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MEASURING THE CONTRIBUTIONS OF NITRIFICATION AND DENITRIFICATION TO THE FLUX OF NITROUS OXIDE FROM SOIL

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Summary—The flux of N_2O from soil can be due to nitrification or denitrification. Since aerobic and anaerobic microsites can develop within the same soil aggregate, nitrification and denitrification could be occurring at the same time. The contribution of nitrification and denitrification to the flux of N_2O can be studied by differentially ^{15}N-labelling the NO_3^- and NH_4^+ pools in soils. By periodically measuring and comparing the enrichments of the N_2O, NH_4^+ and NO_3^- pools, the relative importance of the two processes can be quantified. The conclusions are based on calculations which assume that the ^{15}N atom fractions of the nitrification and denitrification pools remain uniform throughout the incubation. The initial uniformity of the denitrification pool was tested by adding a nitrification-inhibitor, C_2H_2, at time zero and examining the ^{15}N-distribution of the accumulated N_2O at subsequent times. If the ^{15}N distribution in the N_2O is random it originated from one source, but if the ^{15}N distribution is non-random the N_2O originated from two or more sources. Two soil incubation experiments were conducted. In the first experiment fresh sieved soil was incubated over 10 days at 40, 50 and 60% moisture content with $(NH_2)_2CO$ (70 μmol N g^{-1}) and KNO_3 (14 μmol N g^{-1}) differentially labelled at 10 atom% excess ^{15}N. The headspace was sampled daily for N_2O before being refreshed with normal air. Every second day the sizes and enrichments of the NH_4^+ and NO_3^- pools were determined by destructive sampling. In the second experiment the assumption that the method of addition of label created only one denitrifying pool was tested by blocking nitrification with C_2H_2 (10 kPa). Fresh soil was incubated for three incubation times (6, 12 and 24 h) with differentially-labelled NH_4NO_3 (1.46 μmol N g^{-1}) enriched to 20 atom% excess ^{15}N, with glucose (42 and 83 μmol C g^{-1}) to promote denitrification. In the first experiment the enrichment of the N_2O did not match either the enrichment of the NH_4^+ or NO_3^- pools, showing that N_2O was being produced by nitrification and denitrification. Quantification of the fractional contributions of nitrification and denitrification showed that denitrification was the dominant process in the first 2 days, but then nitrification became the dominant process for the rest of the incubation. More N_2O was produced at 50 and 60% moisture than at 40% moisture, but the relative contributions of the two processes were the same at all moisture contents. Nitrification was responsible for 70% of the N_2O flux. In the second experiment examination of the isotopic composition of the N_2O showed that the ^{15}N atoms were randomly distributed throughout the molecules. The N_2O therefore originated from one denitrifying pool, confirming that our method of addition of label initially created one NO_3^- pool for denitrification. There seems to be no feasible way at present to test the uniformity of the nitrification pool. © 1997 Elsevier Science Ltd

INTRODUCTION

The flux of N_2O from soil can be due to nitrification or denitrification (Hutchinson and Davidson, 1993). Nitrification proceeds in most agricultural soils during the growing season, particularly when mineralization rates are high and after NH_4^+-containing fertilizers or manures have been applied. High rates of denitrification are also often measured during the growing season after either NH_4^+- or NO_3^--containing fertilizers have been applied. Since aerobic and anaerobic microsites can develop within the same soil aggregate (Smith, 1980; Renault and Stengel, 1994), nitrification and denitrification could take place at the same time (Kuenen and Robertson, 1994).

The sources of N_2O can be identified using selective inhibitors, sterilization, or by adding substrates (Davidson and Schimel, 1995). Nitrification can be inhibited by various compounds including C_2H_2 (Hynes and Knowles, 1982), nitrapyrin (Rogers and Ashworth, 1982), and methyl fluoride or dimethyl ether (Miller *et al.*, 1993). The disadvantage of nitrification inhibitors is that prevention of NO_3^- for-

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mation may affect the rate of denitrification. Certain nitrifiers also reduce NO_2^- to N_2O directly under conditions of O_2 stress while actively oxidizing NH_4^+ (Poth and Focht, 1985). Sterilization can be used to separate abiotic from biotic sources. Adding NH_4^+ or NO_3^- as substrates cannot provide definitive identification of the sources of N_2O unless the substrates are labelled. The fluctuations in the isotopic composition of N_2O around natural abundance can be used to identify the processes producing it (Yoshida, 1988; Yoshinari, 1990). More potential exists, however, for studying the sources of N_2O using labelled substrates. The use of radioactive ^{13}N-labelled substrates is restricted to a very few laboratories, but has recently been used to study aerobic emissions of N_2O and N_2 from soil cores (Speir *et al.*, 1995a). Using substrates enriched in ^{15}N is more practical for studies on N-fertilized soils, particularly since the analysis of ^{15}N in N_2O by isotope-ratio mass spectrometry (IRMS) has been fully automated (Stevens *et al.*, 1993). The contributions of nitrification and denitrification to the N_2O flux can be studied by differentially labelling the NH_4^+ and NO_3^- pools with ^{15}N. By periodically measuring and comparing the enrichments of the N_2O, NH_4^+ and NO_3^- pools, the relative importance of the two processes can be quantified. If there is only one denitrifying pool of NO_3^-, simultaneous nitrification and denitrification can be confirmed by examining the distribution of ^{15}N atoms within the N_2O molecules, particularly in the treatment pair where NO_3^- is labelled. When the NH_4^+ pool is at natural abundance and the NO_3^- pool is enriched with ^{15}N, nitrification will produce N_2O at natural abundance while denitrification will produce N_2O of the same enrichment as the NO_3^- pool from which it was derived. The distribution of ^{15}N atoms within the mixture of N_2O molecules will be random if there has been only one source of N_2O, but non-random if there has been more than one source.

Two soil incubation experiments were conducted. In the first experiment, favouring nitrification, we show how the contributions of nitrification and denitrification to N_2O flux can be measured in a soil incubated at different moisture contents. The measurements relied on the assumption that the method of application of label resulted in one uniformly-labelled pool of NO_3^- for denitrification. In the second experiment, favouring denitrification, we show how this assumption can be tested using C_2H_2 as a nitrification inhibitor.

MATERIALS AND METHODS

Theoretical basis for measuring the contributions of nitrification and denitrification to nitrous oxide flux

When a ^{15}N-labelled substrate is added, it is assumed to mix fully with the native soil pool to form one uniformly-labelled pool. If N_2O is evolved into a headspace or enclosure containing normal atmosphere, the flux is calculated simply from change in concentration with time. Information about the source and processes producing N_2O can be obtained by measuring and comparing the enrichments of the N_2O, NH_4^+ and NO_3^- pools.

Nitrous oxide is emitted from two pools of different ^{15}N atom fractions, a_d (a denitrification pool, assumed to be equivalent to the NO_3^- pool) and a_n (a nitrification pool, assumed to be equivalent to the NH_4^+ pool), into an atmosphere in which there is initially negligible N_2O. The ^{15}N atom fraction a_m of the resulting mixture is given by

$$a_m = da_d + (1 - d)a_n \quad (1)$$

where d is the fraction of the N_2O flux derived from the denitrification pool and $(1 - d)$ is the fraction of the N_2O flux derived from the nitrification pool. If the ^{15}N atom fractions of the two soil pools and the N_2O mixture are measured, then d can be calculated as:

$$d = (a_m - a_n)/(a_d - a_n). \quad (2)$$

Confirmation that two processes are occurring simultaneously can be obtained by measuring the distribution of ^{15}N atoms in the N_2O molecules. If N_2O with ^{15}N content at natural abundance mixes with N_2O derived from a source enriched in ^{15}N, the distribution of ^{15}N atoms in the N_2O molecules will be non-random. Interpretation of data is easier and more robust when the NO_3^- pool is labelled rather than when the NH_4^+ pool is labelled. When the NH_4^+ pool is labelled, nitrification will enrich the nitrate pool. Hence the distribution of ^{15}N atoms in the N_2O molecules cannot be used to confirm simultaneous nitrification and denitrification. When the NO_3^- pool is labelled, any non-randomness in the distribution of ^{15}N atoms in the N_2O could be due to nitrification and denitrification occurring simultaneously [Fig. 1 (a)] or denitrification only occurring from two pools of different enrichment [Fig. 1 (b)]. The assumption that denitrification is occurring from only one NO_3^- pool can be tested by blocking nitrification.

