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RCM

A novel dual-isotope labelling method for distinguishing between soil sources of N_2O

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We present a novel ^{18}O-^{15}N-enrichment method for the distinction between nitrous oxide (N_2O) from nitrification, nitrifier denitrification and denitrification based on a method with single- and double-^{15}N-labelled ammonium nitrate. We added a new treatment with ^{18}O-labelled water to quantify N_2O from nitrifier denitrification. The theory behind this is that ammonia oxidisers use oxygen (O_2) from soil air for the oxidation of ammonia (NH_3), but use H_2O for the oxidation of the resulting hydroxylamine (NH_2OH) to nitrite (NO_2^-). Thus, N_2O from nitrification would therefore be expected to reflect the ^{18}O signature of soil O_2, whereas the ^{18}O signature of N_2O from nitrifier denitrification would reflect that of both soil O_2 and H_2O. It was assumed that (a) there would be no preferential removal of ^{18}O or ^{16}O during nitrifier denitrification or denitrification, (b) the ^{18}O signature of the applied ^{18}O-labelled water would remain constant over the experimental period, and (c) any O exchange between $H_2^{18}O$ and NO_3^- would be negligible under the chosen experimental conditions. These assumptions were tested and validated for a silt loam soil at 50% water-filled pore space (WFPS) following application of 400 mg N kg^{-1} dry soil. We compared the results of our new method with those of a conventional inhibition method using 0.02% v/v acetylene (C_2H_2) and 80% v/v O_2 in helium. Both the ^{18}O-^{15}N-enrichment and inhibitor methods identified nitrifier denitrification to be a major source of N_2O, accounting for 44 and 40%, respectively, of N_2O production over 24 h. However, compared to our ^{18}O-^{15}N-method, the inhibitor method overestimated the contribution from nitrification at the expense of denitrification, probably due to incomplete inhibition of nitrifier denitrification and denitrification by large concentrations of O_2 and a negative effect of C_2H_2 on denitrification. We consider our new ^{18}O-^{15}N-enrichment method to be more reliable than the use of inhibitors; it enables the distinction between more soil sources of N_2O than was previously possible and has provided the first direct evidence of the significance of nitrifier denitrification as a source of N_2O in fertilised arable soil. Copyright © 2005 John Wiley & Sons, Ltd.

Emissions of nitrous oxide (N_2O) are of concern due to the high global warming potential of this gas, its long atmospheric lifetime, and its involvement in the destruction of stratospheric ozone.[1] Agricultural soils are the main global source of N_2O,[2] especially when fertilised. Several different microbial processes produce N_2O in soil, the most significant of which are thought to be nitrification, nitrifier denitrification and denitrification.[3]

Nitrification and nitrifier denitrification are both carried out by autotrophic nitrifying bacteria. In ammonia oxidation, the first stage of nitrification, ammonia (NH_3) is oxidised to nitrite (NO_2^-) and N_2O can develop as a by-product. As oxygen (O_2) is required for this process, it takes place in aerobic microsites of soils. Nitrifier denitrification is a pathway that ammonia oxidisers are thought to turn to under short-term O_2 limitation whereby NO_2^- is reduced to molecular nitrogen (N_2) via N_2O.[4] The ability to undertake this process may be a universal trait in the betaproteobacterial ammonia-oxidising bacteria.[5] This reduction is thought to be similar to denitrification, whereby heterotrophic denitrifiers use nitrate (NO_3^-) or NO_2^- as an electron acceptor under low O_2 conditions. Although the conditions conducive for nitrification, nitrifier denitrification and denitrification differ, they are thought to take place simultaneously in different microhabitats of the same soil.[6,7]

To derive effective management strategies to mitigate N_2O emissions from soils, the respective contributions of the different microbial processes need to be quantified. To date, any distinctions between N_2O production from nitrifiers, denitrifiers, and 'other sources' encompassing chemodenitrification, heterotrophic nitrification, dissimilatory nitrate reduction to ammonium (DNRA) or aerobic denitrification,

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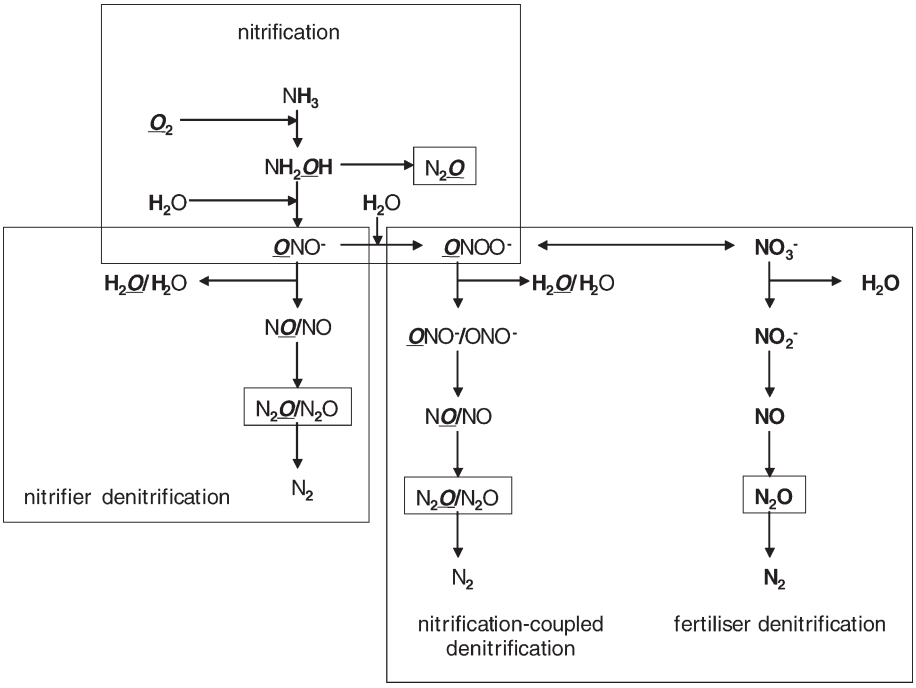

Figure 1. Overview of the sources of nitrogen and oxygen in N_2O from nitrification, nitrifier denitrification and denitrification. The splitting off of water in the reduction reactions is only shown exemplarily. Different font styles indicate different sources. ONO-: nitrite; ONOO-: nitrate (drawn linearly for reasons of simplicity, note that each O atom is bound to the central N atom).

have relied on inhibition methods or stable isotope techniques. An inhibition method involving the use of acetylene (C_2H_2, 0.02% v/v) and O_2 (100% v/v) has been developed to differentiate between nitrification, nitrifier denitrification, denitrification, and 'other sources' of N_2O.[8,9] Unfortunately, these inhibitors are not reliable for all soil types and microorganisms.[10–12] Recent developments in stable isotope techniques have enabled differentiation between, and quantification of, N_2O produced during denitrification and nitrification.[12–14] However, the contribution of nitrifier denitrification to N_2O emissions from soils is still unknown, and stable isotope techniques now need to be extended to enable quantification of N_2O production during this process.

