An improved ^{15}N tracer approach to study denitrification and N turnover in soil incubations

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Abstract

Rationale: Denitrification (the reduction of oxidized forms of inorganic N to N_2O and N_2) from upland soils is considered to be the least well understood process in the global N cycle. The main reason for this lack of understanding is that the terminal product (N_2) of denitrification is extremely difficult to measure against the large atmospheric background.

Methods: We describe a system that combines the ^{15}N-tracer technique with a 40-fold reduced N_2 (2 % v/v) atmosphere in a fully automated incubation set up for direct quantification of N_2 and N_2O emissions. The $\delta^{15}N$ values of the emitted N_2 and N_2O were determined using a custom-built gas preparation unit that was connected to a DELTA V Plus isotope ratio mass spectrometer. The system was tested on a pasture soil from sub-tropical Australia under different soil moisture conditions and combined with ^{15}N tracing in extractable soil N pools to establish a full N balance.

Results: The method proved to be highly sensitive for detecting N_2 (1.12 µg N h^{-1} kg^{-1} dry soil (ds)) and N_2O (0.36 µg N h^{-1} kg^{-1} ds) emissions. The main end product of denitrification in the investigated soil was N_2O for both water contents, with N_2 accounting for only 3% to 13% of the total denitrification losses. Between 90 and 95% of the added ^{15}N fertiliser could be recovered in N gases and extractable soil N pools.

Conclusions: The high and N_2O-dominated denitrification rates found in this study are pointing at both the high ecological and the agronomic importance of denitrification in subtropical pasture soils. The new system allows for a direct and highly sensitive detection of N_2 and N_2O fluxes from soils and may help to significantly improve our mechanistic understanding of N cycling and denitrification in terrestrial agro-ecosystems.

Introduction

Denitrification (the reduction of oxidized forms of inorganic N to N_2O and N_2) from upland soils is considered to be the least well understood process in the global N cycle, and the lack of information on denitrification is a fundamental constraint on the ability of society to address N pollution problems in many areas[1, 2]. The main reason for this lack of understanding is that denitrification is extremely difficult to measure due to methodological limitations of existing methods to quantify the dominant end-product (N_2) of denitrification against the huge atmospheric N_2 background[3]. Nitrous oxide is produced as an intermediate during the denitrification process and can be emitted to the atmosphere causing environmental pollution due to its high global warming potential (298 times that of CO_2) and its contribution to stratospheric ozone depletion[4, 5]. While N_2O fluxes from soils have been extensively studied, the total denitrification losses, N_2 emissions and the N_2O/N_2 partitioning are virtually unknown for most terrestrial ecosystems [3, 6]. Few methods exist for measuring both N_2O and N_2 fluxes from soil. Currently, there are two main approaches for the direct quantification of denitrification gas formation, including N_2 and N_2O: (1) The He gas flow soil core method, which directly measures N_2 and N_2O formation in an almost N_2-free atmosphere with a N_2 background of a few ppm only[7], and (2) the ^{15}N tracing technique, which requires $^{15}NO_3^-$ application to soil followed by the determination of $^{15}N_2$ and/or $^{15}N_2O$ in the gaseous product pool[8]. The major drawbacks of the He soil core technique are the extreme technical effort to reach the required gas tightness against the intrusion of atmospheric N_2, and the time needed to establish an N_2-free atmosphere, which is mainly determined by outgassing of N_2 dissolved in soil water. In contrast, the major drawback of the ^{15}N tracing technique is the low sensitivity of N_2 flux measurements due to the large atmospheric N_2 background and the rather high $^{30}N_2$ detection limits using current isotope ratio mass spectrometry (IRMS) instruments due to significant interferences at *m/z* 30 arising from NO^+ ions formed in the ion source[9].

Here, we present an improved ^{15}N tracer approach that is based on previously developed methods of combining the ^{15}N-tracer technique with an artificial atmosphere [10-12]. The method uses the background air in soil incubation vessels that is replaced with a helium–oxygen gas mixture with a 40-fold reduced N_2 background (2 % v/v) and combines it with IRMS analysis allowing for direct quantification of N_2 and N_2O emissions from soil. This approach allows for the direct IRMS analysis of the change in the $\delta^{15}N$ values of the headspace N_2 with sufficient sensitivity, but does not require the addition of high levels of highly enriched $^{15}NO_3^-$. Moreover, it minimises the sensitivity to both intrusion of atmospheric N_2 and outgassing of water-dissolved N_2, as the contribution of these two processes is very small at the background N_2 concentration used in the new method (2% v/v) [10].

In this study, we describe a system that was further refined for the use in soil incubations in a laboratory setup (a) using a fully automated gas purging and sampling system that was linked to (b) a custom-built gas preparation unit connected to an isotope ratio mass spectrometer, to measure changes in the concentration and isotopic signature of N_2 and N_2O in the incubation vessel's headspace over time. Finally, we used the system to investigate the influence of

different soil moisture conditions on N_2 and N_2O emissions and gross N turnover rates from a subtropical pasture soil.