Soil

Soil was obtained by coring (3×7.5 cm deep) at random from the surface of a plot receiving 100 kg N ha^{-1} yr^{-1} as ammonium nitrate–calcium carbonate (27% N) at the Agricultural Research Institute, Hillsborough, N. Ireland and bulked. The soil is an acid brown earth (48% sand, 31% silt, 20% clay), of pH 6.0, containing 11.6% organic matter (on an oven dry soil basis). Soil was collected in October 1994 for Experiment 1 and in January 1995 for Experiment 2. It was partially air-dried in a glass-house at 20°C for 2 days until it could be sieved through a 5-mm sieve without

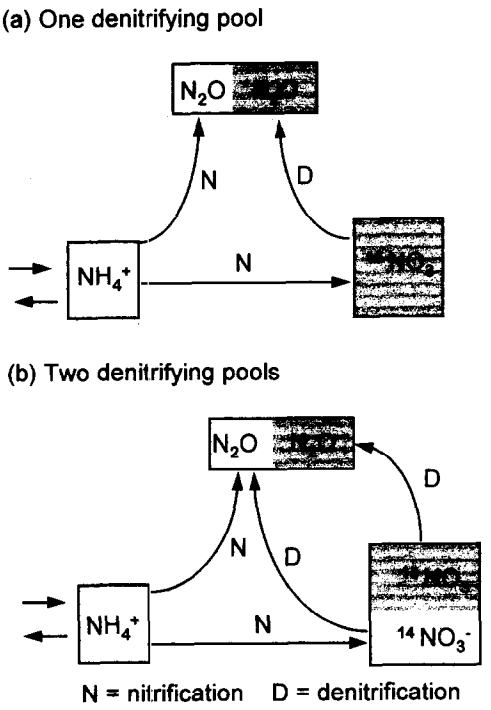

Fig. 1. Possible sources and processes affecting the enrichment of the N_2O produced when $^{15}NO_3^-$ is added to soil: (a) one denitrifying pool; (b) two denitrifying pools.

smearing. Experiments were performed on this soil within 2 days of sieving.

Experiment 1—measuring the contributions of nitrification and denitrification to nitrous oxide flux

The flux of N_2O during a 10 d incubation period was measured from soil incubated under well-aerated conditions. Soil moisture content was varied to try to alter the ratio of nitrification-to-denitrification. The experiment has been previously described in detail by Burns *et al.* (1996) for studying the processes responsible for NO_2^- formation.

Fresh soil (equivalent to 80 g on an oven-dried basis) was weighed into 500 ml Kilner jars. There were three N treatments: (i) control; (ii) soil treated with natural abundance KNO_3 and ^{15}N-labelled $(NH_2)_2CO$; and (iii) soil treated with natural abundance $(NH_2)_2CO$ and ^{15}N-labelled KNO_3. The labelled substrates (or distilled water in the case of control treatments) were added by pipetting solutions uniformly over the soil surface, so that the resulting moisture contents of the soil were 40, 50 and 60% (oven-dry basis). Air-filled porosities at these moisture contents were 60, 50 and 40%, respectively. Urea was applied at 70 μmol N g^{-1} oven-dried soil and KNO_3 at 14 μmol N g^{-1} oven dry soil. All ^{15}N-labelled materials were at 10 atom% excess. The jars were sealed and kept at 20°C in the dark. There were three replicate jars per treatment per sampling occasion, giving a total of 162 jars for the experiment. Headspace samples were taken before the jars were aerated by removing the lids for 5 min each day. A 15 ml sample was transferred using a 20 ml gas-tight syringe fitted with a push-button valve to an evacuated (< 100 Pa), septum-capped vial (9 ml) for CO_2 analysis by gas chromatography. A 12 ml sample was transferred to an evacuated (< 100 Pa), septum-capped vial (12 ml) for analysis of ^{15}N in N_2O by isotope-ratio mass spectrometry.

Soil from three replicate jars per treatment was extracted with 200 ml of 2 M KCl every second day over the 10 day period. Jars containing soil-KCl slurries were shaken for 1 h on an orbital shaker. The extracts were then filtered (Whatman GF/D) and stored at 4°C, prior to analysis within 1 week for concentrations and ^{15}N contents of NH_4^+ and NO_3^-.

Experiment 2—testing the assumption that there was only one uniformly-labelled nitrate pool being denitrified

The ability of the method of application of ^{15}N used in Experiment 1 to uniformly label the NO_3^- pool undergoing denitrification was tested by blocking nitrification and N_2O reductase with C_2H_2. Acetylene at 10 kPa blocks nitrification and the reduction of N_2O to N_2 (Davidson *et al.*, 1986), so that enough N_2O is produced for isotopic analysis. Glucose was added to promote denitrification. A ^{15}N-labelled NH_4^+ treatment was included as well as the ^{15}N-labelled NO_3^- treatment so that the efficacy of C_2H_2 for blocking nitrification could be checked.

The treatments applied in factorial combination were two forms of ^{15}N ($^{15}NH_4NO_3$ and $NH_4^{15}NO_3$) at the same N rate (1.46 μmol N g^{-1} of oven-dry soil), two rates of glucose (42 and 83 μmol C g^{-1} of oven-dry soil), three incubation times (6, 12 and 24 h), and with or without C_2H_2. Each treatment was replicated six times; replicates being arranged randomly during incubation at 20°C. Control treatments without N or C addition were included for time zero and each incubation time.

Fresh 5-mm sieved soil (200 g containing 0.458 g H_2O g^{-1} oven-dry soil) was placed in each of 186 (144 for N and C treatments and 42 for controls) 500 ml Kilner jars. The required amounts of N and C were dissolved in 20 ml of water and dispensed uniformly over the soil surface using a syringe. Control treatments received 20 ml of water, bringing all soil moisture contents to 60% (oven-dry basis). Immediately after liquid addition, a nylon lid with a gas-sampling septum was fitted to each jar using an O-ring to form a gas-tight seal. The volume of headspace in each jar containing treated soil was 363 ml. For the treatments with C_2H_2, 36 ml of C_2H_2 at atmospheric pressure were added to the headspace after removing an equal amount of air. The C_2H_2 had been scrubbed

through water to remove acetone and other impurities (Gross and Bremner, 1992). For the treatments without C_2H_2, He was used instead of C_2H_2 to maintain the same mass of N_2 (306 mg) and O_2 in the headspace of each jar. At the end of each incubation period the headspace of each jar was sampled for analyses by G.C. and by IRMS. A 15 ml sample was transferred using a 20 ml gas-tight syringe fitted with a push-button valve to an evacuated (< 100 Pa), septum-capped vial (9 ml) for CO_2 and C_2H_2 analysis by G.C. A 12 ml sample from headspaces without C_2H_2 was transferred to a septum-capped vial (12 ml) which had been flushed with He and evacuated to < 100 Pa. For the headspaces with C_2H_2 a 13.6 ml sample was transferred to a similar tube containing a 40 x 40 mm piece of glass-fibre filter paper (Grade GF/D, Whatman International Ltd, Kent, UK). A 1 ml aliquot of 0.1 M $KMnO_4$ in 1.0 M KOH solution de-gassed with He was then injected on to each filter paper. The alkaline $KMnO_4$ oxidized the C_2H_2 to CO_2 which was absorbed by the KOH.

Within 30 min after gas sampling all of the soil in each jar was extracted by the blending procedure of Stevens and Laughlin (1995). Soil was transferred to a 1 l food homogeniser, 200 ml of 3 M KCl and 12 ml of 2 M KOH added, and the mixture blended for 30 s. A 200 ml portion of each suspension was centrifuged immediately at $2000 \times g$ for 5 min and the supernatant filtered sequentially through a GF/D and a GF/F (Whatman International Ltd). Filtrates were stored at 4°C prior to analysis within 1 week for concentrations and ^{15}N contents of NH_4^+ and NO_3^-.

