanted pots) or every 3–7 d to the others ('infrequent' watering): see Van Vuuren et al. (1997). Randomly selected pots in each treatment (including the unplanted controls) were weighed daily to determine water losses. It was assumed that the losses from weighed pots reflected those from other pots in the same treatment. Losses were replaced by manual watering to restore the soil wetness to  $0.4 \text{ g g}^{-1}$  dry soil in each treatment. This method was found to be inaccurate until daily water loss exceeded ca. 10 g per pot, and this occurred after 16 d.

Planted pots were arranged in blocks within chambers to minimise position effects, each block containing five pots per watering treatment. Both chambers were maintained at  $25^\circ\text{C}$ , day,  $20^\circ\text{C}$  night. Photosynthetically active radiation was  $750 \mu\text{mol m}^{-2} \text{ s}^{-1}$  at pot height, and daylength 12 h. Relative humidity was  $>90\%$ .

## 2.3. Sampling and analyses

Five planted pots and one unplanted pot were sampled from each treatment 21, 28, 36 and 41 d after sowing. Plant material was bulked to form three samples — leaves, stems and roots — per pot. Plants remained vegetative throughout. Shoots were cut at soil level and divided into leaves and stems. Roots were removed manually from soil and washed. All plant material was weighed fresh and oven-dry.

Root-free soil from planted pots, and samples from unplanted controls, were weighed fresh and after oven-drying to estimate soil wetness at harvest. From each pot, a sub-sample of root-free soil (10 g fresh wt) was shaken for 1 h with 50 ml 2 M KCl solution to extract  $\text{NO}_3^-$  and  $\text{NH}_4^+$ .  $\text{NO}_3^-$  and  $\text{NH}_4^+$  concentrations in the KCl extracts were determined colorimetrically (Rand et al., 1976) using a flow-injection analyser (Flow Solution 111, Alpkem, NSW).

$\text{NO}_3^-$  in each extract was concentrated for  $\delta^{15}\text{N}$  analysis using a reduction — microdiffusion procedure. Extracts containing  $<50 \mu\text{g NO}_3\text{-N}$  (the minimum weight of N for reliable  $\delta^{15}\text{N}$  determination by CF-IRMS: Scrimgeour and Robinson, 1999) were discarded. For each remaining extract, that volume which contained  $50 \mu\text{g NO}_3\text{-N}$  (as determined colorimetrically) was shaken with MgO and Devarda's alloy for 72 h in a sealed container to quantitatively reduce  $\text{NO}_3^-$  to  $\text{NH}_3$ .  $\text{KNO}_3$  standards containing  $50 \mu\text{g NO}_3\text{-N}$  were treated similarly. Liberated  $\text{NH}_3$  was trapped as  $\text{NH}_4^+$  on an acidic glass fibre trap sealed in polytetrafluoroethylene (PTFE) tape floated on the surface of each extract (Sørensen and Jensen, 1991; Stark and Hart, 1996; Holdus and Wishart, pers. comm.). The PTFE tape was removed from each trap and the latter dried in a desiccator for five days. The  $\delta^{15}\text{N}$  value of each trap, i.e., the  $\delta^{15}\text{N}$  of soil  $\text{NO}_3^-$  ( $\delta_{\text{nitrate}}$ ), was determined by CF-IRMS (Europa Scientific, Crewe, U.K.). Analytical precision (SD) was  $<1\%$  for  $\text{NO}_3^-$  standards.

The Devarda's alloy–MgO reduction is not specific to  $\text{NO}_3^-$ . Other nitrogenous anions may also be reduced to volatile derivatives and so contaminate N trapped on the glass fibre. The resulting  $\delta^{15}\text{N}$  of the glass fibre is then not necessarily  $\delta_{\text{nitrate}}$  (Scrimgeour and Handley, pers. comm.). To account for this, only those samples in which recoveries of N (as measured by CF-IRMS) from the reduction — microdiffusion procedure were within 10% of those expected from colorimetric analysis (which was specific for  $\text{NO}_3^-$ ), and were from runs in which recoveries of standards were quantitative, were accepted. Quantitative recovery was essential to avoid generating isotopic artefacts during the microdiffusion step. If some  $\text{NH}_4^+$  in solution was not volatilised, or some volatilised  $\text{NH}_3$  not trapped on the glass fibre, the  $\delta^{15}\text{N}$  value of any trapped N could have been very different from the true  $\delta_{\text{nitrate}}$ . Fractionations of up to 34‰ can occur when  $\text{NH}_4^+ \leftrightarrow \text{NH}_3$  (see Handley and Raven, 1992), so artefacts may easily occur. These stringent criteria led to the acceptance of only 33  $\delta_{\text{nitrate}}$  data out of a possible 98. Published data for  $\delta_{\text{nitrate}}$  obtained using the Devarda's alloy–MgO reduction which have not been screened for isotopic artefacts should be treated with caution.

## 2.4. Data analysis

The amounts of water added to each pot were converted into amounts of water used by the plants by deducting amounts lost by evaporation from unplanted pots. Cumulative water use was calculated by summing the amounts of water used between consecutive waterings. Estimates of variation in water use among replicates could not be calculated. Water use was estimated

as water lost by only one selected pot per treatment (after adjusting for evaporation from unplanted pots). The dry weight of plant material in each pot was obtained by summing the dry weights of leaves, stems and roots.