Materials and Methods

Automated gas sampling system

Direct and sensitive measurement of both N_2 and N_2O emission from incubated soil with the improved ^{15}N tracer approach requires the replacement of the atmosphere in the incubation vessel with a helium–oxygen gas mixture with a 40-fold reduced N_2 background (2 % v/v) and subsequent sampling and analysis of the vessel's headspace air by IRMS over time. The following two steps were carried out in a fully automated way by a gas sampling system, as shown in Fig. 1:

- purging of the incubation vessel with an N_2-reduced artificial atmosphere (2% N_2 and 20.5% O_2 in Helium)
- headspace sampling at defined intervals to measure changes in the concentration and isotopic signature of N_2 and N_2O in the vessel's headspace over time.

The system accommodated seven 1000 mL Duran® incubation flasks (DURAN Group GmbH, Wertheim, Germany), which were capped with a metal plate with two ¼ in stainless steel lead through tubes each (inlet and outlet). The plates were sealed to the flask via screw caps and greased Teflon® rings. The flask inlets were connected to the automated flushing and sampling unit via a tenfold valve array (Bürkert Fluid Control Systems, Ingelfingen, Germany) (Fig. 1). The flask outlets were connected to magnetic 2/2-way valves each (Huba Control AG, Würenlos, Switzerland). The flasks could be both sampled (5 mL/min) and purged (1 L/min) through two ¼ in. stainless steel tubes.

Flasks 1-6 were used for soil incubations, while flask number 7 remained empty to be used as reference/standard. To minimise any diffusion of atmospheric N_2 into the incubation vessels, the sampling system was housed in a sealed aluminium box (55 cm x 35 cm x 40 cm) (Zarges GmbH, Weilheim, Germany), which was continuously flushed with argon during the operation of the system. To ensure temperature control over the course of the experiment, the aluminium box was placed into a thermostated water bath (0-40º C). At the onset of the incubation experiment the reduced N_2 background was established by repeated cycles of flushing flasks 1-6 with a gas mix containing 2% N_2 ($\delta^{15}N$ = -15.09 ‰ vs air-N_2) and 20.5% O_2 in He (Air Liquide Deutschland GmbH, Frankfurt, Germany). Overall flushing time was ten minutes per flask with a flow rate of 1 L min^{-1}. Flask 7 was used as reference and flushed the same way with a N_2O calibration standard containing 50 ppm N_2O in helium ($\delta^{15}N$ = -0.18 ‰ vs. air-N_2) (Air Liquide).

Once the flushing was completed, the automated gas sampling procedure started with flask 1 by extracting 100 mL (5 mL/min) of headspace gas using a gas pump (MB-41E) (Metal Bellows, Sharon, MA, USA) and injecting it into the isotope ratio mass spectrometer sample preparation unit (Fig. 2). The gas pump was connected to two parallel flow resistance capillaries to provide constant flow rates of 1 mL/min for N_2 sampling and 4 mL/min for N_2O collecting.

Since concentration analysis is very sensitive to pressure changes inside the incubation vessel the removed gas volume was replaced immediately with the respective gas mixture that was

used for flushing the flasks. Gas samples were taken sequentially from each incubation flask (1-6) followed by a calibration sample (flask 7). In total, each flask was sampled three times per day during the incubation experiment.

IRMS analysis for N_2 and N_2O took 3445 s per sample. Consequently, it took 6.7 hours to sample all seven flasks one time and 20 hours to complete a full cycle of three samples per flask (21 measurements).

Gas preparation unit and IRMS analysis

For the analysis of the headspace gas samples the automated sampling system was linked to a custom-built gas preparation unit that was connected to a DELTA V Plus isotope ratio mass spectrometer (Thermo Scientific, Waltham, MA, USA). The schematic setup of the automated gas sampling and preparation unit is shown in Fig. 2.

A headspace gas sample of 100 mL (5 mL/min) was pumped from the incubation flask through two water/CO_2 traps based on Sicapent® (Elementar, Hanau, Germany) and NaOH on support (VWR, Radnor, PA, USA) filling from the gas sample to the sample preparation unit. The "H_2O/CO_2 Trap 1" (Fig. 2) removes the moisture and CO_2 of the sample coming out of the flasks. The "H_2O/CO_2 Trap 2" is placed ahead the first cryo trap to remove remaining traces of water and CO_2 from the calibration gas and sample flasks (Fig. 2).

Two liquid nitrogen traps controlled by pneumatic pistons were used for the trapping and focusing of the N_2O in the gas sample. The first trap ("Cryo Trap 1") was connected to an 8-port valve (VICI AG International, Schenkon, Switzerland) and was used to freeze N_2O and separate it from the other gases in the gas sample. A parallel gas sample bypass at the 8-port valve was used for the transfer of 1/5 of the sample (1 mL/min) to a sampling loop (length 180 cm, inner diameter 0.5 mm, volume 353 µL) within a 6-port valve (VICI AG) for direct N_2 analysis, while 4/5 of the gas sample (4 mL/min) was led through "Cryo Trap 1" to collect N_2O. The sequence was as follows (see Fig. 2). First, Cryo Trap 1 was submerged in liquid nitrogen to trap N_2O, and at the same time Cryo Trap 2 was exposed to room atmosphere in order to direct N_2 to the separation columns ("mode 1"). In a second step ("mode 2"), Cryo Trap 1 was exposed to room atmosphere and Cryo Trap 2 was submerged in liquid nitrogen to focus N_2O. Then, by lifting Cryo Trap 2 out of the liquid nitrogen, N_2O was directed towards the separation columns.