Gas analyses

The concentrations of CO_2 and C_2H_2 were determined in each vial using a Varian Genesis headspace auto-sampler to transfer 0.5 ml aliquots to a Perkin Elmer Model 8500 G.C. fitted with a 5 m x 2 mm column of Poropak QS and a thermal conductivity detector. The concentration and ^{15}N content in N_2O were determined by automated continuous-flow IRMS. The IRMS system was as described by Stevens *et al.* (1993) with a segment of I_2O_5 activated by H_2SO_4 (Gastec tube, no. 1HH.) included in the scrubber tube between the $Mg(ClO_4)_2$ and the Ascarite to remove any residual C_2H_2. The ion currents (I) at m/z 44, 45, and 46 enabled ^{45}R ($^{45}I/^{44}I$) and ^{46}R ($^{46}I/^{44}I$) to be calculated for N_2O. The ^{15}N content of the N_2O was calculated from either ^{45}R, using eqns (5) and (7), or ^{46}R, using eqns (6) and (7), of Stevens *et al.* (1993). When the ^{15}N distribution in the N_2O was non-random, the ^{15}N content of the N_2O was calculated using both ^{45}R and ^{46}R (Stevens and Laughlin, 1994):

$$\text{Atom\% } ^{15}\text{N in } N_2O = 100(^{45}R + 2\,^{46}R - ^{17}R - 2\,^{18}R)/(2 + 2\,^{45}R + 2\,^{46}R)$$

The concentration of N_2O was calculated as described by Stevens *et al.* (1993) from the measurements of ^{44}I, ^{45}I, and ^{46}I.

Analysis of ammonium and nitrate

Nitrate was determined by flow injection analysis using the Griess-Ilosvay reaction after reduction of NO_3^- to NO_2^- by a Cd column (Tecator Ltd, 1983). The ^{15}N content of NO_3^- was determined by producing N_2O for continuous-flow IRMS (Stevens and Laughlin, 1994). Ammonium was determined by a gas diffusion method adapted to flow-injection analysis (Tecator Ltd, 1982). The ^{15}N content of NH_4^+ was determined by diffusion into HBO_3 (Saghir *et al.*, 1993), acidifying with H_2SO_4, drying to a residue, and then generating N_2 by dry combustion for IRMS (Preston and Owens, 1983).

Calculation of d in Experiment 1

Values of a_d and a_n to match the daily measurements of a_m were calculated by extrapolation linearly from the measured values of a_d and a_n every 2 days. Daily values of d were then calculated using equation 2.

Statistical analyses

In Experiment 1 standard errors for each treatment were calculated on all the daily data relating to gas analyses. Replicates varied from 15, for days 1 and 2, to 3, for days 9 and 10, due to destructive sampling.

In both experiments analysis of variance was used to determine the significance of treatments on the flux of CO_2 and on the size and enrichments of the NH_4^+, NO_3^- and N_2O pools. Only the results of gas analyses from the three replicate jars of each treatment that had been sampled on each of the 10 days were used in the analysis of variance for Experiment 1.

RESULTS AND DISCUSSION

Production of nitrous oxide

Analysis of the headspaces each day for N_2O and CO_2 concentration showed that there was no significant difference ($P > 0.05$) between the ^{15}N-labelled $(NH_2)_2CO$ and ^{15}N-labelled KNO_3 treatments. Fluxes of N_2O and CO_2 averaged over the two label types are shown in Fig. 2 (a)–(c) for the three soil moisture contents. Nitrous oxide was being produced and the rate of production increased during the incubation. Throughout the incubation period, significantly ($P < 0.001$) more N_2O was produced when the soil moisture content was 50 or 60% than

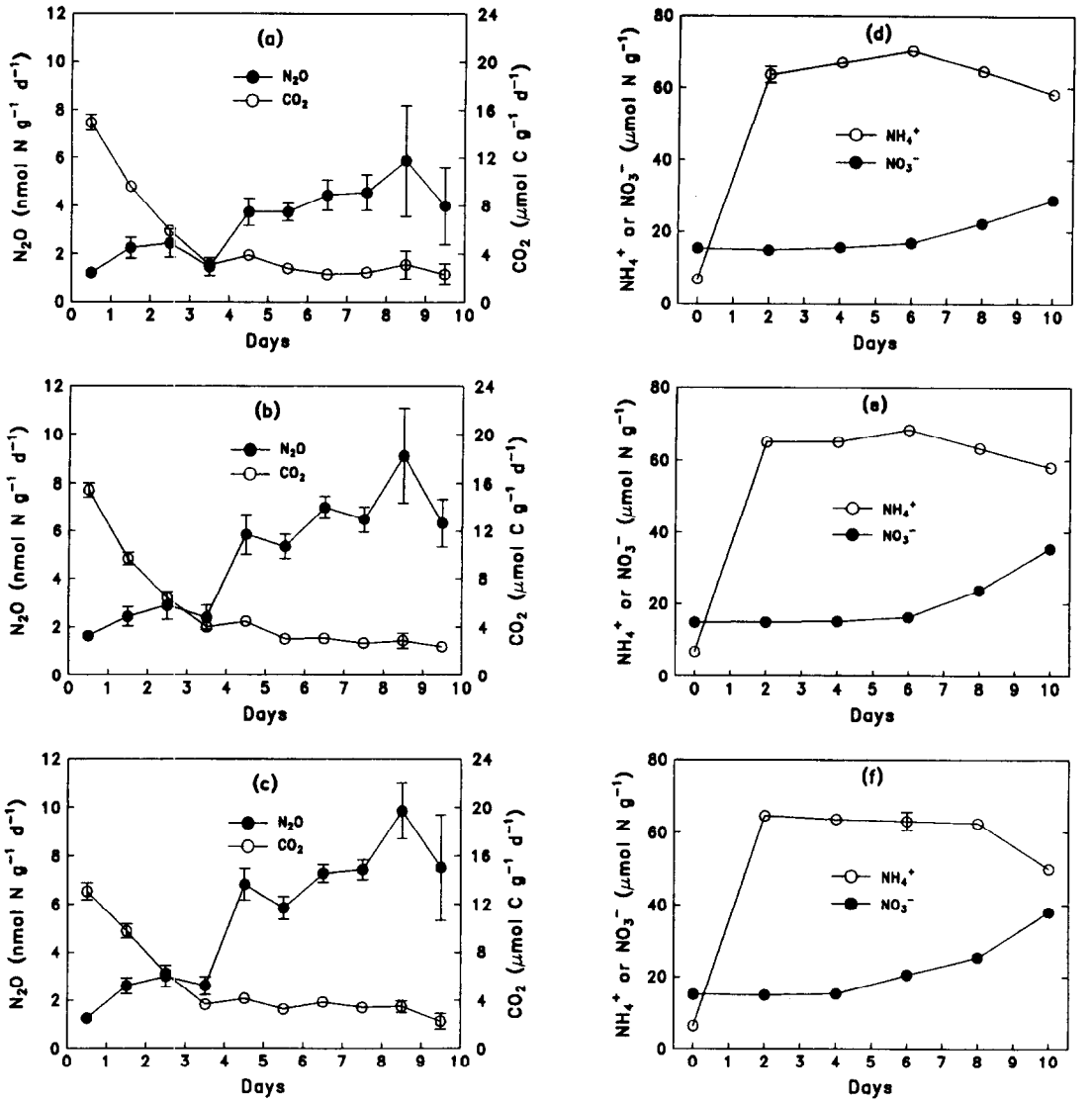

Fig. 2. N_2O and CO_2 production at (a) 40% (b) 50% and (c) 60% moisture content, together with NH_4^+ and NO_3^- concentrations at (d) 40% (e) 50% and (f) 60% moisture content in soil incubated with $(NH_2)_2CO$ and KNO_3. Error bars are the standard errors of means (n ranges from 30, for days 1 and 2, to 6 for days 9 and 10) or are smaller than the symbols.

when the soil moisture content was 40%. Soil respiration rate, indicated by CO_2 concentration, was similar ($P > 0.05$) at all moisture contents throughout the incubation. Carbon dioxide production was greatest at the start of the incubation, declining rapidly over the first 4 days to a constant value of about 3 μmol CO_2-C g^{-1} d^{-1}. Ammonium and NO_3^- concentrations averaged over the two label types [Fig. 2 (d)–(f)] showed that rapid $(NH_2)_2CO$ hydrolysis followed by net nitrification occurred during the incubation. The mineral N data have been more fully discussed by Burns *et al.* (1996) in relation to NO_2^- formation. Nitrous oxide production could have been associated with denitrification induced by respiration or with nitrification.