Values of  $\delta_{\text{nitrate}}$  were converted to  $^{15}\text{N}/^{14}\text{N}$  ratios (using Eq. (1)) for statistical analysis (to allow log-transformation of negative  $\delta_{\text{nitrate}}$  values). Treatment, harvest and block effects were tested by analysis of variance of log-transformed data (to normalise variances) of soil wetness, plant and root dry weight,  $[\text{NO}_3^-]$  and  $^{15}\text{N}/^{14}\text{N}$  ratio of soil  $\text{NO}_3^-$ . Differences between means were compared using Scheffé's post-hoc test (Sokal and Rohlf, 1995, p. 254). Data quoted in the text are back-transformed means and 95% confidence limits.

The net  $^{15}\text{N}/^{14}\text{N}$  fractionation factor ( $\alpha$ ) describing changes in the  $^{15}\text{N}/^{14}\text{N}$  ratio of soil  $\text{NO}_3^-$  in relation to its concentration in the soil solution was estimated using the Rayleigh equation (Mariotti et al., 1981; Hoefs, 1987, p. 11):

$$R^* = f^{(1/\alpha)-1} \quad (2)$$

where  $R^*$  is the  $^{15}\text{N}/^{14}\text{N}$  ratio of  $\text{NO}_3^-$  as a fraction of the initial  $^{15}\text{N}/^{14}\text{N}$  ratio,  $f$  is the fraction of the initial  $\text{NO}_3^-$  remaining in the soil at the time of measurement.  $\alpha$  was estimated from a zero-intercept regression of  $\ln R^*$  on  $\ln f$ . From Eq. (2), the slope of this regression was, by definition,  $(1/\alpha)-1$ , from which a value for  $\alpha$  was obtained (see, e.g., Mariotti et al., 1981; Koba et al., 1997).  $\alpha$  was calculated separately for each treatment. A convenient transformation of  $\alpha$  onto the ‰ scale allowed direct comparison with  $\delta$  values:

$$\varepsilon = (\alpha - 1) \times 1000 \quad (3)$$

Positive values of  $\varepsilon$  denote a discrimination against  $^{15}\text{N}$  in a substrate compared with a product.

### 3. Results

#### 3.1. Plant growth and water use

Treatments influenced total dry matter production at only the last two harvests (Fig. 1). By the end of the experiment, plants grown in ambient  $[\text{CO}_2]$  were significantly smaller when watered infrequently ( $p \leq 0.001$ ). Watering frequency had no effect on total dry matter production at elevated  $[\text{CO}_2]$ . With frequent watering, there was no difference in the final dry weight of whole plants grown in ambient or elevated  $[\text{CO}_2]$ . The allocation of dry matter to roots was 15–40% of total dry matter production (Fig. 1).

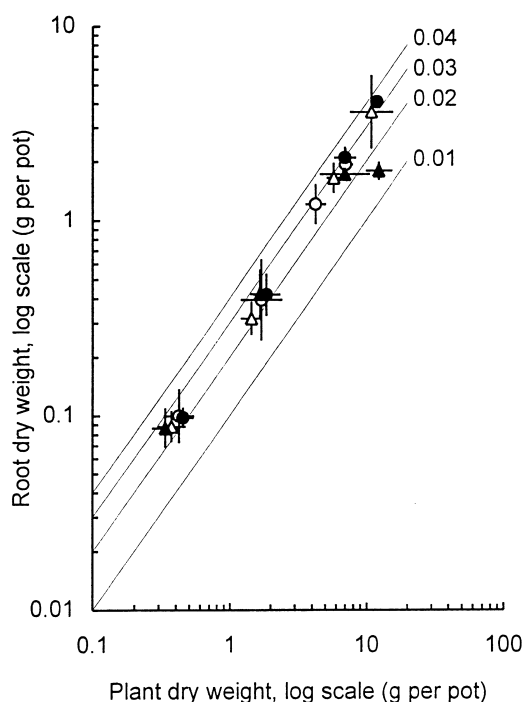


Fig. 1. Root dry weight and total dry weight, per pot. Symbols are means  $\pm 95\%$  confidence limits. Open symbols, atmospheric  $[\text{CO}_2] = 350 \mu\text{l l}^{-1}$ ; closed symbols,  $[\text{CO}_2] = 1000 \mu\text{l l}^{-1}$ ; triangles, frequently watered; circles, infrequently watered. Each sloping line is a constant root dry weight per total dry weight, i.e., root weight ratio, with a value as shown. The values of both variates increased with time, i.e., those sampled on day 21 are on the lower left, those on day 41, on the top right of the graph.

Differences among treatments in allocation to roots generally followed those for total dry matter.

For a given plant dry weight, cumulative water use was always smaller in elevated  $[\text{CO}_2]$  compared with ambient  $[\text{CO}_2]$  (Fig. 2). Differences in water use between frequently and infrequently watered plants were negligible at elevated  $[\text{CO}_2]$ . In ambient  $[\text{CO}_2]$ , infrequently watered plants used slightly less water. During the experiment, the amount of water used  $\text{g}^{-1}$  plant dry weight produced fell from 600–1000 to 200–400  $\text{g g}^{-1}$ .

#### 3.2. $[\text{NO}_3^-]$ and $\delta_{\text{nitrate}}$

Within treatments,  $[\text{NO}_3^-]$  and  $\delta_{\text{nitrate}}$  were not significantly different among harvests, so these data were averaged across harvests. In unplanted soil,  $[\text{NO}_3^-]$  was  $42 \pm 13 \text{ mM}$ , regardless of  $[\text{CO}_2]$  or watering treatment (Fig. 3), the same as it was initially. Likewise,  $\delta_{\text{nitrate}}$  in unplanted soil was the same as the initial  $\delta_{\text{nitrate}}$  of  $-2.4\text{‰}$ .