In mode 1, N_2 and O_2 were separated by use of a GC column (molecular sieve, 5Å, 60/80 mesh, 200 cm length 1/16 in. o.d., 0.5 mm i.d. at a temperature of 10 °C).

Between the molecular sieve column and the isotope ratio mass spectrometer, a magnetic 3/2-way valve (Fluid Automation Systems GmbH, Fellbach, Germany) was installed in order to dilute the sample, thus reducing the amplitude of the O_2 peaks. The dilution flow was 33 mL/min and the gas sample flow was 2 mL/min. Finally, the N_2 quantity and the $\delta^{15}N$ values of the N_2 in the sample were measured by IRMS.

After N_2 was analysed, the system was switched to mode 2. The trapped N_2O was released by moving "Cryo Trap 1" out of the liquid nitrogen and focused in the sampling loop of the 6 port valve, which had been submerged into the liquid nitrogen and hence served as "Cryo

Trap 2". Nitrous oxide was transferred from "Cryo Trap 1" to "Cryo Trap 2" in the helium flow. After finishing the N_2O transfer, "Cryo Trap 2" was lifted out of the liquid nitrogen and N_2O was transferred through a CP-Porabond Q fused silica capillary GC column at 10 °C (Agilent Technologies Inc., Santa Clara, CA, USA) (25m x 0.32 mm i.d), where N_2O was separated from any remaining contaminants (NO, CO_2) in the gas sample before measurement of the N_2O concentration and $\delta^{15}N$ value of the N_2O via IRMS. Switching between N_2 separation in the molecular sieve column (mode 1: collecting of N_2O, analysis of N_2) and N_2O separation in the Porabond GC column (mode 2: analysis of N_2O) was deployed by use of a further 8-port valve (VICI AG). Finally, the N_2O quantity and the $\delta^{15}N$ value of the N_2O in the sample were determined by IRMS.

Calculation of the gas fluxes

N_2 and N_2O production

The N_2O production rates per mass of incubated soil were obtained from the measured N_2O concentration changes observed over the sampling points in time. Based on the N_2O peak area it is possible to determine the N_2O concentration in the headspace of the incubation flasks. The calibration of this peak area, as a sum of the peaks of *m/z* 44, 45 and 46, was performed with the standard of known N_2O mixing ratio (50 µL L^{-1}) in flask 7. N_2O emissions were calculated from the slope of the linear increase of the three gas headspace concentrations over time.

$$F = \frac{\Delta C_{N_2O} \cdot V_{head} \cdot MW}{M_{ds} \cdot MV \cdot 10^3} \cdot \frac{273.15}{(273.15+T)} \quad (1)$$

where F is the N_2O emission (µg N kg^{-1}ds); ΔC_{N2O} is the change in mixing ratio over time (µL L^{-1} h^{-1}); MW is the molecular weight of N in N_2O (28 g mol^{-1}); M_{ds} is the dry weight of the soil (kg); MV is the molar volume of the gas at 273 K and 1013 hPa (L mol^{-1}); and T is the incubation temperature (ºC). The R^2 values of the linear regression of the N_2O mixing ratio increased over time, ranging from 0.91 to 1.00, indicating that the linear regression approach was appropriate and that no fluxes had to be discarded.

The N_2 production was calculated on the basis of the $\delta^{15}N$ values of N_2O and N_2 in the headspace air observed at the 3 sampling points in time.

$$N_{2\,formed} = \frac{(\ \delta^{15}N_{\mathrm{N2sample}} \bullet n_{N\,2\,\mathrm{sample}} - \delta^{15}N_{N\,2\,flask} \bullet n_{Nflask})}{\delta^{15}N_{N\,2Osample}} \quad (2)$$

where $\delta^{15}N_{2\ \mathrm{sample}}$ and $\delta^{15}N_{\mathrm{N2O\ sample}}$ are the $\delta^{15}N$ values of N_2 and N_2O, respectively, measured in the headspace gas sample (‰), and $\delta^{15}N_{2\ \mathrm{flask}}$ is the $\delta^{15}N$ value of the reduced N_2 background in the flask headspace at the start of the incubation. $n_{N2\ \mathrm{sample}}$ is the measured quantity of N in the flask headspace (mol), and n_{Nflask} the quantity of N in the flask headspace (mol) at the start of the incubation.

This procedure of calculating the N_2 production is based on the assumptions that:

i. N_2 is always derived from the reduction of N_2O.
ii. Isotopic discrimination during N_2O reduction is negligible at ^{15}N-enriched levels.

Correction of gas sample dilution

For the analysis of headspace gas concentrations a total of 100 mL of gas was withdrawn from the sample flasks and replaced immediately with the gas mixture that was used for flushing the flasks. The dilution effect caused by this replacement in the flasks headspace was accounted for in the calculation by a simple mixing model. The N_2O concentrations were corrected by:

$$C_{corr} = C_{meas} \cdot \frac{V_{flask} - V_{sample}}{V_{flask}} \quad (3)$$

where C_{corr} is the N_2O concentration in the flask before refilling (mol mL^{-1}), C_{meas} the measured is N_2O concentration (mol mL^{-1}), V_{flask} the total gas volume of the flasks (mL) and V_{sample} the volume of the sample withdrawn (100 mL).