In this paper, we present a new ^{18}O-^{15}N-enrichment technique that enables distinction between N_2O production from nitrification, nitrifier denitrification and denitrification. It is modified from the method of Baggs *et al.*,[13] which uses single- and double-^{15}N-labelled ammonium nitrate to distinguish between nitrifier and denitrifier pathways. We added a treatment with ^{18}O-labelled water to distinguish between nitrification and nitrifier denitrification. Ammonia oxidisers use O_2 from soil air for the oxidation of NH_3, but H_2O for the oxidation of the resulting hydroxylamine to NO_2^-.[15,16] Thus, the $^{16/18}O$ signature of N_2O from nitrification was hypothesised to reflect that of soil O_2, while the $^{16/18}O$ signature of N_2O from nitrifier denitrification was assumed to reflect that 50% of the O_2 was derived from soil O_2 and 50% from H_2O (Fig. 1). If these assumptions hold true then application of ^{18}O-labelled water to soil can be used to distinguish between nitrified and nitrifier-denitrified N_2O. The new technique was tested and the method applied for the first time in a short-term experiment. Results were compared with those obtained with a C_2H_2 and O_2 inhibition method.[9]

EXPERIMENTAL

Set-up

Soil (0–15 cm depth) was sampled from an arable field on the Imperial College London Estate at Wye in July 2004. The soil was a brown earth silt loam (17% sand, 68% silt, 15% clay, total carbon 2.3%, total N 0.3%, pH (H_2O) 6.8, bulk density 1.14 g cm^{-3}) of the Coombe series classified as a Cambisol (FAO classification). The soil was air-dried, sieved (2 mm) and stored at 4°C until establishment of the experiments.

For the experiments, 200 g air-dried soil was weighed into 500 mL Kilner jars with gas-tight lids containing a gas-sampling port. The gravimetric water content of the soil was determined in three subsamples after drying at 105°C for 24 h. The soil was conditioned at approximately 30% water-filled pore space (WFPS) for 4 days prior to the experiments and kept in the dark at 21°C. Soil WFPS was calculated based on soil bulk density, gravimetric water content and particle density. All treatments were set up as a fully randomised design with two sets of three replicates per treatment, allowing for gas sampling and mineral N analysis at 6 and 24 h after fertiliser application.

Assumption testing

A preliminary experiment was carried out to test the assumptions underlying the new enrichment method. These were that (a) there would be no preferential removal of ^{16}O or ^{18}O during either nitrifier denitrification or nitrification-coupled denitrification, (b) the ^{18}O signature of the applied

labelled water would remain constant over the experimental period, and (c) oxygen exchange between NO_3^- and added ^{18}O-labelled water would be negligible under the chosen experimental conditions. Without preferential removal of ^{16}O or ^{18}O during nitrifier denitrification or nitrification-coupled denitrification, and with a constant $H_2^{18}O$ signal, the isotopic signature of N_2O produced after 6 and 24 h would therefore reflect the ^{18}O enrichment of the H_2O applied. If there was no oxygen exchange between water and NO_3^-, the ^{18}O signature of N_2O from denitrification would be at natural abundance levels after addition of ^{18}O-labelled water to soils where ammonia oxidation and N_2O reduction in denitrification were inhibited by large concentrations of C_2H_2 (5% v/v) (Fig. 1).

The conditioned soil was brought to 50% WFPS with ^{18}O-labelled water at 0.1, 0.5 and 1.0 atom % excess ^{18}O. The soil was stirred well after each 10 mL addition of water to ensure uniform distribution. Unlabelled NH_4NO_3 was applied with the water at 400 mg N kg^{-1} dry soil. Each treatment was replicated three times. After closing the Kilner jars, C_2H_2 (5% v/v) was added to the headspace of half of the jars to enable testing of assumption (c). All jars were incubated at 21°C in the dark. After 6 and 24 h, gas samples (12 mL) for total ($^{14+15}N$-)N_2O analysis were transferred from the headspace of the jars to evacuated gas vials (Labco, UK). Samples (125 mL) for ^{18}O-N_2O analysis were transferred to 125 mL gas-tight glass bottles (Supelco, USA) that had been flushed with helium (He) and evacuated. After gas sampling, soil was sampled for mineral N analysis (see below).

^{18}O-^{15}N-enrichment method

Soil was wetted to 50% WFPS and treatments were established according to Table 1. Each treatment was replicated three times. The ^{15}N-labelled NH_4NO_3 (400 mg N kg^{-1} dry soil; 10 atom % excess ^{15}N) was dissolved in the water and the solution mixed into the soil after every 10 mL addition to ensure uniform distribution. Treatments 5 to 7 comprised the inhibition method of Webster and Hopkins,[9] which was compared with our proposed method (see calculations below). To check the reliability of C_2H_2 and O_2 as inhibitors of N_2O-producing pathways in this soil, $NH_4^{15}NO_3$ was applied in these treatments. To establish treatments with large O_2 concentrations (TR 5 and 7), the Kilner jars were flushed with 80% O_2 in He twice for 2 min each time.

Table 1. Treatments established for the enrichment experiment. NH_4NO_3 was added at a concentration of 400 mg N kg^{-1} dry soil (10 at% excess ^{15}N where applicable); H_2O: unlabelled water, $H_2^{18}O$: addition of ^{18}O-labelled water (10 atom % excess ^{18}O) to achieve a final enrichment of 1 atom % excess ^{18}O; C_2H_2: 0.02% (v/v); O_2: 80% (v/v)

	Fertiliser	Water	Inhibitor
TR1	$^{15}NH_4^{15}NO_3$	H_2O	—
TR2	$^{14}NH_4^{15}NO_3$	H_2O	—
TR3	$^{15}NH_4^{14}NO_3$	H_2O	—
TR4	$^{14}NH_4^{14}NO_3$	$H_2^{18}O$	—
TR5	$^{14}NH_4^{15}NO_3$	H_2O	C_2H_2
TR6	$^{14}NH_4^{15}NO_3$	H_2O	O_2
TR7	$^{14}NH_4^{15}NO_3$	H_2O	C_2H_2, O_2

Where appropriate, C_2H_2 was added at a concentration of 0.02% (v/v) to ensure inhibition of ammonia oxidation without influencing denitrification.[17,18]

The Kilner jars were kept at 21°C in the dark during the incubation. Gas and soil samplings were undertaken 6 and 24 h after addition of NH_4NO_3 as described above.