In planted soil, however, decreases in  $[\text{NO}_3^-]$  occurred and were associated with significant  $^{15}\text{N}$  enrichments in residual soil  $\text{NO}_3^-$ . These effects were greatest in the frequently watered, ambient and elev-

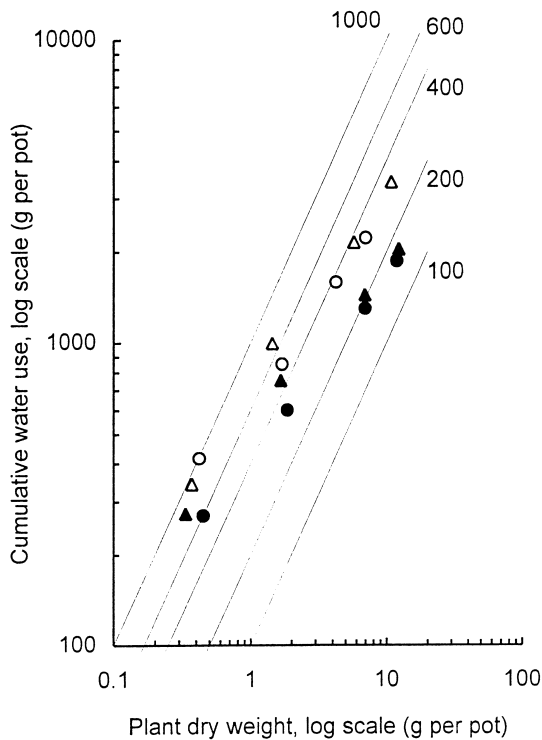


Fig. 2. Cumulative water use and total plant dry weight, per pot. Symbols are means, and are as defined in the legend to Fig. 1. 95% confidence limits for dry weight are as shown in Fig. 1, but have been omitted here for clarity. None could be calculated for water use. Water use was estimated as water lost by one selected pot per treatment (after adjusting for evaporation from unplanted pots). As the same amount of water was added to each pot in a treatment, there was no variation in water use when calculated on that basis. Each sloping line is a constant water use per total dry weight, with a value (in g [water] g<sup>-1</sup> [dry weight]) as shown. The values of both variates increased with time, i.e., those sampled on day 21 are on the lower left of the graph, those on day 41, on the top right.

ated [CO<sub>2</sub>] treatments where [NO<sub>3</sub><sup>-</sup>] fell to < 3 mM and  $\delta_{\text{nitrate}}$  increased to  $+9.5 \pm 2.2\%$ . [NO<sub>3</sub><sup>-</sup>] decrease and <sup>15</sup>N enrichment in soil NO<sub>3</sub><sup>-</sup> were slightly smaller in the infrequently watered, elevated [CO<sub>2</sub>] treatment, and least of all in the infrequently watered, ambient [CO<sub>2</sub>] treatment. Even in the latter,  $\delta_{\text{nitrate}}$  changed significantly from its initial value ( $-2.4\%$ ) to  $+1.4 \pm 2.0\%$ .

Values of  $\epsilon$  (Eq. (3)) were not significantly different among treatments (including the unplanted control) and ranged from  $+2.7$  to  $+5.8\%$ . When averaged across all treatments, the mean value of  $\epsilon$  was  $+4.0 \pm 0.6\%$ , as derived from all the  $\delta_{\text{nitrate}}$  data ( $n = 33$ ,  $r = -0.843$ ,  $p < 0.001$ ).

$\delta_{\text{nitrate}}$  was correlated with soil wetness before watering as averaged for all harvests ( $r = +0.960$ ,  $p < 0.01$ ; Fig. 4). The smallest change in  $\delta_{\text{nitrate}}$  occurred in what was, on average, the driest soil, in the infrequently watered, ambient [CO<sub>2</sub>] treatment. The difference in soil wetness between the infrequently watered

treatments is explained by the smaller water use under elevated [CO<sub>2</sub>] (Fig. 1).

#### 4. Discussion

Our results can be summarised as follows. Soil NO<sub>3</sub><sup>-</sup> became <sup>15</sup>N-enriched during the experiment but only in the presence of plants. The degree of enrichment depended on treatment. When frequently watered, the <sup>15</sup>N-enrichment of NO<sub>3</sub><sup>-</sup> was large, whether plants were grown in ambient or elevated [CO<sub>2</sub>]. [NO<sub>3</sub><sup>-</sup>] was correspondingly decreased. When infrequently watered, a large <sup>15</sup>N-enrichment of NO<sub>3</sub><sup>-</sup> occurred only under elevated [CO<sub>2</sub>]; in ambient [CO<sub>2</sub>], infrequent watering was associated with a smaller, but still significant, enrichment and with a smaller decrease in [NO<sub>3</sub><sup>-</sup>]. The most significant changes in [NO<sub>3</sub><sup>-</sup>] and  $\delta_{\text{nitrate}}$  occurred before the first harvest. To explain  $\delta^{15}\text{N}$  patterns in terms of underlying processes, lengthy and detailed arguments are inevitable as the issues involved are