The $\delta^{15}N$ value of the N_2 in the headspace was corrected by:

$$\delta^{15}N_{corr} = \frac{(n_{meas} \bullet \delta^{15}N_{N2meas}) - (n_{sample} \bullet \delta^{15}N_{N2sub})}{n_{meas} - n_{sample}} \quad (4)$$

where $\delta^{15}N_{corr}$ is the $\delta^{15}N$ value of the N_2 in the flask before refilling, $\delta^{15}N_{N2meas}$ the measured $\delta^{15}N$ value of N_2 (‰), n_{meas} the quantity of N_2 at the time of sampling (mol), n_{sample} the quantity of N_2 replaced with the substitution gas (mol), and $\delta 15N_{N2sub}$ the $\delta^{15}N$ value of the N_2 in the substitution gas (‰).

Analytical error and precision, linearity of N_2O concentrations, and detection limits of N_2 and N_2O emissions

The analytical errors of the $\delta^{15}N$ values measured for N_2 and N_2O as well as of the N_2O concentrations were determined as the standard deviations gained from repeated injections of a N_2-reduced artificial atmosphere standard (2% N_2 and 20.5% O_2 in helium) of N_2 (n = 20) and of a 2.39 µL L^{-1} N_2O standard (Air Liquide) (n = 35). The analytical precision of the $\delta^{15}N$ values was on average 0.08‰ for N_2 analysis and 0.42‰ for N_2O analysis. The standard deviation of the N_2O concentration analysis was on average 0.047 µL L^{-1}. The detection limit of the N_2 and N_2O was defined as the three-fold standard deviation of the mean of the measured concentrations and the $\delta^{15}N$ values of N_2 and N_2O. With the used experimental setup, these values corresponded to a detection limit of 1.12 µg N h^{-1} kg^{-1}ds and 0.36 µg N h^{-1} kg^{-1} ds for N_2 and N_2O emissions, respectively.

The linearity of the IRMS measurement of the N_2O concentration was checked by diluting a 50 ppm N_2O standard gas in helium (Air Liquide) and analysing this dilution series with N_2O

concentrations in the range from 0.064 to 47 ppm (Fig. 3). This test showed linearity of analysis over this wide concentration range, which was never exceeded by our samples.

Soil properties and experimental design

To test the incubation setup, we used arable soil from a sub-tropical dairy pasture in Gympie, Australia[13, 14]. The general soil properties are given in Table 1. Following sampling (April 2014), the soil was air-dried and sieved (2 mm); and stones, roots, and crop residues were removed. Before the start of the incubation experiments, soil subsamples of 40 g dry weight were packed into the incubation flasks and remoistened with deionized water to approx. 45% water-filled pore space (WFPS) and pre-incubated at 20º C for 7 days. The water content was held stable during pre-incubation by replacing the evaporative water loss following daily weighing. Immediately before the start of the incubation, the soil was labelled with an equivalent of 50 µg-N g^{-1} dry soil (ds) by broadcast application of a KNO_3 solution (4 at.% ^{15}N) and wetted with deionized water to 80% and 100% WFPS, respectively. Overall, 1.82 µg ^{15}N-excess g^{-1} ds was added to the soil. Three replicate flasks of each water treatment were then purged with the helium–oxygen gas mixture with a 40-fold reduced N_2 background (2 % v/v) and incubated at 20ºC in the sealed incubation box. Immediately after purging, the automated gas sampling procedure was started, i.e., the headspace gas was sampled and analysed from each incubation flask over a period of three days as described above.

Soil analysis and calculation of gross soil N turnover rates

At the start and end of the incubation period, triplicated subsamples of 30 g each were extracted with 60 mL K_2SO_4 for measurement of ammonium-N (NH_4^+-N), nitrate-N (NO_3^--N) and dissolved organic carbon (DOC) concentrations[15]. The soil extracts were immediately frozen until colorimetric measurement of the NO_3^- and NH_4^+ concentrations by a commercial laboratory (Dr. Janssen, Gillersheim, Germany). The total organic C and total N (TN) concentrations in extracts were determined using an infrared gas analyser and a chemoluminescence detector[16]. The organic C concentrations in the extracts are referred to as DOC concentrations.

A further subset of triplicated soil samples was fumigated with chloroform for subsequent extraction and measurement of organic C and total N. These data were used to calculate the microbial biomass C and N using the chloroform-fumigation extraction method[16]. The microbial biomass C and N were calculated from the difference in TN and organic C in unfumigated and fumigated soils without application of correction factors in order to obtain conservative estimates of the active part of microbial biomass[17].

In addition to the concentrations of the mentioned N compounds and pools, we also determined the ^{15}N enrichment. For this purpose we used a 30 mL subsample of each soil extract and conducted sequential micro diffusion of NH_4^+-N, NO_3^--N and dissolved organic N (DON-N) on acid filter traps[15]. For the determination of ^{15}N enrichment in microbial biomass N (MBN), the TN in the extracts from unfumigated and fumigated samples was diffused on filter traps[15].

Gross rates of nitrification were calculated based on the concentration and ^{15}N enrichment of the extractable soil NO_3^- at the beginning and end of the incubation period[18]. Dissimilatory nitrate reduction to ammonium (DNRA) was calculated by $^{15}NO_3^-$ tracing into the NH_4^+ pool over the 72hr incubation period[19].

The data on ^{15}N enrichment in soil NH_4^+-N, NO_3^--N, DON and MBN were also used to calculate the percentage of recovered ^{15}N excess in these N pools at the beginning and end of the incubation period in relation to the ^{15}N isotopic excess applied via the $^{15}NO_3^-$ label[15].