Gas analysis

Gas samples were analysed for total ($^{14+15}N$-)N_2O on an Agilent 6890 gas chromatograph fitted with an electron capture detector (column and detector temperatures 40 and 250°C, respectively). Samples (125 mL) were analysed for ^{15}N-N_2O and ^{18}O-N_2O on a SerCon 20/20 isotope ratio mass spectrometer (IRMS) following cryofocusing in an ANCA TGII gas preparation module (SerCon, UK). Samples containing 5% C_2H_2 were treated with potassium permanganate before isotopic measurement to decrease C_2H_2 concentrations.[19] To avoid memory effects, natural abundance samples were measured before analysis of enriched samples. As laboratory standards, ambient air and 5 ppm N_2O in N_2 (BOC Specialty Gases, UK) were used. Linearity of the measurement of ^{15}N-enriched samples was checked with dilutions of 98 atom % ^{15}N-N_2O (Isotec, Sigma-Aldrich). Samples for cross-calibrations of ^{15}N- and ^{18}O-N_2O were analysed at UC Davis, California, USA.

Mineral N analysis

For mineral N analysis, 20 g soil was extracted with 75 mL 1 M KCl and filtered through Whatman No. 1 filter paper (first drops discarded). The extracts were stored at 4°C until analysis. Concentrations of NH_4^+-N, NO_3^--N and NO_2^--N in the KCl extracts were determined by colorimetric analysis on a FIAstar spectrophotometer 5023. The ^{15}N enrichments of NH_4^+ and NO_3^- were determined by microdiffusion[20] and subsequent analysis on the IRMS. Flour was used as a laboratory standard for ^{15}N. Memory effects were prevented by measuring at least one flour sample between the analysis of differently enriched mineral N samples. The linearity of the IRMS for ^{15}N measurements was checked with $^{15}NH_4^{15}NO_3$. NO_2^- in the extracts from the method testing experiment was converted into N_2O following the method described by Stevens and Laughlin.[21] The KCl extract (20 mL) was made up to 50 mL with 2 M KCl in 125 mL amber gas-tight glass bottles (Supelco, USA). Then, 1 mL 1 M HCl and 0.5 mL 0.04 M hydroxylamine solution (hydroxylamine hydrochloride, BDH, UK) were added and the bottles shaken for 18 h. Gas samples (approximately 20 mL) were transferred to fresh He-flushed and evacuated 125 mL gas-tight bottles and analysed on the IRMS.

Calculations and statistical analysis

Equations (1) to (4) were used to calculate sources of N_2O based on treatments with the dual isotope method (subscript 'DI'; Fig. 1):

$$N_2O_{FD.DI} = c^{15}N_2O_{TR2} \quad (1)$$

$$N_2O_{NCD.DI} = (c^{15}N_2O_{TR2}/c^{15}NO_3^-{}_{TR2}) \times c^{15}NO_3^-{}_{TR3} \quad (2)$$

$$N_2O_{ND.DI} = (cN_2^{18}O_{TR4} \times cf - 2/3N_2O_{NCD.DI}) \times 2 \quad (3)$$

$$N_2O_{N.DI} = c^{15}N_2O_{TR1} - N_2O_{FD.DI} - N_2O_{NCD.DI} - N_2O_{ND.DI} \quad (4)$$

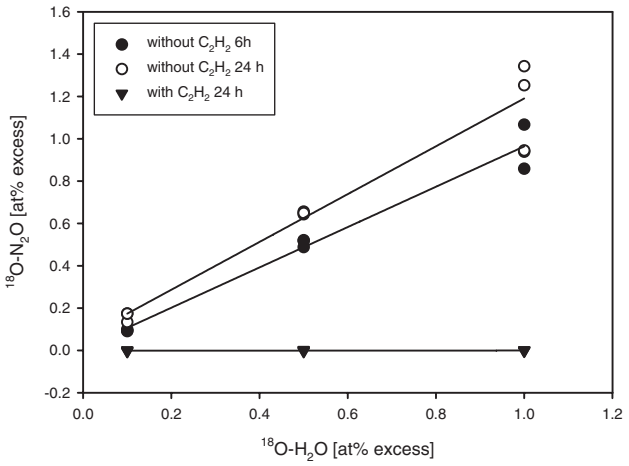

Figure 2. ^{18}O enrichment in N_2O after 6 and 24 h versus initial ^{18}O enrichment of water added at the start of the experiment with and without C_2H_2 (assumption testing). C_2H_2 was added where applicable at 5% (v/v). Each treatment received 400 mg NH_4NO_3-N kg^{-1} dry soil.

where '$c^{(15)}N_2^{(18)}O$' or '$c^{(15)}NO_3^-$' is the measured concentration in μmol or mmol kg^{-1} dry soil of (isotopically enriched) N_2O or NO_3^-, respectively. Subscripts 'TR' after N_2O indicate treatment numbers according to Table 1. Other subscripts after N_2O indicate sources of N_2O: 'FD' fertiliser-derived denitrification, 'NCD' nitrification-coupled denitrification, 'ND' nitrifier denitrification, and 'N' nitrification. 'cf' denotes a conversion factor to account for the application of $H_2^{18}O$ at 1 atom % excess ^{18}O while ^{15}N-labelled NH_4NO_3 was applied at 10 atom % excess ^{15}N. This factor (3.7) was derived from the ratio of $N_2^{18}O$ values of treatments with 1 atom % excess to 0.1 atom % excess $H_2^{18}O$ from the testing of assumptions (a) and (b).

Equations (5) to (8) were used to calculate sources of N_2O based on treatments with inhibitors (subscript 'IN'):

$$N_2O_{D.IN} = cN_2O_{TR5} - cN_2O_{TR7} \quad (5)$$

$$N_2O_{N.IN} = cN_2O_{TR6} - cN_2O_{TR7} \quad (6)$$

$$N_2O_{ND.IN} = (cN_2O_{TR1+2+3+4})/4 - cN_2O_{TR5} - cN_2O_{TR6} - cN_2O_{TR7} \quad (7)$$

$$N_2O_{'other\ sources'.IN} = c(N_2O_{TR7}) \quad (8)$$

where 'cN_2O' is a measured concentration of N_2O in μmol kg^{-1} dry soil. The subscripts are the same as explained above, with 'D' being total denitrification (fertiliser-derived and nitrification-coupled). 'Other sources' are sources of N_2O other than nitrification, denitrification and nitrifier denitrification.