Sources of nitrous oxide

The enrichments of the N_2O, NH_4^+ and NO_3^- pools for each treatment are shown in Fig. 3. Within each of the labelled pairs, results were similar at each moisture content. In the labelled $(NH_2)_2CO$ treatments, the $(NH_2)_2CO$ was enriched to 10 atom% excess ^{15}N. Rapid $(NH_2)_2CO$ hydrolysis resulted in uniform enrichment of the NH_4^+ pool to 9.2 atom% excess during the incubation. The NO_3^- pool gradually became enriched after day 4 due to nitrification producing labelled NO_3^- to mix with the native soil NO_3^-. In the labelled NO_3^- treatments, the $(NH_2)_2CO$ added was unlabelled and the NH_4^+ pool remained unlabelled throughout the incubation. The NO_3^- added was labelled at

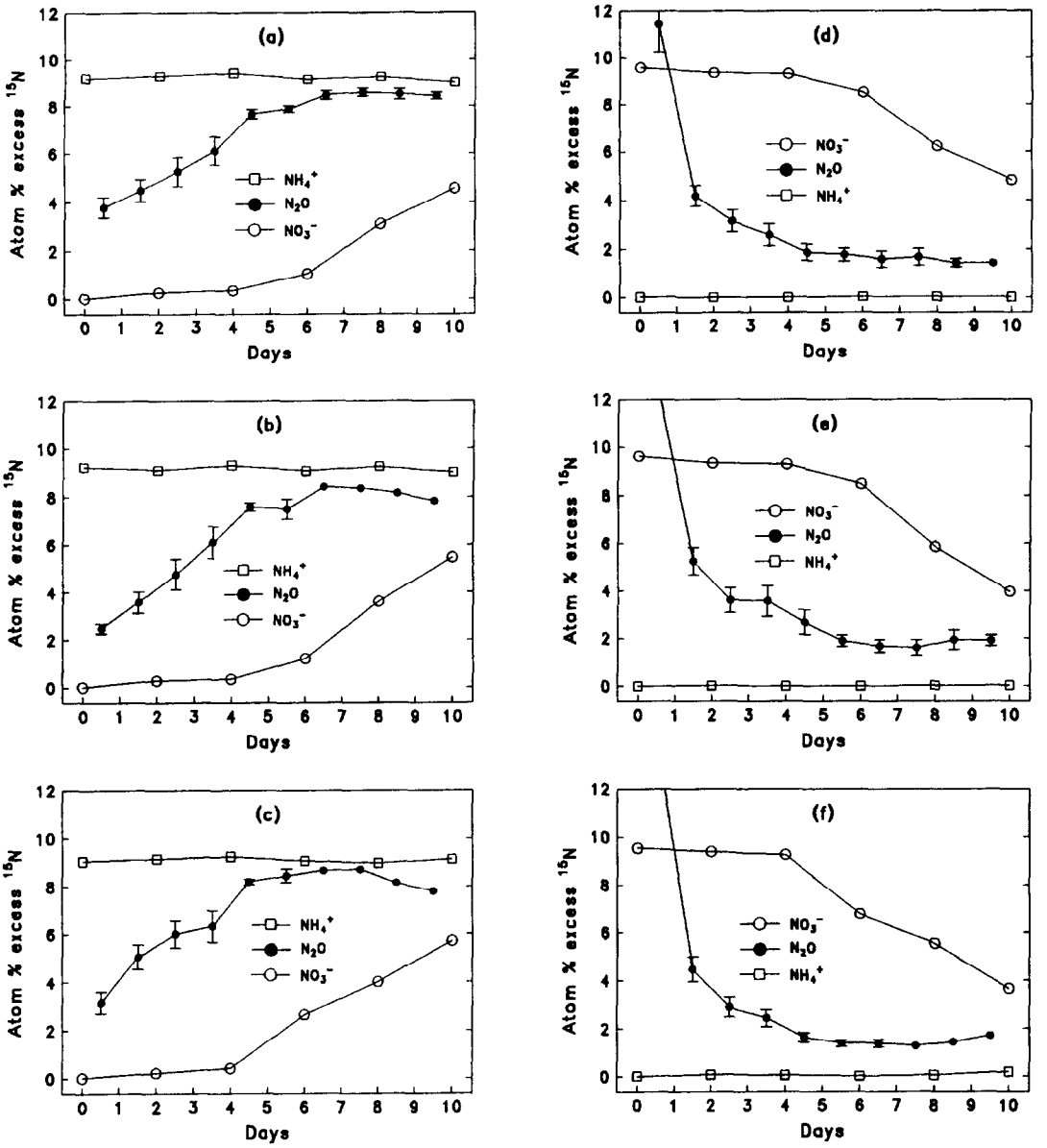

Fig. 3. The ^{15}N atom% excess in NH_4^+, NO_3^- and N_2O from soil incubated with ^{15}N-labelled $(NH_2)_2CO$ and KNO_3 (natural abundance) at (a) 40% (b) 50% and (c) 60% moisture content, and with $(NH_2)_2CO$ (natural abundance) and ^{15}N-labelled KNO_3 at (d) 40% (e) 50% and (f) 60% moisture content. Error bars are the standard errors of means (n = 3) or are smaller than the symbols.

10 atom% excess and after day 4 becomes diluted due to natural abundance NO_3^- from nitrification of the unlabelled NH_4^+. The enrichment of the N_2O does not match exactly the enrichment of either the NH_4^+ or the NO_3^- pools. If N_2O had been produced solely during nitrification of NH_4^+, it should have been enriched to 9.2 atom% excess in the ^{15}N-labelled $(NH_2)_2CO$ treatments, and it should have been at natural abundance in the ^{15}N-labelled NO_3^- treatments. Conversely, if N_2O had been produced solely by denitrification, its enrichment should have matched the enrichment of NO_3^- in either of the labelled treatments. Nitrous oxide was, therefore, being produced by nitrification and denitrification occurring simultaneously, provided that denitrification was occurring from only one uniformly-labelled NO_3^- pool.

Until day 2, nitrification and denitrification were about equally important for producing N_2O. From day 6, nitrification was the dominant process producing N_2O. Most of the readily-available C had been metabolised by day 4 [Fig. 2 (a)–(c)] so denitrification as the dominant N_2O source was less likely from day 4 onwards. As shown by net NO_3^- production [Fig. 2 (d)–(f)] nitrification increased from day 4 onwards. Nitrification could therefore

have been the dominant N_2O producing process after day 4.

The reason for enrichment of the N_2O exceeding the enrichment of the NO_3^- pool in the ^{15}N-labelled NO_3^- treatments at day 1 was because of ^{15}N-labelled NO_2^- impurity in the enriched KNO_3. This added NO_2^- would have been metabolised within the first day (Burns *et al.*, 1995), so would not have interfered with observations in subsequent days. To prepare the ^{15}N-labelled NO_3^- treatment, $K^{15}NO_3$ labelled at 99 atom% ^{15}N was diluted with KNO_3 at natural abundance. A stock solution of 0.1 M $K^{15}NO_3$ labelled at 10 atom% excess ^{15}N contained $^{15}NO_2^-$ at a concentration of 0.82 mM labelled at >90 atom% ^{15}N. Dilution of the NO_3^- enrichment by natural abundance NO_3^- did not dilute the enrichment of the NO_2^-. When the ^{15}N-labelled NO_3^- treatments were added to soil, denitrification or chemo-denitrification of the enriched NO_2^- pool could have produced N_2O whose ^{15}N enrichment was more than 10 atom% excess. A method for oxidizing the NO_2^- impurity in $K^{15}NO_3$ solutions to NO_3^- has been developed in our laboratory.