Results

N gas emissions and N-balance

The temporal course of the N_2 and N_2O emissions of the two treatments is displayed in Fig. 4. Over the 3-day incubation, substantial N_2 and N_2O fluxes could be observed in all measurements and both treatments. The measured N_2O emissions in the 80% WFPS treatment ranged from 20 to 340 µg N h^{-1} kg^{-1}, while the N_2O emission in the 100% WFPS treatment were significantly higher, ranging from 425 to 627 µg N h^{-1} kg^{-1}. There was no significant difference in the N_2O emissions between the three days in the 80% WFPS treatment. In the 100% WFPS treatment the N_2O emissions stayed stable over day 1 and day 2, but declined significantly by 40% on day 3.

The N_2 emissions were significantly lower than the N_2O emissions (10-30 fold) in both treatments, ranging from 0 to 34 µg N h^{-1} kg^{-1} and 8 to 49 µg N h^{-1} kg^{-1} in the 80% and 100% WFPS treatments, respectively. In both treatments there was no significant difference in the N_2 fluxes over the first 2 days, but the N_2 emissions were higher on day 3, with the highest N_2 fluxes reaching 37.4 µg N h^{-1} kg^{-1} on day 3 at 100% WFPS.

Due to the dominant N_2O emissions, the $N_2O/(N_2 + N_2O)$ molar ratio ranged from 0.90 to 0.97 during the incubation period (Fig. 4).

Table 2 provides an overview of the changes in soil inorganic N pools, the ^{15}N enrichment in the inorganic N pools, DOC, MBN, and microbial biomass C (MBC) during the incubation experiment. Soil nitrate and ammonium levels were high after the 7-day pre-incubation period, and the nitrate content increased by 50 µg-N g^{-1} ds after the application of the ^{15}N-labelled KNO_3 solution (4 at.% ^{15}N), i.e., corresponding precisely to the NO_3^--N addition rate. There was no significant change in the soil nitrate and ammonium content in the 80% WFPS treatment over the 3-day incubation experiment, while there was a significant decrease in soil nitrate and a significant increase in soil ammonium in the 100% WFPS treatment. The application of the labelled KNO_3 solution (4 at.% ^{15}N) resulted in an average enrichment of 1.74 at.% ^{15}N in the soil nitrate pool at the start of the incubation, which declined to 1.49 at.% ^{15}N and 1.55 at.% ^{15}N at 80% and 100% WFPS, respectively, at the end of the incubation. There was no ^{15}N enrichment in the soil ammonium pool at the start of the incubation (0.37 at.% ^{15}N), but in both treatments the soil ammonium pool was slightly ^{15}N-enriched at the end of the incubations (0.42 at.% ^{15}N), showing a transfer of the added ^{15}N nitrate into the

ammonium pool. The soil microbial biomass C and N concentrations did not significantly differ at the end of the incubation period between the two soil treatments, and there was no significant enrichment of ^{15}N in the microbial biomass N pool.

The recovery of the added ^{15}N isotopic excess as NH_4^+, NO_3^- and gaseous N losses (N_2 and N_2O) from denitrification at the start and the end of the incubation is illustrated in Table 3. At the start of the incubation, all excess ^{15}N was present in the soil NO_3^- pool. Over the course of the incubation, there was ^{15}N tracer redistribution from the soil NO_3^- pool to the soil NH_4^+ pool and to N gases. After 3 days, 87% of the added ^{15}N still remained in the soil NO_3^- pool in the 80% WFPS treatment, and 67% in the 100% WFPS treatment. 1% and 2% of the initially added ^{15}N excess was recovered in the NH_4^+ pool, while 7% and 21% were lost via denitrification in the 80% and 100% WFPS treatment, respectively. In total, 89% and 95% of the initially added ^{15}N excess could be recovered in the different N pools at the end of the experiment (Table 3).

Soil N transformation rates

From the ^{15}N measurements we were able to calculate average gross NO_3^- turnover rates over the 3-day incubation period, as shown in Table 4. There was a significant effect of soil moisture on NO_3^- turnover, with denitrification rates being 3 times higher at 100% WFPS (9.9 µN g^{-1}ds day^{-1}) than at 80% WFPS (3.4 µN g^{-1}ds). The gross nitrification over the 3 days was significantly higher at 80% WFPS (8.6 µN g^{-1}ds day^{-1}) than at 100% WFPS (5.7 µN g^{-1}ds day^{-1}), while the DNRA was an order of magnitude lower, amounting to 0.4 µN g^{-1}ds and 0.6 µN g^{-1}ds at 80% and 100%WFPS, respectively.

Gross nitrification minus denitrification and DNRA equalled about +15 and -15 µg N g^{-1} ds over the three-day incubation period (Table 4) and thus – considering the measured uncertainties – fitted to the observed soil NO_3^- pool changes in this time span (Table 3). Hence, these processes appeared to largely control changes of the soil NO_3^-pool with little importance of other consumption processes such as microbial nitrate immobilization or NO emissions by denitrification.