Statistical analyses were performed with SPSS for Windows 10.0. Normality was tested using the Kolmogorov-Smirnov test. In normally distributed data, differences between treatments were analysed using analysis of variance (ANOVA, $\alpha = 0.05$). In the few cases where inhomogeneity of variances was detected using Levene's test, data were log-transformed before analysis. The $LSD_{0.05}$ statistics was used for multiple comparisons between means. Where the data was not normally distributed, the Kruskal-Wallis test was used to evaluate differences ($\alpha = 0.05$), with Schaich-Hamerle analysis as a post hoc test.

RESULTS

Assumption testing

The N_2O concentration in treatments without C_2H_2 increased linearly over the 24 h measurement period (data not shown). The average N_2O production from all treatments without C_2H_2 was 3.19 ± 0.38 μmol kg^{-1} dry soil day^{-1}. C_2H_2 decreased N_2O production by about 98% to an average of 0.06 ± 0.01 μmol kg^{-1} dry soil day^{-1}. There were no significant differences in N_2O production between ^{18}O treatments either in the presence or in the absence of C_2H_2.

In the treatments without C_2H_2, the atom % enrichment of ^{18}O in N_2O was positively correlated with the ^{18}O enrichment of the added water ($R^2 = 0.98$–0.99; $P < 0.001$; Fig. 2). When 5% C_2H_2 was added to inhibit both ammonia oxidation and reduction of N_2O to N_2 in denitrification, no significant ^{18}O enrichment in N_2O was detected.

There was no significant difference in concentrations of available NH_4^+ and NO_3^- between treatments (Fig. 3), or between 6 and 24 h. NO_2^- concentrations increased in the first 6 h in treatments without C_2H_2, and later decreased. Concentrations were significantly lower in all treatments with C_2H_2, but there was no significant effect of the ^{18}O enrichment of water. ^{15}N in NH_4^+ and NO_3^- remained at background levels throughout the experiment (0.41 ± 0.14 and 0.38 ± 0.02 atom % ^{15}N for NH_4^+ and NO_3^-, respectively), and was not significantly different from the unlabelled NH_4NO_3 used as a blank for the diffusion technique.

^{18}O-^{15}N-enrichment method

N_2O production

Total N_2O production was linear in all treatments during the 24 h experiment (data not shown). There were no significant differences between treatments without inhibitors (N_2O production averaged 1.47 ± 0.11 μmol N_2O-N kg^{-1} dry soil day^{-1}; Fig. 4(a)). Flushing with O_2 at the beginning of the experiment significantly ($P < 0.05$) decreased N_2O production (0.84 ± 0.07 μmol N_2O-N kg^{-1} dry soil day^{-1}). In both treatments with C_2H_2, N_2O production was significantly lower ($P < 0.001$) than in all other treatments (0.16 ± 0.02 and 0.12 ± 0.03 μmol N_2O-N kg^{-1} dry soil day^{-1} for TR5 and TR7, respectively).

The ^{15}N atom % enrichment of N_2O differed between treatments (Fig. 4(b)). Enrichment was higher ($P < 0.001$) in the treatment receiving $^{15}NH_4^{15}NO_3$ (TR1) than in that receiving $^{15}NH_4NO_3$ (TR3), and higher ($P < 0.001$) where the NH_4^+ component was labelled (TR3) than where the NO_3^- was labelled (TR2). The use of inhibitors (TR 5–7) further decreased ($P < 0.05$) the ^{15}N enrichment of N_2O after application of $^{14}NH_4^{15}NO_3$. There were no significant differences between the inhibitor treatments. As expected, the ^{15}N enrichment of N_2O after addition of unlabelled NH_4NO_3 (TR4) was at natural abundance levels. The trends were similar for ^{15}N-N_2O concentrations, but differences between measurements after 6 and 24 h were more pronounced. The ^{15}N-N_2O concentrations in TR 5–7 with inhibitors were not

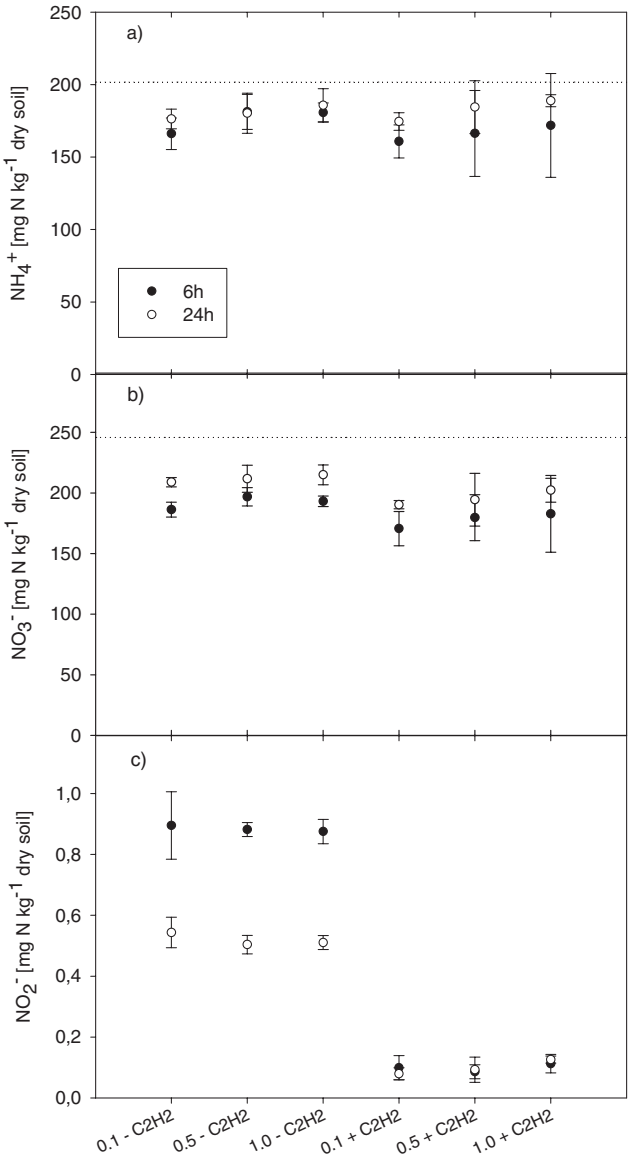

Figure 3. Soil mineral nitrogen concentrations at 6 and 24 h (assumption testing): 0.1, 0.5, or 1.0: 0.1, 0.5, or 1.0 atom % excess ^{18}O-H_2O, respectively; -C2H2: no C_2H_2, +C2H2: with 5% (v/v) C_2H_2. Dotted lines indicate initial concentrations immediately after fertiliser application.