Confirmation that N_2O was produced from two sources can be obtained by examining the ^{15}N distribution in the N_2O. For either of the labelled treatments, the ^{15}N content of the N_2O calculated from ^{45}R was not the same as the ^{15}N content calculated from ^{46}R, particularly during the first 4 days of the incubation (Fig. 4). The distribution of the ^{15}N atoms in the N_2O molecules was therefore not random, because it was a mixture originating from sources with different enrichments. Interpretation of the results from the ^{15}N-labelled NO_3^- treatments is more diagnostic than from the ^{15}N-labelled $(NH_2)_2CO$ treatments, since one of the possible sources of N_2O, i.e. the NH_4^+ pool, was always unlabelled. In the ^{15}N-labelled $(NH_2)_2CO$ treatments both possible sources of N_2O became labelled during the incubation (Fig. 3).

Quantifying the nitrous oxide flux due to nitrification and denitrification

The fractional contributions of nitrification and denitrification to the N_2O flux were quantified for all treatments (Fig. 5). Denitrification was the dominant process in the first 2 days but then nitrification became the dominant process for the remainder of the incubation. For all moisture contents, the fraction of the N_2O flux due to nitrification increased steadily for the first 4 days, stabilized for all moisture contents, and then declined from day 8 when the moisture content was 50 or 60%. The fraction of the N_2O flux due to denitrification was the converse of that due to nitrification.

The contributions of nitrification and denitrification to the N_2O flux were quantified for all treatments (Fig. 6). More N_2O was produced at 50 and 60% moisture content than at 40% moisture content, but the relative contributions of the two processes were the same at all moisture contents. Nitrification was responsible for 70% of the N_2O flux during the incubation.

Was there only one uniformly-labelled pool of nitrate being denitrified

The quantification of the contributions of nitrification and denitrification described for Experiment 1 rely on the assumption that the substrate for each of the processes exists in only one uniformly-labelled pool. If the labelled NO_3^- did not mix with native soil NO_3^- to form one uniformly-labelled pool, then denitrification alone could result in N_2O whose ^{15}N distribution was non-random, and whose ^{15}N content was between that of NO_3^- and NH_4^+. Similarly, if the labelled NH_4^+ did not mix with native soil NH_4^+ to form one uniformly-labelled pool, then nitrification alone could result in N_2O whose ^{15}N distribution was non-random, and whose ^{15}N content was between that of NO_3^- and NH_4^+. In Experiment 1, the size of the native NH_4^+ pool in soil was small (0.14 μmol g^{-1}) compared to the amount of $(NH_2)_2CO$ added (70 μmol g^{-1}), so even if there had been incomplete mixing it would have had little effect on the validity of the results for N_2O. The size of the native NO_3^- pool in soil was 0.83 μmol g^{-1} compared with an amount of NO_3^- added (14 μmol g^{-1}), so again even if there had been incomplete mixing it should have had little effect on the validity of the results for N_2O. Since added N may not be in excess of native N in other experiments, we conducted Experiment 2 to test that our procedure for adding labelled NO_3^- resulted in one uniformly-labelled NO_3^- pool for denitrification.

Testing that only one uniformly-labelled nitrate pool was denitrifying

The effect of C_2H_2 on the size and enrichment of the NO_3^- pool is shown in Table 1 for each treatment and incubation time. When $^{15}NH_4NO_3$ was added, the NO_3^- pool became enriched in the absence of C_2H_2, but remained unlabelled in the presence of C_2H_2. When NH_4 $^{15}NO_3$ was added, the rate of dilution of label was faster in the absence of C_2H_2 than in the presence of C_2H_2. Nitrification therefore occurred in the absence of C_2H_2, and C_2H_2 blocked nitrification effectively.

Nitrous oxide production was the same ($P > 0.05$) for $^{15}NH_4NO_3$ and NH_4 $^{15}NO_3$ treatments, averaging 3.8 nmol N g^{-1} h^{-1} in the presence of C_2H_2 and 1.5 nmol N g^{-1} h^{-1} in the absence of C_2H_2. The isotopic composition of N_2O during each incubation period sampled is shown in Table 2 for each treatment. In the presence of C_2H_2 the N_2O was not enriched in ^{15}N when $^{15}NH_4NO_3$ was added, but was enriched to 29 atom% excess on average when NH_4 $^{15}NO_3$ was added. Without C_2H_2