Discussion

Performance of the novel soil incubation system

The use of a fully automated sampling system has the advantage of eliminating any errors associated with the manual extraction and injection of gas samples. It also minimises the intrusion of atmospheric N_2 during syringe sampling and transfer of the sample gas from the incubation vessel to the isotope ratio mass spectrometer, as done by Meyer, et al. [10] A custom-built gas preparation unit allowed for simultaneous measurement of both the concentration and the isotopic signature of N_2 and N_2O in the sample air with high precision. This resulted in a detection limit of 1.12 µg N h^{-1} kg^{-1}ds and 0.36 µg N h^{-1} kg^{-1}ds for N_2 and N_2O emissions, respectively. Assuming a soil surface area of 78.5 cm^2 in the incubation vessel, these detection limits of emission rates corresponded to 5.8 µg N m^{-2} hr^{-1} (1.3 g N ha^{-1}

day^{-1}) and 1.8 µg N m^{-2} hr^{-1} (0.4 g N ha^{-1} day^{-1}) for N_2 and N_2O emissions, respectively. These detection limits are at the lower end of those reported for gas flow soil core incubation systems for N_2 detection, i.e. 1.3 g N ha^{-1} day^{-1} in our study vs 1.9 to 42.0 g N ha^{-1} day^{-1} for N_2 in He gas flow soil core incubation systems[7, 20-22]. For the N_2O emissions, the detection limit of our system of 0.4 g N ha^{-1} day^{-1} vs 0.1 to 2.3 g N ha^{-1} day^{-1} is, however, at the higher end of laboratory-based setups[7, 20-22], but well in the range of automated N_2O field chamber systems, which are also suitable for measuring typical N_2O fluxes from low-emitting natural systems[23]. It needs to be noted that the detection limit of such a system depends on the sensitivity of the IRMS analysis, the ^{15}N enrichment of the soil NO_3^- pool, the headspace volume and N_2 concentration of the gas incubation vessel, the surface area and density of the soil sample, and the measurement interval. Thus, the detection limit can potentially be lowered by (1) reducing the vessel headspace, (2) using a higher ^{15}N enrichment of the applied tracer, (3) increasing the measurement interval, and (4) by a further reduction of the N_2 background concentrations. However, these parameters need to be adjusted according to the expected N_2 and N_2O emission range. Our analytical system needed at least 100 mL of sample air for the detection of ambient N_2O concentration. Consequently, a headspace of at least 500 mL was required. Furthermore, there is a trade-off in the sensitivity of the IRMS system between N_2 and N_2O flux measurements. A higher ^{15}N enrichment of the tracer will lower the detection limit for the N_2 flux, but may result in a high enrichment of the N_2O that exceeds the detection range of the IRMS instrument. These factors need to be optimised for each soil and experimental setup but, when adjusted appropriately, this method can be used to quantify N_2 and N_2O emissions from high-emitting fertilised agricultural soils as well as from low-emitting natural systems.

A key advantage of this approach over previously established gas flow methods is that it greatly reduces the sensitivity against N_2-diffusion into the incubation vessels, as the magnitude of N_2-diffusion is very low compared with the background N_2 concentration used in the new method (2% v/v). In earlier studies with conventional gas-flow-soil-core incubation systems without isotope-specific N_2 measurements, N_2 diffusion into incubation vessels resulted in a high detection limit for N_2 fluxes and measurement of very low concentration of N_2 (10-50 ppmv) were highly constrained by leakage[24, 25]. Another advantage is the short time needed to purge the incubation vessel with an N_2-reduced artificial atmosphere. The entire purging process of the seven incubation vessels was completed within less than 1 hr, and the measurements could be started immediately after purging. Conventional gas-flow-soil-core incubation systems require 24–48 hr to complete the soil gas exchange process, during which quantification of N_2 emissions is not possible[7, 20]. This is problematic, since the highest emission often occur at the onset of the experiment (in particular when N fertiliser has been applied) and cumulative losses of N gases can be severely underestimated.

An advantage of our approach over conventional ^{15}N tracer methods is that it does not require the addition of high levels of highly enriched $^{15}NO_3^-$. Hence, the chemical modification of the soil resulting from tracer N additions can be reduced, and small increases in the soil NO_3^-

pool by tracer level N additions may trigger only minor alterations of N transformation rates and N gas product ratios[26].

Furthermore, the most problematic aspect of the conventional ^{15}N tracer method is the determination of the enrichment of the source pool (NO_3^-). Direct measurement of the enrichment of this pool using extraction and diffusion of soil NO_3^- does not reflect the enrichment of actual denitrifying sites and substrates in the soil, and the use of these data in denitrification rate calculations has been shown to result in unrealistically high N_2 flux rates[22]. In our approach, we calculate the N_2 flux by dividing the $^{15}N_2$ flux by the ^{15}N atom % enrichment of the N_2O pool, assuming that total N_2 ($^{14/15}N_2$) is produced from the reduction of N_2O derived from both NH_4^+ and NO_3^-[27]. Thus, a direct determination of the enrichment of the NO_3^- pool is not required.

Finally, another major challenge in accurately measuring ^{15}N-N_2 is the rather high $^{30}N_2$ detection limits on current mass spectrometers, mainly due to significant interferences at *m/z* 30 arising from NO^+ ions formed in the ion source[9]. This is a major constraint of the ^{15}N gas flux method where samples with non-random distribution of isotopologues require the accurate determination of *m/z* 28, 29 and 30 (corresponding to $^{14}N^{14}N$, $^{15}N^{14}N$ and $^{15}N^{15}N$, respectively) [27]. However, for low ^{15}N enrichment (below 5% ^{15}N) the contribution of *m/z* 30 to the $\delta^{15}N$ value of N_2 is negligible[8]. Hence, in our approach using a 1.74 at.% ^{15}N enrichment in the soil nitrate pool at the start of the incubation, the $\delta^{15}N$ value of N_2 can be calculated with high precision using only the *m/z* 28 and 29 peaks from the isotope ratio mass spectrometer.