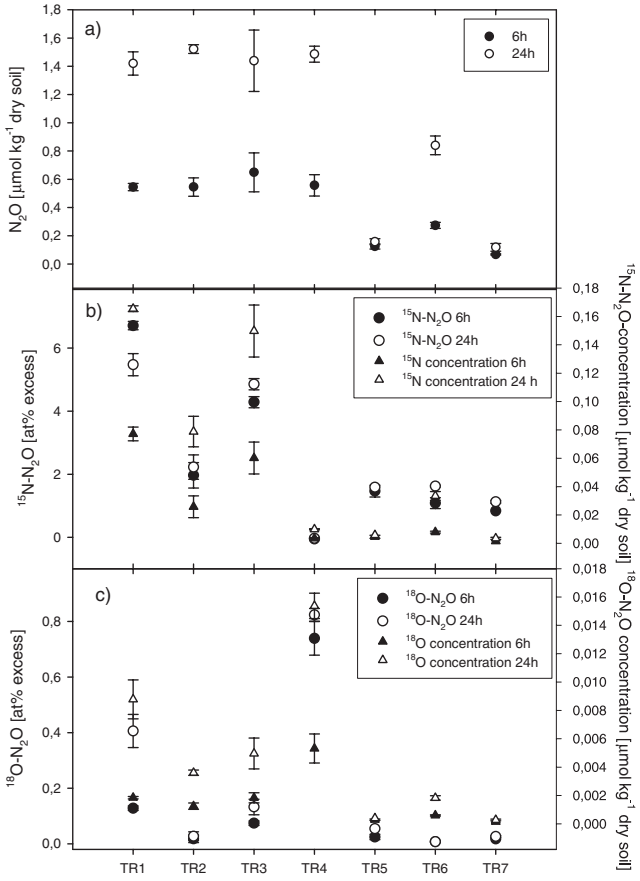

Figure 4. N_2O production (a), atom % enrichment of ^{15}N-N_2O and concentration of ^{15}N-N_2O (b) and atom % enrichment of ^{18}O-N_2O and concentration of ^{18}O-N_2O (c) at 6 and 24 h of incubation (enrichment experiment). The treatment labels on the x-axis correspond to those in Table 1.

significantly different from that with unlabelled NH_4NO_3 (TR4).

The ^{18}O enrichment of N_2O was highest ($P < 0.005$) in the treatment that had received ^{18}O-labelled H_2O (TR4; Fig. 4(c)), with 0.74 and 0.82 atom % excess ^{18}O at 6 and 24 h, respectively. However, N_2O from treatments to which ^{15}N-labelled NH_4^+ had been applied (TR 1 and 3) were also enriched in ^{18}O and this enrichment was slightly, but not significantly, higher in the double-labelled $^{15}NH_4^{15}NO_3$ (0.12 and 0.41 atom % excess ^{18}O at 6 and 24 h, respectively) than in the single-labelled $^{15}NH_4NO_3$ treatment (0.07 and 0.13 atom % excess ^{18}O at 6 and 24 h, respectively). The same trend was observed in ^{18}O-N_2O concentrations, especially after 24 h. All treatments fertilised with $NH_4^{15}NO_3$ produced N_2O with the same ^{18}O signature (0.02 and 0.03 atom % excess ^{18}O at 6 and 24 h, respectively), regardless of the use of inhibitors.

Soil mineral N

There were no significant differences in NH_4^+ and NO_3^- concentrations between treatments or between sampling times (Fig. 5). Concentrations of NO_2^- decreased between 6 and 24 h, but there were no significant differences between treatments, except in those receiving C_2H_2 (TR 5 and 7), where concentrations of NO_2^- were significantly lower ($P < 0.001$) than in the other treatments.

The measured ^{15}N enrichment of NH_4^+ in treatments TR1 and TR3 (12.0 ± 2.0 atom % excess ^{15}N) was not significantly different from the 10 atom % excess of the $^{15}NH_4^+$ added (Fig. 6). No significant differences were detected between the other treatments. NO_3^- was enriched in ^{15}N in all treatments receiving ^{15}N-NO_3^- (TR 1, 2, 5, 6, and 7), with no significant differences between these treatments. The measured enrichment was lower than the 10 atom % excess of the ^{15}N-NO_3^- applied (6.7 ± 0.9 atom % excess). ^{15}N enrichment of NO_2^- varied between treatments ($P < 0.001$). The highest enrichment was measured in the double-labelled treatment (TR1, 7.2 ± 1.8 and 6.1 ± 0.1 atom % excess ^{15}N at 6 and 24 h, respectively). At 6 h, the enrichment of $^{15}NO_2^-$ was only slightly lower in the $^{15}NH_4^+$ treatment (5.9 ± 1.8 atom % excess ^{15}N), but decreased significantly ($P < 0.001$) thereafter to only 1.8 ± 0.2 atom % excess ^{15}N at 24 h. Addition of

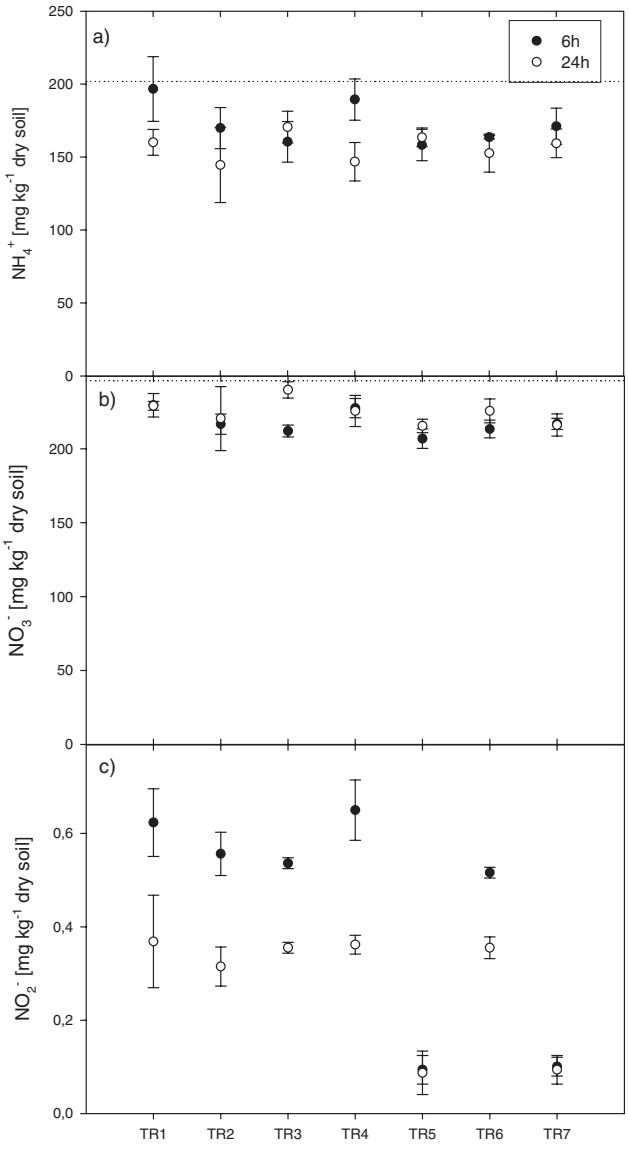

Figure 5. Mineral nitrogen concentrations in the soil after 6 and 24 h (enrichment experiment). Dotted lines indicate initial concentrations. Treatment labels (x-axis) correspond to those in Table 1.