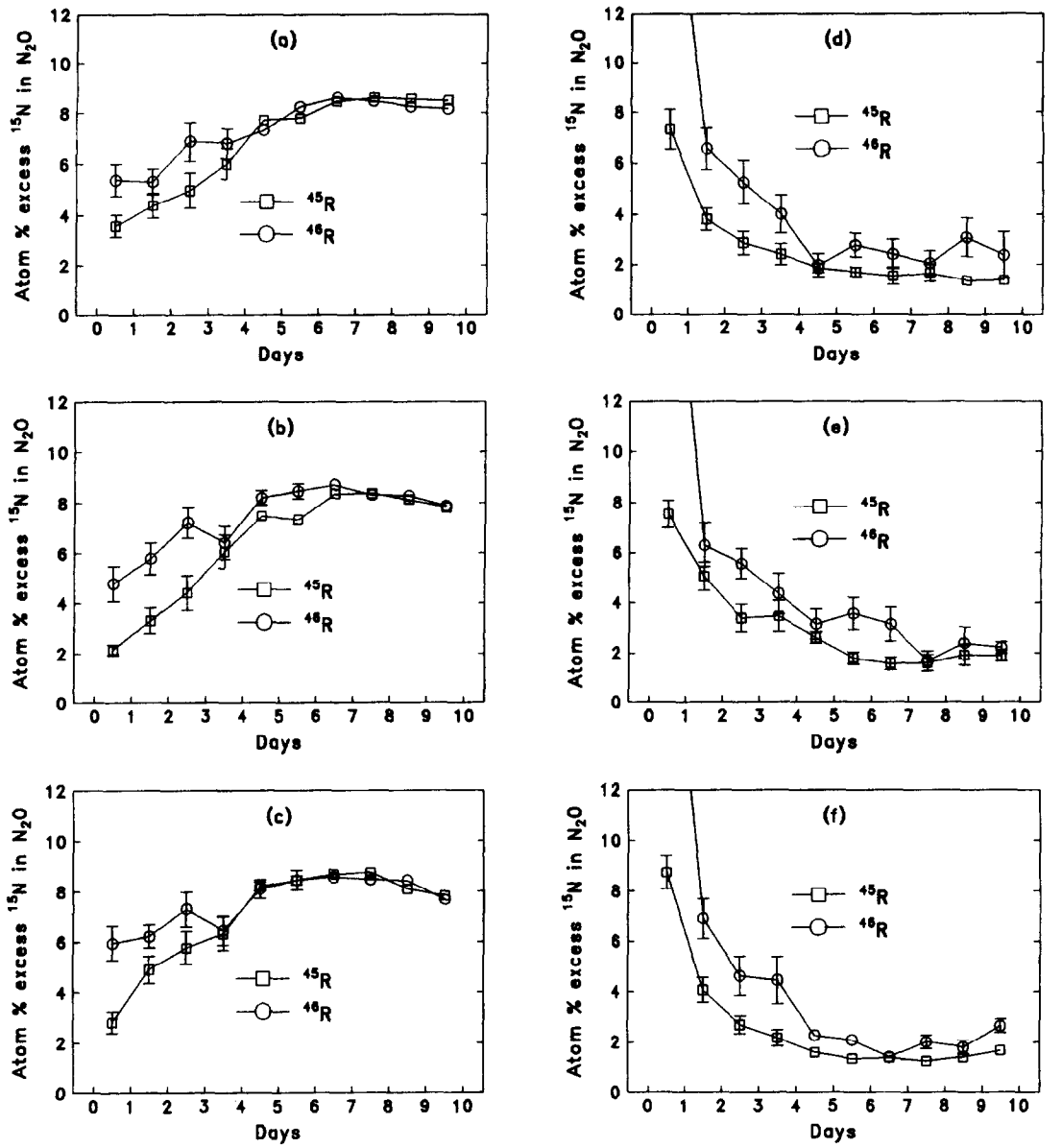

Fig. 4. The ^{15}N atom% excess calculated from ^{45}R or ^{46}R for N_2O from soil incubated with ^{15}N-labelled $(NH_2)_2CO$ and KNO_3 (natural abundance) at (a) 40% (b) 50% and (c) 60% moisture content, and with $(NH_2)_2CO$ (natural abundance) and ^{15}N-labelled KNO_3 at (d) 40% (e) 50% and (f) 60% moisture content. Error bars are the standard errors of means (n = 3) or are smaller than the symbols.

the N_2O was enriched to 9 atom% excess on average when $^{15}NH_4NO_3$ was added, and to 22 atom% excess when NH_4 $^{15}NO_3$ was added. The ^{15}N contents of the N_2O were calculated from either ^{45}R or ^{46}R (Table 2). When the calculated values agree, the ^{15}N distribution in the N_2O was random and, hence, the N_2O originated from a single source, but when the values differed the ^{15}N distribution was non-random implying that the N_2O originated from two or more sources. The ^{15}N contents of the N_2O were the same ($P < 0.05$) whether they were calculated from ^{45}R or ^{46}R. The N_2O therefore originated predominantly by one process from one pool i.e. by denitrification from a single uniformly-labelled NO_3^- pool. Nitrification occurring in the absence of C_2H_2 appeared to produce little N_2O in this experiment.

If the NH_4 $^{15}NO_3$ added at 40 atom% excess mixed with all of the native soil NO_3^- pool at time zero, the enrichment of the mixture should have been 25.1 atom% excess. When dilution of the NO_3^- pool by nitrification was blocked by C_2H_2, the enrichment of the N_2O produced by denitrification would have been expected to be 25.1 atom% excess also. The enrichment of the N_2O was greater, averaging 28.6 atom% excess over both carbon con-

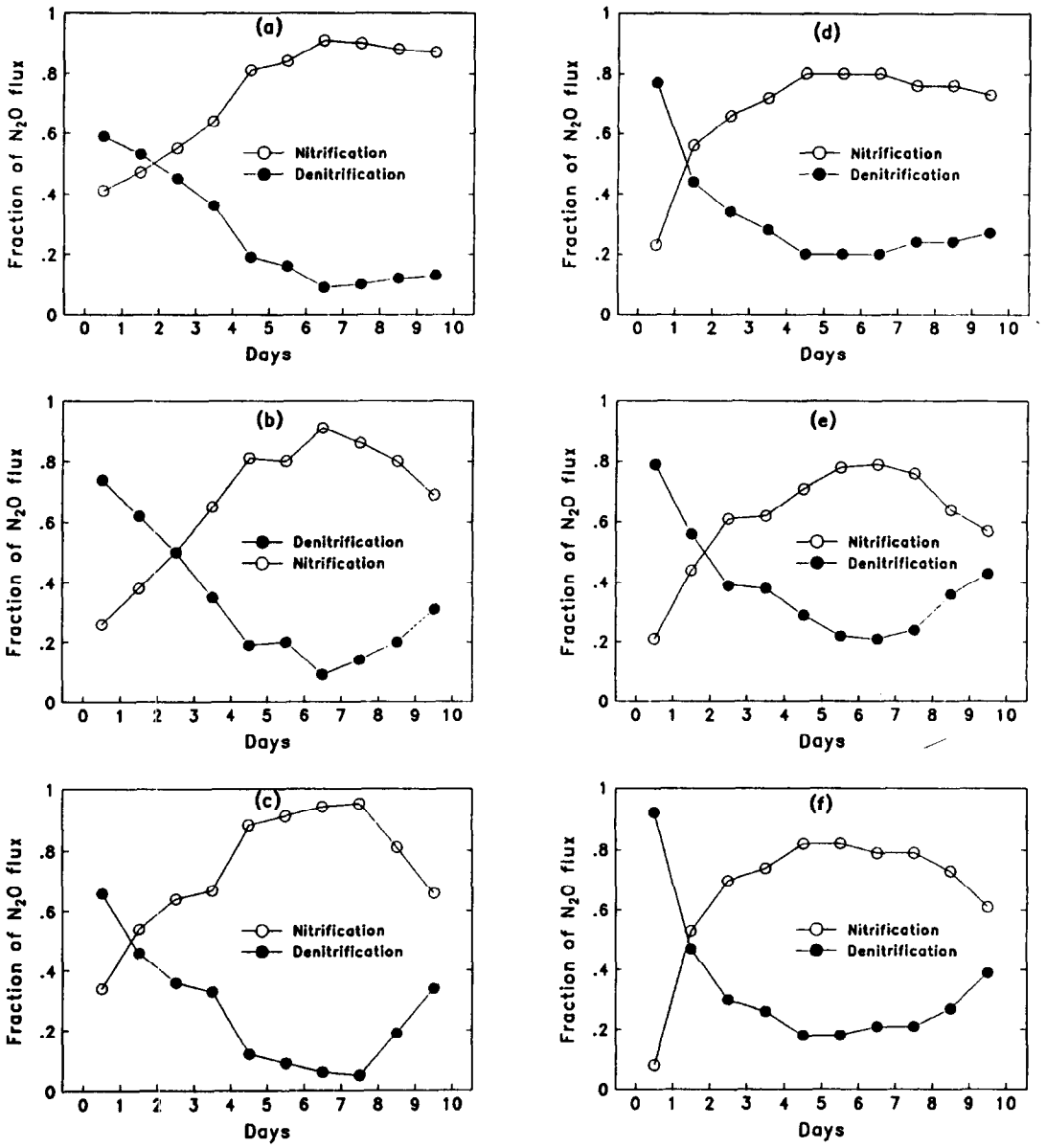

Fig. 5. Fractionation of the N_2O flux between nitrification and denitrification from soil incubated with ^{15}N-labelled $(NH_2)_2CO$ and KNO_3 (natural abundance) at (a) 40% (b) 50% and (c) 60% moisture content, and with $(NH_2)_2CO$ (natural abundance) and ^{15}N-labelled KNO_3 at (d) 40% (e) 50% and (f) 60% moisture content.

tents. Some of the soil NO_3^- appeared to be extractable but not involved in dilution of the NO_3^- pool which was denitrifying. If only two-thirds of the native soil NO_3^- pool had mixed with added NO_3^-, the enrichment of the mixture would have equalled the enrichment of the N_2O. When dilution of the NO_3^- pool was not blocked by C_2H_2, the enrichment of the N_2O produced was less than the theoretical enrichment of the NO_3^- pool at time zero (25.1 atom%excess). This indicates that NO_3^- formed by nitrification was mixing with and diluting the labelled NO_3^- pool which was denitrifying.