A general shortcoming of our approach is that there is still the need to introduce a ^{15}N label to the soil. Despite the fact that only small amounts of the ^{15}N label are required, a major challenge still is achieving and assessing uniform distribution of the labelled N in the soil. It has been shown that denitrification often occurs in soil micro-sites (hotspots) that can account for a high percentage of the total soil denitrification activity[28]. Such hotspots may form around sites with low or high concentrations of the ^{15}N label and thus result in an over- or underestimation of the total N_2 flux. This is a particular problem for the use of this method with intact soil cores, where aggregates can impede a uniform distribution of the label in the soil. The method also assumes that isotopic discrimination during N_2O consumption is negligible at ^{15}N-enriched levels. Isotopic discrimination of ^{15}N during N_2O consumption has been reported to vary between soils but is estimated to be in the range of -2 ‰ to -10 ‰ [30]. It could, therefore, lead to an underestimation of the N_2 fluxes. To ensure that isotopic discrimination is negligible we recommend using an enrichment of the nitrate pool of at least 1.0 at.% ^{15}N. However, the optimal ^{15}N enrichment depends on the N transformation rates and the N_2 and N_2O emission range and should be tested and verified for each soil and experimental setup. Moreover, the method relies on the assumption that all N_2 originates from N_2O and does therefore not account for any N_2 production due to annamox processes.[29]

Final constraints in the use of this method are the complex technology needed to ensure the gas-tightness of the incubation vessel and the high costs associated with the IRMS analysis of

^{15}N=enriched gases and soil extracts, and that it is currently not applicable under field conditions.

N turnover and gas emissions from a subtropical pasture

Over the 3-day incubation, substantial N_2 and N_2O fluxes could be observed in all treatments, highlighting the suitability of this method to study denitrification and N turnover in such an agro-ecosystem. Following the initialisation of the treatments, elevated N gas emissions were observed in both treatments, with N_2O emissions exceeding N_2 emissions by a factor of 10-30. N_2 and N_2O losses were significantly higher at 100% WFPS than at 80% WFPS, which is consistent with many studies reporting greater denitrification rates at higher soil moisture content [31, 32]. The maximum observed rates of N_2O emissions reached 627 µg N h^{-1} kg^{-1} ds in the 100% WFPS treatment. This rate is in the reported range of emissions from cropland soils that were enriched with nitrate (50-100 mg-N kg^{-1} ds) and glucose (300-1000 mg-C kg^{-1} ds) and incubated under anaerobic conditions [7, 33]. The fact that we observed comparable rates without the addition of glucose as a labile carbon source reflects the high microbial activity and N turnover of the investigated subtropical pasture soil.

The $N_2O/(N_2 + N_2O)$ molar ratios (0.90 to 0.98) are at the higher end of reported molar ratios from anaerobic incubation of terrestrial soils. From an arable soil in China incubated under anaerobic conditions, $N_2O/(N_2 + N_2O)$ molar ratios ranging from 0.01 to 0.19 have been reported [7], while for irrigated dryland soils in Uzbekistan a molar ratio of 0.09 under flooded conditions was observed [32]. Thus, we expected that under saturated conditions the main end product of denitrification would be N_2, but over the 3 days of incubation more than 90% of denitrification products occurred as N_2O. However, these molar ratios are known to vary substantially depending on soil conditions and are primarily influenced by the soil nitrate content, the availability of easily degradable C substrates, soil moisture, soil oxygen content, the genetic potential for N_2O reduction and soil pH[34-37]. Higher N_2O than N_2 emissions have also been reported from other cropland soils that were enriched with nitrate and a labile carbon source and incubated under anaerobic conditions, in particular during the first few days of the incubation [21, 33]. Overall, the highest $N_2O/(N_2 + N_2O)$ molar ratios are expected at low availability of labile C and high soil nitrate levels, providing a preferable electron acceptor at the expense of N_2O, that have been shown to inhibit the conversion of N_2O to N_2[37, 38]. In our study the nitrate concentrations stayed high in the 80% WFPS treatment and declined slowly in the 100% WFPS treatment, while the DOC concentrations slowly increased in both treatments. We hypothesize that the extraordinarily high $N_2O/(N_2 + N_2O)$ molar ratios in our study were primarily caused by a combination of two effects: (i) the high soil nitrate concentration and (ii) a reduced N_2O reductase activity after re-wetting of the soil. It is known that the ability of soils to reduce N_2O to N_2 depends largely on their NO_3^- content, and NO_3^- concentrations in excess of 50 µg-N g^{-1} ds inhibit anaerobic reduction of N_2O to N_2 by soil microorganisms [38]. Hence, the extraordinarily high NO_3^- concentration (>100 µg-N g^{-1} ds) in our treatments inhibited the conversion of N_2O to N_2 to an extent that N_2O was the predominant product of denitrification. Moreover, it has been shown that the antecedent soil moisture conditions affect the activity of the reduction enzymes involved in the denitrification process[39]. In our study the soil was stored air-dried for 30 days and then pre-

incubated at 45% WFPS for 7 days. Therefore, the N_2O reductase activity could have been inhibited, and *de novo* synthesis of this enzyme has been shown to require 16-48 hrs[40], resulting in a high $N_2O/(N_2 + N_2O)$ molar ratio over the 3-day incubation. This hypothesis is further supported by the fact that the N_2O emissions declined and the N_2 emissions increased significantly on day 3 in the 100% WFPS treatment, when nitrate levels had declined by about 20%.