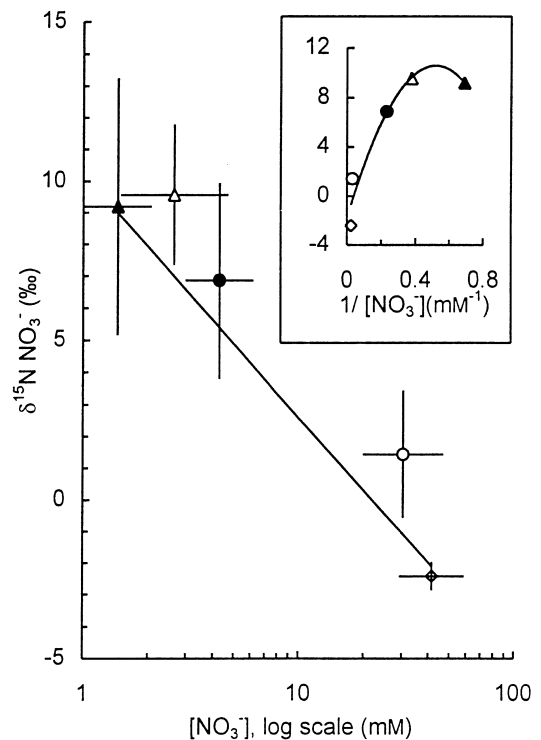


Fig. 3.  $\delta^{15}\text{N}$  of soil NO<sub>3</sub><sup>-</sup> and the concentration of NO<sub>3</sub><sup>-</sup> in soil solution. Symbols are as defined in the legend to Fig. 1, with the addition of: diamond, unplanted pots, all [CO<sub>2</sub>] and watering treatments. Data are averages for the four harvests as no significant differences were found for either variate among harvests. The linear regression is  $y = -0.2773x + 9.3833$  ( $r = -0.986$ ,  $p < 0.01$ ) and is characteristic of a Rayleigh process (Mariotti et al., 1988). Inset: The same data plotted as  $\delta^{15}\text{N}$  vs.  $[\text{NO}_3^-]^{-1}$ . The regression  $y = -46.42x^2 + 48.03x - 1.851$  ( $r = +0.972$ ,  $p < 0.01$ ) indicates that the relation is not linear and, therefore, unlikely to result from a mixing process (cf. Fig. 5 in Mariotti et al., 1988).

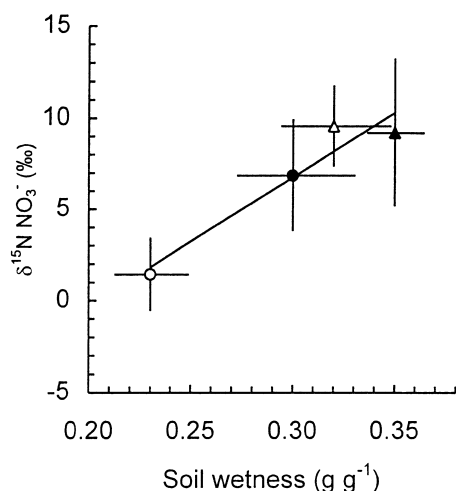


Fig. 4.  $\delta^{15}\text{N}$  of  $\text{NO}_3^-$  and mass wetness of soil at harvest. Symbols are means for all harvests, and are as defined in the legend to Fig. 1. The regression  $y = 70.6x - 14.42$  ( $r = +0.960$ ) is significant at  $p < 0.01$ .

complex (e.g., Mariotti et al., 1988). And, because the same  $\delta^{15}\text{N}$  data may often be interpreted in alternative ways, it is important that all possibilities are examined critically to assess which provide(s) the most likely explanations for the data. We begin with denitrification.

#### 4.1. Denitrification

Denitrification — an anaerobic process — is stimulated by hypoxia, other things being equal and, sometimes, by plants (Smith and Tiedje, 1979; Klemetsson et al., 1987; Bakken, 1988). Denitrification in the field is episodic and localised, being restricted to times when and places where  $\text{NO}_3^-$  and C are readily available and  $\text{O}_2$  is not.  $\text{N}_2\text{O}$  derived from denitrification is often further reduced to  $\text{N}_2$  as  $[\text{O}_2]$  approaches zero. Denitrification discriminates against  $^{15}\text{N}$  in  $\text{NO}_3^-$ . Provided that some  $\text{NO}_3^-$  remains unreduced, denitrification results in  $\delta_{\text{nitrate}}$  becoming more positive (Chien et al., 1977).  $\epsilon$  values for denitrification from unplanted, waterlogged soils amended with glucose range from +4‰ (Chien et al., 1977) to +33‰ (Mariotti et al., 1982a).

Had significant denitrification occurred in the 3 weeks between the addition of fertiliser and sowing,  $\delta_{\text{nitrate}}$  would have become more positive than the  $\delta^{15}\text{N}$  value of fertiliser  $\text{NO}_3^-$ , and  $[\text{NO}_3^-]$  would have decreased. There was a decrease (from 3.1 to 1.2 g) in the amount of  $\text{NO}_3\text{-N}$  in each pot, consistent with a denitrification loss of  $\text{NO}_3^-$ . This was not, however, accompanied by any  $^{15}\text{N}$ -enrichment in residual  $\text{NO}_3^-$ . Processes other than denitrification (e.g., immobilisation) were more likely to have caused the change in  $[\text{NO}_3^-]$  in unplanted soil before 0 d.