inhibitors significantly decreased the ^{15}N enrichment of NO_2^- compared to TR2, which had also received $^{14}NH_4^{15}NO_3$, but no inhibitors. This effect was stronger for the treatments with C_2H_2 (TR 5 and 7) than for the treatment with only O_2 (TR6). The trends for ^{15}N concentrations of mineral N were the same as for the atom % enrichments.

Sources of N_2O

Calculation of the sources of N_2O according to the proposed new enrichment method (Eqns. (1)–(4)) identified nitrifier denitrification and fertiliser denitrification to be the main sources of N_2O (Fig. 7), each accounting for 44% of the total N_2O emission over 24 h. Nitrification accounted for 30% of N_2O production within the first 6 h, but had become less important by 24 h (2% of N_2O production). In contrast, the contribution from nitrification-coupled denitrification increased between 6 and 24 h from 4 to 10% of total production.

Calculation of the sources of N_2O from treatments with inhibitors (Eqns. (5)–(8)) identified nitrification and nitrifier denitrification as the main sources of N_2O (Fig. 7). Together, these processes were responsible for almost 80% of the production in the first 6 h, and 90% over 24 h. Fertiliser denitrification contributed about 10% to the N_2O production after 6 h and less than 3% after 24 h. 'Other sources' were responsible for about 10% of the N_2O production at both measurement times.

DISCUSSION

Assumptions associated with addition of ^{18}O-H_2O

Enrichment of ^{18}O-N_2O increased with addition of increasingly enriched ^{18}O-H_2O (Fig. 2; n = 9). As the gradients of the regression lines after 6 and 24 h were not significantly different from 1, it could be concluded that no preferential removal of ^{16}O or ^{18}O took place in this soil over 24 h. Thus our first assumption (a) that there would be no preferential removal of ^{18}O or ^{16}O in nitrifier denitrification and denitrification could be confirmed. Furthermore, the stability of the response over 24 h indicates that the enrichment of the H_2O pool remained constant over this time period, confirming assumption (b).

The third assumption (c) that there would be no oxygen exchange between ^{18}O-labelled H_2O and NO_3^- was validated by the absence of an increase in enrichment of ^{18}O-N_2O with increasing ^{18}O enrichment of the added water in C_2H_2-treated soils. The ^{18}O enrichment of N_2O remained stable over the 24 h and not significantly different from natural abundance levels in ambient air (Fig. 2, n = 9). Other authors have previously found no oxygen exchange when growing pure cultures of denitrifiers on NO_3^-.[22] Casciotti *et al.*[23] reported that oxygen atoms from ^{18}O-H_2O contributed less than 10%, and often less than 3%, to the oxygen atoms in N_2O produced by *Pseudomonas aureofaciens*. However, oxygen exchange has been shown to be more important when pure cultures of denitrifiers were grown on NO_2^- or NO.[22,24,25] Since NO_3^- was readily available in our soil, denitrifiers probably used this as substrate, leading to the observed negligible ^{18}O exchange between H_2O and NO_3^-. This relationship may be expected to vary depending on soil type and N availability, and so we recommend that this assumption always be tested prior to application of the ^{18}O-^{15}N method we present here.

As all assumptions were confirmed here, we could use the addition of ^{18}O-H_2O along with the existing ^{15}N technique[13] to distinguish between nitrification, nitrifier denitrification and denitrification as sources of N_2O production in this soil.

N_2O source determinations

Both our new ^{18}O-^{15}N-enrichment method and the inhibition (0.02% C_2H_2 and 80% O_2, v/v) method identified nitrifier denitrification as the predominant source of N_2O in our soil (Fig. 7). However, the methods differed in their estimation of the relative contribution of the other microbial sources, with fertiliser denitrification being important with the enrichment method and nitrification being the main N_2O source with the inhibition method. In our opinion, these differences between