Simultaneous nitrification and denitrification

Nitrification and denitrification were taking place simultaneously in both soil incubation Experiments. Nitrification was the dominant process producing N_2O in Experiment 1 where the soil was predominantly aerobic, but denitrification was the dominant process in Experiment 2 where denitrification was favoured. Other soil incubation studies have shown that nitrification was the dominant process producing N_2O in aerobic soils (Bremner and Blackmer, 1979; Robertson and Tiedje, 1987; Skiba *et al.*, 1993). In aerobic agricultural soils N_2O can be pro-

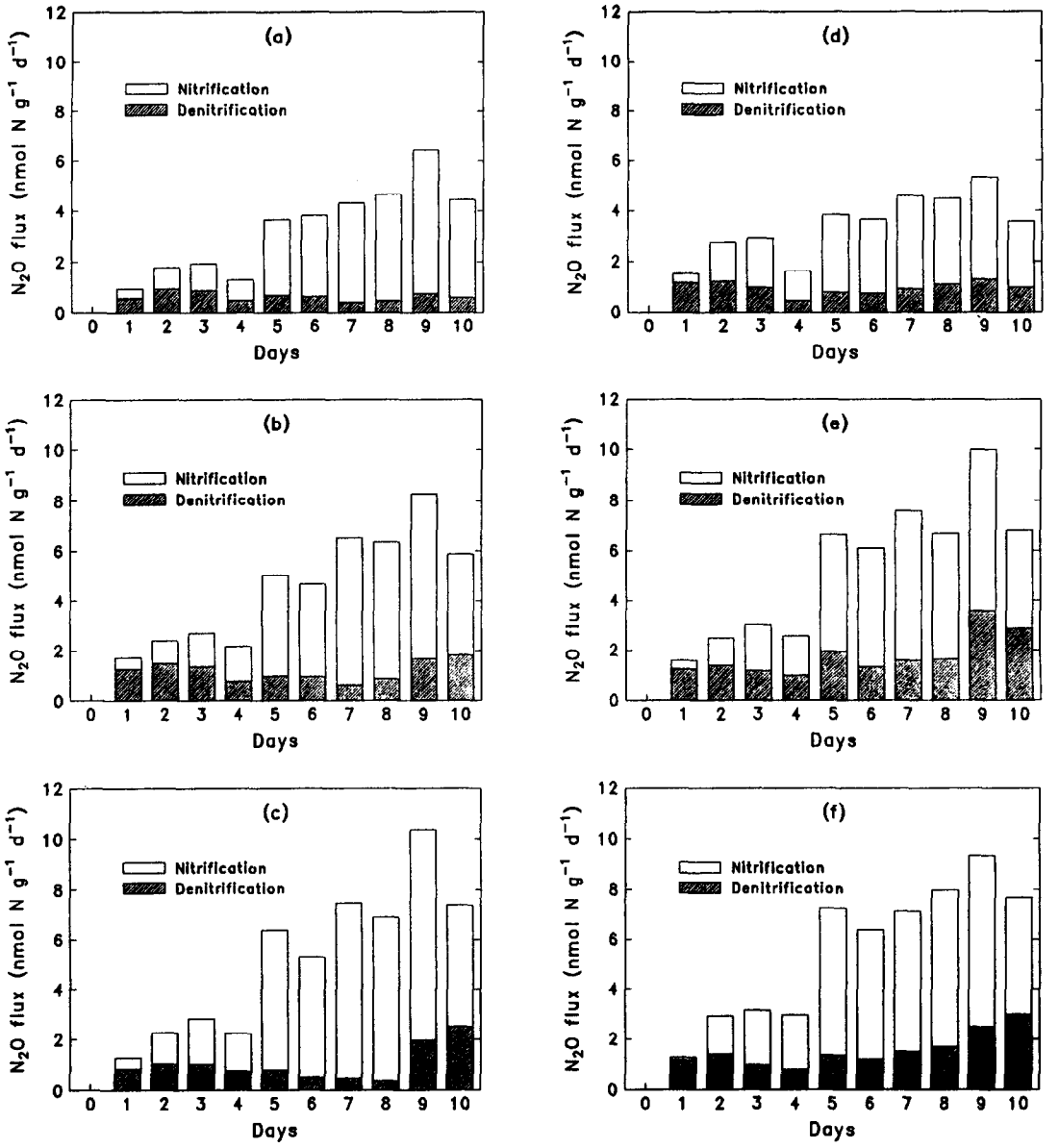

Fig. 6. Quantification of the N_2O flux due to nitrification and denitrification from soil incubated with ^{15}N-labelled $(NH_2)_2CO$ and KNO_3 (natural abundance) at (a) 40% (b) 50% and (c) 60% moisture content, and with $(NH_2)_2CO$ (natural abundance) and ^{15}N-labelled KNO_3 at (d) 40% (e) 50% and (f) 60% moisture content.

duced, however, by denitrification due to anaerobic microsites within soil aggregates (Smith, 1980; Renault and Stengel, 1994) or by aerobic denitrification (Lloyd, 1993; Robertson and Kuenen, 1984). Nitrous oxide flux increased with soil moisture content. Similar results have been found by Goodroad and Keeney (1984) and Klemedtsson *et al.* (1988). Moisture content affects microbial processes by affecting diffusion of both substrates and gases (Skopp *et al.*, 1990).

Previous studies have attempted to quantify the contributions of nitrification and denitrification to N_2O flux by using inhibitors to block nitrification (Davidson *et al.*, 1986; Robertson and Tiedje, 1987; Skiba *et al.*, 1993). The use of variations in isotopic composition around natural abundance (Yoshida, 1988; Yoshinari, 1990) and ^{13}N techniques (Speir *et al.*, 1995a) may have the potential to assess the relative importance of nitrification and denitrification to N_2O flux. Substrates labelled with ^{15}N can be used without inhibitors to quantify the sources of N_2O. Addition of substrates, however, will stimulate the processes so the technique is relevant to studies on fertilized soils. Only with the extra sensitivity of detection of ^{13}N can studies be conducted on natural systems (Speir *et al.*, 1995b). At

Table 1. The effect of glucose and C_2H_2 on the size and enrichment of the mineral N pools in soil treated with differentially-labelled NH_4NO_3

Time (h)	Nitrogen label	Glucose (μmol C g^{-1})	C_2H_2 (% v/v)	NH_4^+ – N	NO_3^- – N	NH_4^+ – N	NO_3^- – N
				(μmol N g^{-1})		(atom% excess ^{15}N)	
0	$^{15}NH_4NO_3$	42	0	0.78*	1.16*	37.7*	0.0*
0–6	$^{15}NH_4NO_3$	42	0	0.24	1.03	15.0	4.7
0–12	$^{15}NH_4NO_3$	42	0	0.06	1.03	2.2	6.8
0–24	$^{15}NH_4NO_3$	42	0	0.06	0.63	0.6	4.9
0	$^{15}NH_4NO_3$	83	0	0.78*	1.16	37.7*	0.0*
0–6	$^{15}NH_4NO_3$	83	0	0.27	1.03	13.6	4.5
0–12	$^{15}NH_4NO_3$	83	0	0.10	0.87	2.5	6.8
0–24	$^{15}NH_4NO_3$	83	0	0.09	0.16	0.4	0.9
0	$NH_4\ ^{15}NO_3$	42	0	0.78*	1.16*	0.0*	25.1*
0–6	$NH_4\ ^{15}NO_3$	42	0	0.26	1.03	0.1	20.6
0–12	$NH_4\ ^{15}NO_3$	42	0	0.09	0.99	0.2	18.4
0–24	$NH_4\ ^{15}NO_3$	42	0	0.07	0.63	0.3	16.1
0	$NH_4\ ^{15}NO_3$	83	0	0.78*	1.16*	0.0*	25.1*
0–6	$NH_4\ ^{15}NO_3$	83	0	0.28	1.02	0.2	21.0
0–12	$NH_4\ ^{15}NO_3$	83	0	0.08	0.84	0.3	18.7
0–24	$NH_4\ ^{15}NO_3$	83	0	0.06	0.17	0.3	7.9
0	$^{15}NH_4NO_3$	42	10	0.78*	1.16*	37.7*	0.0*
0–6	$^{15}NH_4NO_3$	42	10	0.35	0.90	18.6	0.1
0–12	$^{15}NH_4NO_3$	42	10	0.14	0.80	8.5	0.1
0–24	$^{15}NH_4NO_3$	42	10	0.08	0.54	0.7	0.1
0	$^{15}NH_4NO_3$	83	10	0.78*	1.16*	37.7*	0.0*
0–6	$^{15}NH_4NO_3$	83	10	0.34	0.89	18.5	0.1
0–12	$^{15}NH_4NO_3$	83	10	0.16	0.68	7.3	0.1
0–24	$^{15}NH_4NO_3$	83	10	0.06	0.15	0.6	0.1
0	$NH_4\ ^{15}NO_3$	42	10	0.78*	1.16*	0.0*	25.1*
0–6	$NH_4\ ^{15}NO_3$	42	10	0.33	0.86	0.2	24.1
0–12	$NH_4\ ^{15}NO_3$	42	10	0.15	0.76	0.2	23.0
0–24	$NH_4\ ^{15}NO_3$	42	10	0.10	0.55	0.2	20.1
0	$NH_4\ ^{15}NO_3$	83	10	0.78*	1.16*	0.0*	25.1*
0–6	$NH_4\ ^{15}NO_3$	83	10	0.36	0.86	0.1	24.2
0–12	$NH_4\ ^{15}NO_3$	83	10	0.16	0.67	0.2	23.7
0–24	$NH_4\ ^{15}NO_3$	83	10	0.07	0.15	0.3	8.4
	SEM (120 d.f., n = 6)			0.108	0.133	0.40	0.16