There was, however, evidence for denitrification having taken place after 0 d, particularly in planted pots. Five pieces of evidence support this possibility. First, there was a  $^{15}\text{N}$ -enrichment of up to 12‰ in soil  $\text{NO}_3^-$  compared with the initial  $\delta_{\text{nitrate}}$ . This occurred with an  $\epsilon$  value of +4‰, at the lower end of the range expected for denitrification. Turner et al. (1987) found that the  $\delta^{15}\text{N}$  value of  $\text{NO}_3^- + \text{NH}_4^+$  extracted from an Australian arable soil became up to 11‰ more positive as  $[\text{NO}_3^-]$  and  $[\text{NH}_4^+]$  decreased. Soil inorganic N became most  $^{15}\text{N}$ -enriched in planted soil, as in our study.  $\epsilon$  values between +6 and +10‰ can be calculated from Turner *et al.*'s data. Field studies in which denitrification was suspected of  $^{15}\text{N}$ -enriching residual  $\text{NO}_3^-$  have produced  $\epsilon$  values of +5 to +6‰ (Mariotti et al., 1988; Koba et al., 1997).

Second, the linear relation between  $\ln[\text{NO}_3^-]$  and  $\delta_{\text{nitrate}}$  (Fig. 3) was characteristic of a Rayleigh process (Mariotti et al., 1982a). Rayleigh kinetics apply strictly to isotopic changes in 'closed' systems, i.e., those with a finite supply of substrate which is consumed as a product accumulates. A Rayleigh equation can describe isotopic fractionations in an 'open' system if one rate-limiting process dominates, as it often does in biological systems (O'Leary, 1988). So, for example, Mariotti et al. (1988) used Rayleigh theory to show that denitrification, and not mixing, probably dominated isotopic changes in  $\text{NO}_3^-$  in a French aquifer. Koba et al. (1997) used a similar approach to infer the occurrence of intermittent denitrification in Japanese forest soils. Fig. 3 suggests that denitrification occurred in our experiment. Had significant mixing of  $\text{NO}_3^-$  pools occurred (e.g., as a result of nitrification: see below), the relation between  $[\text{NO}_3^-]$  and  $\delta_{\text{nitrate}}$  (Fig. 3, inset) would have been linear, not curved (Mariotti et al., 1988).

Third, the denitrification rates required to produce the observed changes in  $\delta_{\text{nitrate}}$  were plausible. We know the amount of  $\text{NO}_3^-$  initially available and that present at the end of the experiment. We also know  $\delta_{\text{nitrate}}$  (Fig. 3), and estimated a mean value for  $\epsilon$  (+4‰, i.e.,  $\alpha = 1.004$ ). Inserting these data into Eq. (2) and solving for  $f$ , we obtain  $1 - f$ , the fraction of the initial  $\text{NO}_3^-$  lost from the system, having first allowed for  $\text{NO}_3^-$  uptake by the plants. The fraction  $1 - f$  was 0.61–0.95, equivalent to mean daily denitrification rates of 5.0–6.4 kg N ha $^{-1}$ . These rates are large compared with those measured in cool, temperate soils. Addiscott et al. (1991, p. 50) quoted peak daily rates of 30 kg ha $^{-1}$ , and a rate one-tenth of this as more usual.

Fourth, denitrification is stimulated by hypoxia. If denitrification did cause the observed changes in  $\delta_{\text{nitrate}}$ , we would expect a positive correlation between soil wetness and  $\delta_{\text{nitrate}}$ , and this was found (Fig. 4).

Fifth, the effect on  $\delta_{\text{nitrate}}$  was greatest in soil whose  $[\text{NO}_3^-]$  had been decreased most and which supported the largest plants. It was least in unplanted soil. Stimulations of denitrification in  $\text{NO}_3^-$ -rich, planted soil have been attributed to the provision of root-derived C (Smith and Tiedje, 1979) or to  $\text{O}_2$  depletion (Klemetsson et al., 1987). It would be reasonable, therefore, to expect a positive correlation between root mass and the change in  $\delta_{\text{nitrate}}$ . There was no overall correlation between root dry weight and  $\delta_{\text{nitrate}}$  in our experiment ( $r = -0.10$ ,  $p < 0.05$ ). But there was a difference in root growth between the infrequently watered treatments; root systems under elevated atmospheric  $[\text{CO}_2]$  were ultimately larger than in ambient  $[\text{CO}_2]$  ( $p < 0.01$ ).

The  $\epsilon$  value estimated for unplanted soil was the same as that in the planted treatments. This might suggest that whatever process(es) changed  $\delta_{\text{nitrate}}$  in the planted soils also changed it in unplanted, so eliminating *P. coloratum* as an agent of change. However, there was no statistical change in  $[\text{NO}_3^-]$  in unplanted soil compared with the large changes in the planted treatments (Fig. 3). This illustrates that  $\delta^{15}\text{N}$  data alone cannot always provide a clear picture of N transformations: they must, ideally, be combined with information about the amounts of N involved in those transformations.