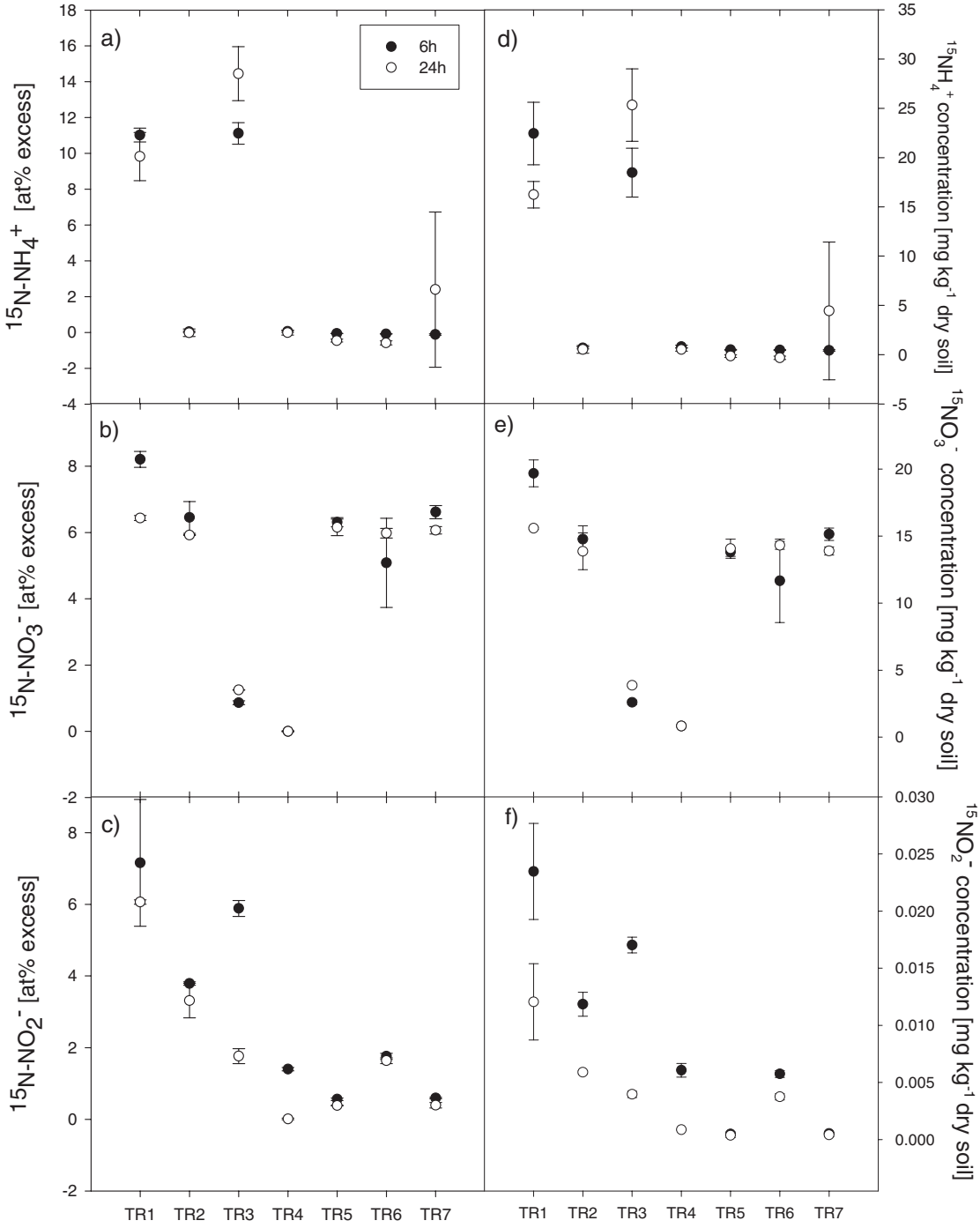

Figure 6. ^{15}N-enrichment (a–c) and ^{15}N-concentrations (d–f) of mineral nitrogen in the enrichment experiment. Treatment labels (x-axis) correspond to those in Table 1.

methods may in part be attributed to known problems associated with the reliance on inhibitors. We now discuss these in more detail.

Problems associated with inhibitors

An overestimation of nitrification and underestimation of denitrification can be caused by incomplete inhibition of denitrification and nitrifier denitrification by large concentrations of O_2.[10] That denitrification was indeed not fully inhibited by O_2 in this study can be concluded from the enrichment of ^{15}N-NO_2^-: In treatments with $^{14}NH_4^{15}NO_3$ and 80% O_2 application (TR6), NO_2^- was significantly enriched in ^{15}N (Fig. 6(c)). Thus, NO_2^- must have been produced from NO_3^-, either in denitrification or via DNRA and subsequent ammonia oxidation. As DNRA was considered to be negligible since no ^{15}N-NH_4^+ was detected in TR2 at 6 or 24 h after addition of $NH_4^{15}NO_3$ (Fig. 6(a)), we conclude that denitrification was not completely inhibited by 80% O_2. Incomplete inhibition of denitrification by O_2 was confirmed by the ^{15}N enrichment of N_2O from TR6 to which $^{14}NH_4^{15}NO_3$ had been applied. The enrichment of ^{15}N-N_2O from TR6 was only slightly lower than that from TR2, which had also received

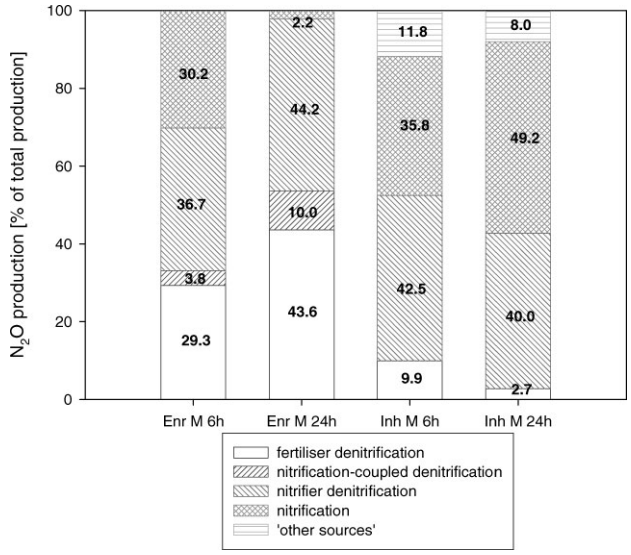

Figure 7. N_2O production by different processes, measured at 6 and 24 h with the new ^{18}O-^{15}N-enrichment method (Enr M) or the inhibition (C_2H_2+O_2) method after Webster and Hopkins[9] (Inh M). The percentage of total N_2O production is given per process.

$^{14}NH_4^{15}NO_3$, but no inhibitors, and was significantly greater than the enrichment in TR4, which had received $^{14}NH_4^{14}NO_3$. This incomplete inhibition of denitrification by O_2 would have resulted in an overestimation of the contribution of 'other sources' to N_2O production. It is possible that inhibition was incomplete as only 80% O_2 instead of 100% O_2 was used, but even application of 100% O_2 has previously been suspected to result in incomplete inhibition.[10] Although problems with the use of large concentrations of O_2 as an inhibitor have been described previously,[10–12] this is to our knowledge the first time that incomplete inhibition of fertiliser denitrification by large concentrations of O_2 has been directly measured in soil. This incomplete inhibition may in part be attributable to aerobic denitrification, which has previously been indicated to occur in this arable soil.[14]

The effect of 80% O_2 on nitrifier denitrification could not be directly measured in this study. However, we speculate that the effect of large concentrations of O_2 on denitrification and nitrifier denitrification should be similar, as the enzymes involved in these processes are thought to be similar.[26] In that case, nitrifier denitrification would have been underestimated by the inhibition method and nitrification overestimated.