*Theoretical value calculated from control measurements at time zero (NH_4^+-N = 0.044 μmol N g^{-1}; NO_3^--N = 0.430 μmol N g^{-1}) and nitrogen addition.

present our technique with ^{15}N and automated gas-phase mass spectrometry is the best practical method for quantifying the sources of N_2O in fertilized soils.

The mechanism for N_2O production by denitrification is well understood, the process proceeding in four stages, the chemical intermediates being NO_2^-, NO and N_2O (Cole, 1994). There are at least two possible mechanisms for N_2O production during nitrification. Certain nitrifying organisms generate N_2O from the reduction of NO_2^-, which they produce under O_2-limiting conditions (Ritchie and Nicholas, 1972; Poth and Focht, 1985). Ritchie and Nicholas (1972) further concluded that NH_4^+-oxidizers reduce NO_2^- to N_2O to minimize intracellular accumulation of toxic amounts of NO_2^-. Alternatively, N_2O can be produced by various reactions of the intermediates formed during NH_4^+ oxidation (Yoshida and Alexander, 1970; Ritchie and Nicholas, 1972). Although NO_2^- is a common intermediate in the production of N_2O by both nitrification and denitrification, there is evidence from Experiment 1 that NO_2^- exists in soil as two separate pools. The ^{15}N content of the NO_2^- pool in Experiment 1 has already been discussed in detail by Burns *et al.* (1995). When the ^{15}N content of the NO_2^- pool was compared with the ^{15}N content of the N_2O pool, it did not match for either the $^{15}NH_4^+$- or $^{15}NO_3^-$-labelled treatments (Fig. 7). The N_2O was therefore not derived from one uniformly-labelled NO_2^- pool. For the $^{15}NH_4NO_3$ treatment, the enrichment of the N_2O was greater than the enrichment of the NO_2^-. Conversely for the $NH_4\ ^{15}NO_3$ treatment, the enrichment of the N_2O was less than the enrichment of the NO_2^-. This could be explained by the existence of two NO_2^- pools, the larger of which was derived from nitrification. The efficiency of production of N_2O from the NO_2^- pool derived from nitrification must be less than the efficiency of production of N_2O from the NO_2^- pool derived from denitrification.

Table 2. The effect of glucose and C_2H_2 on the isotopic composition of the N_2O evolved from soil treated with differentially-labelled NH_4NO_3

Time (h)	Nitrogen label	Glucose (μmol C g^{-1})	C_2H_2 (%v/v)	Atom% ^{15}N in N_2O		
				^{45}R	^{46}R	^{45}R and ^{46}R
0–6	$^{15}NH_4NO_3$	42	0	7.9	9.6	8.2
0–12	$^{15}NH_4NO_3$	42	0	9.3	10.4	9.5
0–24	$^{15}NH_4NO_3$	42	0	10.0	10.6	10.1
0–6	$^{15}NH_4NO_3$	83	0	7.5	9.4	7.8
0–12	$^{15}NH_4NO_3$	83	0	8.7	9.7	8.9
0–24	$^{15}NH_4NO_3$	83	0	9.2	9.9	9.3
0–6	$NH_4{}^{15}NO_3$	42	0	22.4	23.5	22.9
0–12	$NH_4{}^{15}NO_3$	42	0	21.2	21.7	21.4
0–24	$NH_4{}^{15}NO_3$	42	0	21.6	21.8	21.7
0–6	$NH_4{}^{15}NO_3$	83	0	22.3	23.4	22.8
0–12	$NH_4{}^{15}NO_3$	83	0	22.8	22.9	22.8
0–24	$NH_4{}^{15}NO_3$	83	0	21.7	21.9	21.8
0–6	$^{15}NH_4NO_3$	42	10	0.1	0.4	0.1
0–12	$^{15}NH_4NO_3$	42	10	0.1	0.6	0.1
0–24	$^{15}NH_4NO_3$	42	10	0.0	0.2	0.0
0–6	$^{15}NH_4NO_3$	83	10	0.1	0.7	0.1
0–12	$^{15}NH_4NO_3$	83	10	0.1	0.4	0.1
0–24	$^{15}NH_4NO_3$	83	10	0.0	0.2	0.0
0–6	$NH_4{}^{15}NO_3$	42	10	27.9	28.3	28.3
0–12	$NH_4{}^{15}NO_3$	42	10	29.5	29.7	29.7
0–24	$NH_4{}^{15}NO_3$	42	10	29.1	29.2	29.3
0–6	$NH_4{}^{15}NO_3$	83	10	26.9	27.4	27.4
0–12	$NH_4{}^{15}NO_3$	83	10	27.5	27.8	27.8
0–24	$NH_4{}^{15}NO_3$	83	10	29.0	29.2	29.3
	SEM (d.f. = 120, n = 6)			0.31	0.28	0.28

Tracer techniques with ^{15}N for direct measurement of denitrification in soil are based on the hypothesis that the NO_3^- undergoing denitrification exists in a single pool that is isotopically uniform. In reality the NO_3^- being denitrified may exist in multiple pools having different ^{15}N enrichments (Boast *et al.*, 1988). When using our technique to measure the fractional fluxes of N_2O due to nitrification and denitrification, it is necessary to ensure that the N_2O is being produced by the two processes rather than by denitrification of two NO_3^- pools with different enrichments. A treatment using C_2H_2 as a nitrification inhibitor and as a block of N_2O reductase should therefore be included as an integral part of subsequent experiments. Nitrification is inhibited by an C_2H_2 concentration of 10 Pa, but a concentration of 10 kPa is required to block N_2O reductase (Davidson *et al.*, 1986). Although a C_2H_2 treatment was not included in Experiment 1, the method of adding ^{15}N was the same as in Experiment 2. Results from Experiment 2 demonstrated that our method of pipetting the labelled solution evenly over the soil surface initially created a single uniformly-labelled pool for denitrification.

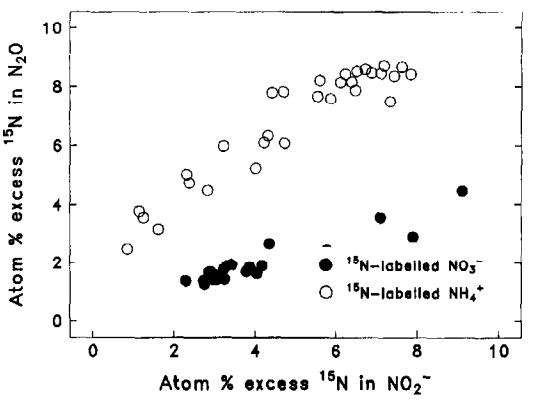

Fig. 7. Comparison of the enrichment of the NO_2^- and N_2O pools for soil incubated with ^{15}N-labelled $(NH_2)_2CO$ and KNO_3 (natural abundance) or ^{15}N-labelled KNO_3 and $(NH_2)_2CO$ (natural abundance).

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