It was striking that we found no significant differences in  $[\text{NO}_3^-]$  and  $\delta_{\text{nitrate}}$  after the first harvest. The major changes in  $[\text{NO}_3^-]$  and  $\delta_{\text{nitrate}}$  occurred in planted soil between 0 and 21 d. Despite the evidence in the preceding paragraphs, it might be argued that the effects on  $\delta_{\text{nitrate}}$  could not have been related to the presence of *P. coloratum* because the largest effects occurred when the plants were relatively small. This does argue that whatever effect *P. coloratum* did have on soil N early in the experiment, it was probably not related to the provision of root-derived C. Assuming a root dry weight of 30 mg per pot at 14 d (extrapolating back from the measurements for 21 d in Fig. 1), a root [C] of 40% and that 10% of root C was transferred into the soil, that C transfer would amount to ca. 0.1 mmol. About 2 mol C are needed to reduce 1 mol  $\text{NO}_3^-$ . So, root-derived C could have reduced ca. 0.05 mmol  $\text{NO}_3^-$  by 14 d. This is equivalent to a daily, plant-induced denitrification rate of only  $< 0.11 \text{ kg ha}^{-1}$ . (This is an under-estimate. It would have been slightly larger had exponential growth of the root system and cumulative inputs of root-derived C into the soil been assumed). The  $\delta_{\text{nitrate}}$  data are consistent with much faster denitrification rates of  $> 5 \text{ kg ha}^{-1} \text{ d}^{-1}$  (see above). Even if specific rates of plant-to-soil C transfer were increased by elevated atmospheric  $[\text{CO}_2]$  (and there is little evidence that this happens: see Darrah, 1996), changes in  $\delta_{\text{nitrate}}$  were unlikely to have been caused primarily by the pro-

vision of C from roots. Nor was the effect on  $\delta_{\text{nitrate}}$  likely to have been caused by a stimulation by root-derived C of microbial respiration in general. Aerobic respiration consumes approximately equimolar amounts of C and  $\text{O}_2$ . Root-derived C could have driven the consumption of only ca. 0.1 mmol of  $\text{O}_2$  up to 14 d. This is  $< 1\%$  of the  $\text{O}_2$  in the soil's pore volume (assuming a gas-filled porosity of 0.2), and so would have had a negligible influence on soil  $[\text{O}_2]$ .

The only other possible influence of plants on denitrification was that on soil  $[\text{O}_2]$ , through increased root respiration ( $\text{O}_2$  consumption), decreased water uptake (diffusive impedance), or both. How likely were these effects early in our experiment? The effect of elevated  $[\text{CO}_2]$  on the growth of *P. coloratum* root systems was not apparent at the first two harvests (Fig. 1). Therefore, we would not expect roots of plants grown in elevated  $[\text{CO}_2]$  to have consumed much more  $\text{O}_2$  per pot early in the experiment. Nor is there any evidence that elevated  $[\text{CO}_2]$  alters specific rates of root respiration (Lambers et al., 1996). Unfortunately, we do not have reliable data for water use by *P. coloratum* during (what turned out to be) the critical period between emergence (3 d) and the first harvest (21 d). We cannot say whether the treatments influenced *P. coloratum*'s water use during that period. However, there is evidence that water use by even small seedlings of  $\text{C}_4$  species is significantly decreased by elevated atmospheric  $[\text{CO}_2]$ . For example, when Samarakoon and Gifford (1996) grew *Zea mays* (maize) at an atmospheric  $[\text{CO}_2]$  of  $717 \mu\text{l l}^{-1}$ , its water use was restricted to 60–80% of that at  $362 \mu\text{l l}^{-1}$ . That effect was apparent after only 12 d growth in irrigated or non-irrigated soil and would have established different soil wetnesses, especially in the topsoil where roots would have been most concentrated. It is possible, therefore, that a restricted use of water by *P. coloratum* under elevated atmospheric  $[\text{CO}_2]$  produced soil moisture conditions conducive to denitrification before 21 d. Because  $\text{NO}_3^-$  was plentiful in the soil at the start of the experiment, it is then that large amounts of denitrification were most likely, other things being equal.

Two pieces of recent, independent evidence, obtained using different techniques and materials, corroborate our argument for a positive, plant-mediated effect of elevated atmospheric  $[\text{CO}_2]$  on denitrification. First, Ineson et al. (1998) measured fluxes of  $\text{N}_2\text{O}$  from beneath a *Lolium perenne* (perennial rye-grass) sward grown in ambient or elevated  $[\text{CO}_2]$ . The  $\text{N}_2\text{O}$  flux was 27% greater at an atmospheric  $[\text{CO}_2]$  of  $600 \mu\text{l l}^{-1}$  compared with that at  $350 \mu\text{l l}^{-1}$ . This effect was attributed to the provision of extra root-derived C under elevated  $[\text{CO}_2]$  (although Ineson et al. (1998) presented no arguments to substantiate that possibility or to eliminate others). Second, Arnone and Bohlen (1998) measured doubled fluxes of  $\text{N}_2\text{O}$  from intact

grassland monoliths exposed to elevated  $[\text{CO}_2]$ . They attributed this specifically to an effect on soil wetness mediated by plants' responses to  $[\text{CO}_2]$ .

Elevated atmospheric  $[\text{CO}_2]$  will not always stimulate denitrification. In contrast to the results discussed above, Hungate et al. (1997c) found no effect of elevated  $[\text{CO}_2]$  on short-term (<9 d) denitrification rate in a Californian grassland. Cause-and-effect associations between elevated  $[\text{CO}_2]$  and denitrification may well be site-dependent.