Addition of C_2H_2 should have inhibited nitrification, nitrifier denitrification and nitrification-coupled denitrification, without affecting fertiliser denitrification or 'other sources' of N_2O. Fertiliser denitrification was estimated using our ^{18}O-^{15}N-enrichment method to be responsible for 29.3 or 43.6% of the total $^{14+15}N$-N_2O production after 6 and 24 h, respectively. However, C_2H_2 addition decreased N_2O production to only 21.7 and 10.8% of the total production after 6 and 24 h. It can therefore be assumed that fertiliser denitrification was either overestimated by our enrichment method or in some way affected by the C_2H_2. The ^{15}N-enrichment method for quantifying fertiliser denitrification is an accepted, widely used[13,14,27] and reliable method, as it directly quantifies ^{15}N-N_2O from ^{15}N-NO_3^- (Eqn. (1)). However, our results showed that addition of C_2H_2 decreased NO_2^- production and the ^{15}N enrichment of this NO_2^- significantly to approximately background levels (TR5; Figs. 5(c) and 6(c)). As denitrification of added ^{15}N-NO_3^- in this treatment should have resulted in production of enriched NO_2^-, this suggests an inhibitory effect of C_2H_2 on ^{15}N-NO_3^- reduction in denitrification. Furthermore, application of 5% C_2H_2 in the assumption testing, which should have inhibited the reduction of N_2O to N_2 in denitrification, generally leading to larger N_2O production, instead decreased N_2O production to only about 2% of the total production measured in the absence of C_2H_2 (Fig. 2). We therefore conclude that addition of C_2H_2 had an unwanted effect on fertiliser denitrification in this soil, which would have resulted in an underestimation of the contribution of denitrification and an overestimation of the contribution of nitrification to N_2O production.

The above evidence suggests that addition of C_2H_2 and O_2 in the inhibitor method most probably results in incomplete inhibition of target enzymes or unwanted side effects (see also[10,11]), leading to inaccurate estimates of N_2O sources, and a probable overestimation of nitrification and underestimation of denitrification and nitrifier denitrification.

Advantages and limitations of our ^{18}O-^{15} N-enrichment method

Our new enrichment method resulted in higher and lower apportionations of fertiliser denitrification and nitrification, respectively, as sources of N_2O compared to the inhibitor method. As we concluded above that the latter method probably overestimated nitrification and underestimated denitrification, our enrichment method appears to provide a more reliable determination of the source of N_2O in soil. The enrichment method indicated a reduced importance of nitrification in the last 18 h of incubation and an increase in the importance of nitrifier denitrification and both fertiliser and nitrification-coupled denitrification. This is in accordance with the ^{15}N-NO_2^- enrichment in TR3 after addition of ^{15}N-NH_4^+ being high at 6 h, indicating a production via nitrification from $^{15}NH_4^+$, but lower at 24 h, suggesting a dilution with NO_2^- from fertiliser denitrification (Figs. 6(c) and 6(f)).

N_2O was enriched in ^{18}O not only in the treatment that had received ^{18}O-labelled water (TR4), but also unexpectedly, although to a lesser extent, in the $^{15}NH_4^{15}NO_3$ and $^{15}NH_4^{14}NO_3$ treatments (TR 1 and 3). We believe that a contamination with ^{18}O from $H_2^{18}O$ in these treatments can be excluded due to the special care taken during set-up, sampling and measurement. If at all, contamination might have affected one replicate, but not all. The ^{18}O enrichment in N_2O from TR 1 and 3 was thought to result from ^{18}O enrichment of H_2O during the production of ^{15}N-labelled NH_4NO_3. However, measurement of ^{18}O in single- and double-labelled NH_4NO_3 fertiliser by TC-EA revealed only a slight ^{18}O enrichment in $^{14}NH_4^{15}NO_3$ ($\delta^{18}O_{SMOW} = 12.1 \pm 0.5‰$), not sufficient to account for the ^{18}O enrichment of the N_2O emitted from these treatments. The $^{15}NH_4^{15}NO_3$ fertiliser was even slightly depleted in ^{18}O ($\delta^{18}O_{SMOW} = -25.2 \pm 4.6‰$). Drying of the NH_4NO_3 at 40°C for 24 h before analysis slightly decreased the enrichment to $10.1 \pm 0.7‰$ and $-30.6 \pm 2.6‰$ for

$^{14}NH_4^{15}NO_3$ and $^{15}NH_4^{15}NO_3$, respectively. We do not conclusively know the reason for the ^{18}O enrichment of N_2O in TR 1 and 3. However, since it was associated with the application of isotopically labelled NH_4NO_3, it has not affected the ^{18}O-N_2O in TR4 and, as only the ^{18}O enrichment of N_2O from this treatment with added ^{18}O-H_2O was used in the calculations, artefacts in the other treatments did not affect our N_2O source determination.

Our ^{18}O-^{15}N-enrichment technique enables quantification of N_2O emissions from nitrification, nitrifier denitrification, nitrification-coupled denitrification and fertiliser denitrification without reliance on inhibitors. This provides a significant advance over the ^{15}N-enrichment technique as it facilitates determination of nitrifier denitrification and nitrification-coupled denitrification. If the traditional ^{15}N-enrichment method had been used, calculating the contribution from nitrification as the difference in ^{15}N-N_2O from treatments with $^{15}NH_4^{15}NO_3$ from those with $NH_4^{15}NO_3$, nitrification would have been estimated to have caused 70 and 56% of total N_2O production at 6 and 24 h, respectively. The new ^{18}O-^{15}N method showed that up to 78% of this was in fact due to nitrifier denitrification. The new method thus enabled the first direct evidence of the significance of nitrifier denitrification in N_2O emissions from soils.

We consider our ^{18}O-^{15}N-enrichment method to have several advantages: It enables differentiation between nitrification, nitrifier denitrification, nitrification-coupled denitrification and fertiliser denitrification as sources of N_2O, is relatively easy to use and does not require inhibition of any kind. However, there are also some disadvantages: (1) ^{18}O-labelled H_2O is quite expensive and so the use of ^{18}O-depleted H_2O may be a cheaper and therefore more practical alternative; (2) until a sufficient body of studies has been compiled, we recommend testing assumption (c) that there is no significant ^{18}O exchange between ^{18}O-H_2O and NO_3^- for different soil types and conditions, preferably after thoroughly testing the effect of large concentrations of C_2H_2 on fertiliser denitrification; (3) NO_3^- and NH_4^+ have to be added to the soil, which would be problematic in natural systems; (4) if other sources such as DNRA or aerobic denitrification produce significant quantities of isotopically enriched N_2O, this would lead to an overestimation of N_2O from the processes considered here; and (5) it is not possible with our enrichment method to subdivide the nitrification source into autotrophic and heterotrophic nitrification. For this differentiation, a combination of the enrichment method with enzyme assays might be used.

In conclusion, our ^{18}O-^{15}N-enrichment method enables a separation between more sources of N_2O in soils than was possible before, and has provided the first direct evidence for the significance of nitrifier denitrification as an N_2O-producing process in soils. We consider isotopic labelling of pathways of N_2O production to provide a more reliable and less disruptive alternative to the use of inhibitors for quantifying sources of N_2O in soil, and to be associated with fewer conceptual uncertainties.

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