#### 4.2. Nitrification

Nitrification — an aerobic process — is depressed by hypoxia, and inhibited by anoxia. Nitrification rates are usually zero in waterlogged soil, but some production of  $\text{NO}_3^-$  may occur in oxic sites within otherwise wet soil (Patrick, 1982). It is also depressed in very dry soil (Schmidt, 1982) and by the presence of plants (Wheatley et al., 1990, 1997; Verhagen et al., 1994). Nitrification, like denitrification, produces  $\text{N}_2\text{O}$ , but by a different biochemical pathway.  $\text{NO}_3^-$  produced by nitrification may be depleted in  $^{15}\text{N}$  compared with  $\text{NH}_4^+$  from which it originated (see Yoneyama, 1996). However,  $\text{NH}_4^+$  produced during mineralisation may be  $^{15}\text{N}$ -enriched compared with the organic N from which it originated (Shi et al., 1992). (A qualification must be added. It is that all published  $\delta^{15}\text{N}$  data for soil inorganic N are potentially in error. None have been obtained with analytical methods specific for  $\text{NH}_4^+$  or  $\text{NO}_3^-$  nor screened rigorously for isotopic artefacts: see above). The  $\delta^{15}\text{N}$  value of  $\text{NO}_3^-$  produced by nitrification may, therefore, be more positive, more negative or the same as that of the soil's total N. It is impossible to predict which applies to any particular soil or set of circumstances.

We note, first, that addition of  $\text{NO}_3^-$  fertiliser with a  $\delta^{15}\text{N}$  value of  $-1.1\text{‰}$  resulted, 3 weeks later, in the soil's  $\delta_{\text{nitrate}}$  becoming slightly more negative than this ( $-2.4\text{‰}$ ). This does not suggest that, in this soil, nitrification produced  $\text{NO}_3^-$  more enriched in  $^{15}\text{N}$  compared with the soil's total N ( $+6.7\text{‰}$ ). Had it done,  $\delta_{\text{nitrate}}$  should have become more positive than the  $\delta^{15}\text{N}$  value of the fertiliser.

Second, suppose that the increase in  $\delta_{\text{nitrate}}$  in planted soil between 0 and 21 d was caused by  $^{15}\text{N}$ -enriched  $\text{NO}_3^-$  derived from nitrification mixing with fertiliser  $\text{NO}_3^-$ . How much  $\text{NO}_3^-$  must have been produced by nitrifiers to cause a change in  $\delta_{\text{nitrate}}$  from  $-2.4\text{‰}$  to  $+10\text{‰}$ ? This can be estimated from an isotopic mass balance. Suppose that  $\text{NO}_3^-$  produced by nitrification had a  $\delta^{15}\text{N}$  value of  $+20\text{‰}$  (i.e., some 13.3‰ more enriched than the soil's total N) and that  $x$  g were produced per pot between 0 and 21 d. This would have mixed with existing  $\text{NO}_3^-$  in the soil (1.2 g of  $\text{NO}_3\text{-N}$  per pot with a  $\delta^{15}\text{N}$  value of  $-2.4\text{‰}$ ). The

isotopic mass balance is:

$$10 = \frac{(-2.4 \times 1.2) + (20 \times x)}{1.2 + x} \quad (4)$$

from which a value of  $x = 1.5$  g  $\text{NO}_3\text{-N}$  can be calculated. This is equivalent to a  $\text{NO}_3\text{-N}$  production rate per unit dry soil of *c.*  $13 \text{ mg kg}^{-1} \text{ d}^{-1}$ , over twice as fast as the maximum rate quoted by Haynes (1986) for a variety of soil types. It would be even greater assuming a less generous (i.e., less positive)  $\delta^{15}\text{N}$  value for  $\text{NO}_3^-$  produced by nitrification.

Third, by 0 d, each pot contained only 1.2 g of  $\text{NO}_3\text{-N}$  even though 3.1 g had been added as fertiliser 3 weeks before. This implies net immobilisation of  $\text{NO}_3^-$ , rather than a net production. Although unusual in a soil with a C-to-N ratio  $<20$ , that is, nevertheless, what our analyses suggest. They do not suggest denitrification in that period, because, again,  $\delta_{\text{nitrate}}$  should have become more  $^{15}\text{N}$ -enriched. This did not happen until later (see above).

Fourth, there was no significant change in  $[\text{NO}_3^-]$  in unplanted soil, irrespective of watering frequency or atmospheric  $[\text{CO}_2]$ . Given that nitrification tends to be inhibited by the presence of plants, we would not expect nitrification to have been any greater in the planted soil nor to vary significantly with  $[\text{CO}_2]$ .

Fifth, the relation between  $[\text{NO}_3^-]^{-1}$  and  $\delta_{\text{nitrate}}$  was not that expected when isotopically distinct  $\text{NO}_3^-$  sources mix (Mariotti et al., 1988). If nitrification had been significant, changes in  $\delta_{\text{nitrate}}$  would have approximated a mixing model more than a fractionation model. There would then have been a linear relation between  $[\text{NO}_3^-]^{-1}$  and  $\delta_{\text{nitrate}}$ , not curved, as in Fig. 3 (inset). We conclude that nitrification was an unlikely explanation for the data in Fig. 3.

#### 4.3. Plant N uptake and assimilation

The uptake of  $\text{NO}_3^-$  by roots cannot explain the increase in  $\delta_{\text{nitrate}}$ . There is no experimental evidence for, nor any theoretical reason to expect, the transport of  $\text{NO}_3^-$  across membranes (as during uptake from the soil solution, for example) to cause  $^{15}\text{N}/^{14}\text{N}$  fractionations (Handley and Raven, 1992). In contrast, N assimilation can cause fractionations of  $^{15}\text{N}/^{14}\text{N}$  with  $\epsilon$  values up to  $+17\text{‰}$  (Mariotti et al., 1982b). Theoretically, at least, this and the internal mixing of plant N, can cause any  $\text{NO}_3^-$  which is effluxed from roots being more  $^{15}\text{N}$ -enriched than the  $\text{NO}_3^-$  originally assimilated, although this depends on many factors (Robinson et al., 1998). Once in the soil, this effluxed N could then mix with less- $^{15}\text{N}$ -enriched  $\text{NO}_3^-$ , so increasing the net  $\delta_{\text{nitrate}}$ . As we saw with Eq. (4), an input per pot of 1.5 g  $\text{NO}_3\text{-N}$  with a  $\delta^{15}\text{N}$  as large as  $+20\text{‰}$  would have been required to cause

the observed shift in  $\delta_{\text{nitrate}}$ . This is over five times as much N as was present in the largest *P. coloratum* plants at the final harvest (data not shown). Robinson et al.'s (1998) calculations imply that ca. 1% of assimilated  $\text{NO}_3^-$  being effluxed is more likely. For *P. coloratum* harvested at the end of our experiment, this would have amounted to only 1.8 mg  $\text{NO}_3\text{-N}$  per pot.

#### 4.4. $\text{NH}_3$ volatilisation

Because of the large potential discrimination (+34%) against  $^{15}\text{N}$  in  $\text{NH}_4^+$  when  $\text{NH}_4^+ \rightarrow \text{NH}_3$ , residual  $\text{NH}_4^+$  may become  $^{15}\text{N}$ -enriched if  $\text{NH}_3$  volatilisation occurs. Should any of that  $^{15}\text{N}$ -enriched  $\text{NH}_4^+$  then be nitrified, the resulting  $\text{NO}_3^-$  would also be enriched.  $\text{NH}_3$  volatilisation from soil was an unlikely explanation for the observed changes in  $\delta_{\text{nitrate}}$  for three reasons. First, little  $\text{NH}_4^+$  was measured in soil extracts. Second, had any  $\text{NH}_4^+$  been present in the soil whose pH was 6.5, only 0.2% of it would have been in the  $\text{NH}_3$  form at equilibrium (Freney et al., 1981). Third, even assuming  $\epsilon = +34\%$ , an isotope mass balance estimates that ca. 10–40% of the  $\text{NO}_3^-$  present initially in the soil would have had to be volatilised (having first been immobilised and re-mineralised as  $\text{NH}_4^+$ ) to account for the change in  $\delta_{\text{nitrate}}$ . These are unreasonably large fractions.

Volatilisation of N (as  $\text{NH}_3$ , amines,  $\text{NO}_x$  or HCN; Wetselaar and Farquhar, 1980) from leaves can also be discounted. Most volatile N losses from plants occur via stomata. Under elevated  $[\text{CO}_2]$ , stomatal conductances of  $C_4$  *Panicum* species are smaller than when under ambient  $[\text{CO}_2]$  (Ghannoum et al., 1997). We should, therefore, expect a smaller  $^{15}\text{N}$  enrichment in plants grown under elevated  $[\text{CO}_2]$  if they had lost N volatiles through their stomata, compared with plants grown in ambient  $[\text{CO}_2]$ . This did not happen. Plants grown at  $1000 \mu\text{l l}^{-1}$  were no less enriched in  $^{15}\text{N}$  than those grown at  $350 \mu\text{l l}^{-1}$  (data not shown). Moreover, to achieve the required isotopic mass balance, unrealistically large amounts of N would need to be volatilised, as much N as was measured in whole plants at the end of the experiment, even if  $\epsilon$  was assumed to be +34%. Bergersen et al. (1988), who also observed  $^{15}\text{N}$ -enrichments in the rooting medium, detected no volatile N emissions from their *Glycine max* (soybean) plants.

#### 4.5. $\text{NO}_3^-$ leaching

Another potential route of N loss was leaching. Leaching *per se* does not discriminate against  $^{15}\text{N}$ . It could not have caused the observed increase in  $\delta^{15}\text{N}$  of residual  $\text{NO}_3^-$ . The occurrence of  $^{15}\text{N}$ -enriched  $\text{NO}_3^-$  several metres below the surface of soil through which  $\text{NO}_3^-$  is being leached has been attributed to denitrifi-

cation at those depths (Herbel and Spalding, 1993), rather than to any preferential retention in soil of  $^{14}\text{NO}_3^-$ .

## 5. Conclusions

The balance of evidence favours denitrification, and none of the alternative processes, as the most likely explanation for the changes in  $\delta_{\text{nitrate}}$  in planted soil. Elevated atmospheric  $[\text{CO}_2]$  reduced the water use by *P. coloratum* seedlings. This left soil wetter between irrigations. If the soil's  $[\text{O}_2]$  then became more depleted in the wetter soils, the large amounts of  $\text{NO}_3^-$  present would have been subject to denitrification, leading to the observed increase in  $\delta_{\text{nitrate}}$ . The provision of more root-derived C as microbial substrates under elevated atmospheric  $[\text{CO}_2]$  was probably small.

If atmospheric  $[\text{CO}_2]$  influences soil  $[\text{O}_2]$  in this way, a positive, plant-mediated effect of  $[\text{CO}_2]$  on the soil-to-atmosphere flux of  $\text{N}_2\text{O}$  derived from denitrification (but not nitrification) is possible. Should that flux be significant at a global scale, the resulting absorption of infra-red radiation by atmospheric  $\text{N}_2\text{O}$  could exacerbate the enhanced greenhouse effect. As the latter is a product of, among other things, elevated atmospheric  $[\text{CO}_2]$ , there is an opportunity for a positive, plant-mediated feedback to occur. This possibility requires explicit testing.

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