The combination of the ^{15}N gas measurements with ^{15}N analysis of the soil mineral N pools allowed for the calculation of gross nitrification and denitrification rates over the 3-day incubation period. There was a significant effect of soil moisture on both rates, confirming the soil moisture status as a main control of soil N turnover. The results highlight the high microbial activity and N turnover potential of the investigated pasture soil. While it is difficult to convert values of incubations to field emissions, assuming a bulk density of 1 g/cm^3 and a 10 cm active soil layer, these rates would correspond to denitrification rates of 9.9 kg-N ha^{-1} $day^{-1}$and 3.4 kg-N ha^{-1} day^{-1} and nitrification rates of 5.7 kg-N ha^{-1} $day^{-1}$and 8.6 kg-N ha^{-1} day^{-1} for 100% and 80% WFPS, respectively. This reveals the high agronomic as well as environmental significance of denitrification as a major pathway of N loss for sub-tropical pastures at high WFPS and after fertilization, with little importance of NO_3^- retention pathways such as DNRA and microbial assimilation. Thus, our findings provide a mechanistic explanation for the low fertiliser N use efficiency commonly observed for these agro-ecosystems[14].

Conclusions

The improved approach combined with a fully automated sampling system allows for a direct and highly sensitive detection of both N_2 and N_2O fluxes from soils. The precision and accuracy of this approach ares comparable with or better than those of previously established methods, while it overcomes some of the major constraints. Combined with soil extraction and ^{15}N pool dilution and tracing methods it allows for the simultaneous and sensitive determination of gross soil N turnover processes and denitrification, including denitrification N gas product ratios in agricultural and – with minimized constraints – in natural systems. Thus, it represents a tool that may help to significantly improve our mechanistic understanding of N cycling and denitrification in terrestrial ecosystems.

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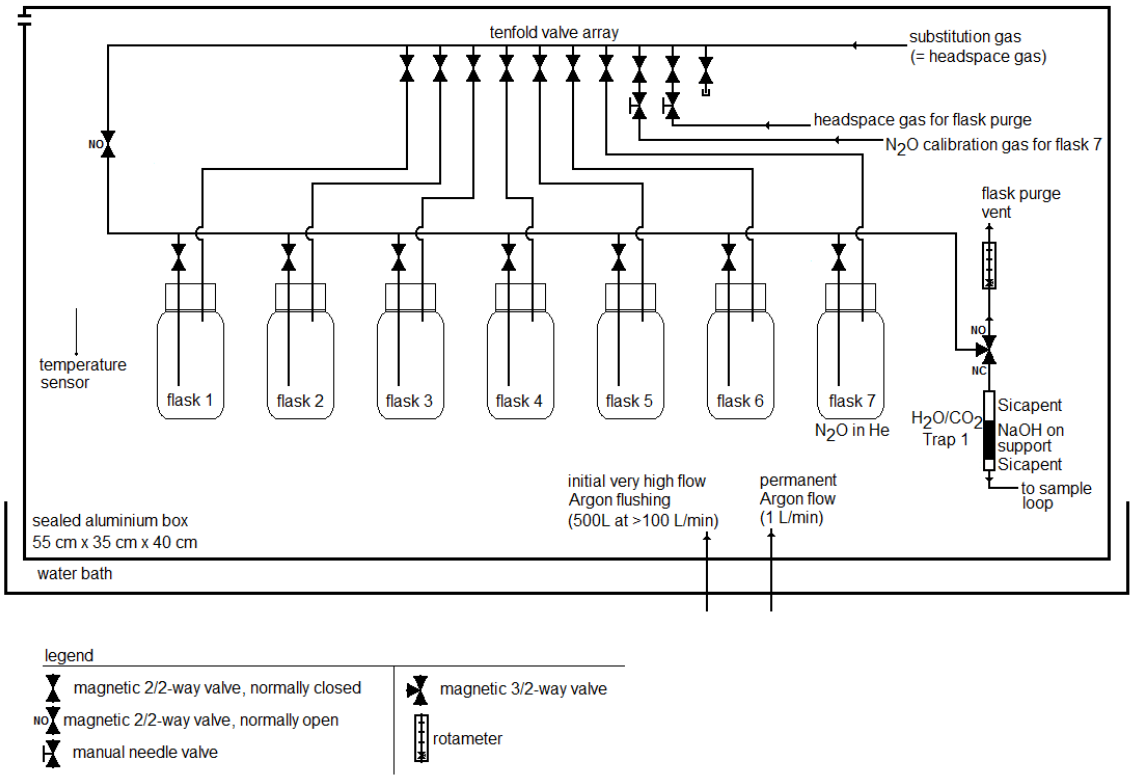

Figure 1. Schematic diagram of the sample preparation unit.

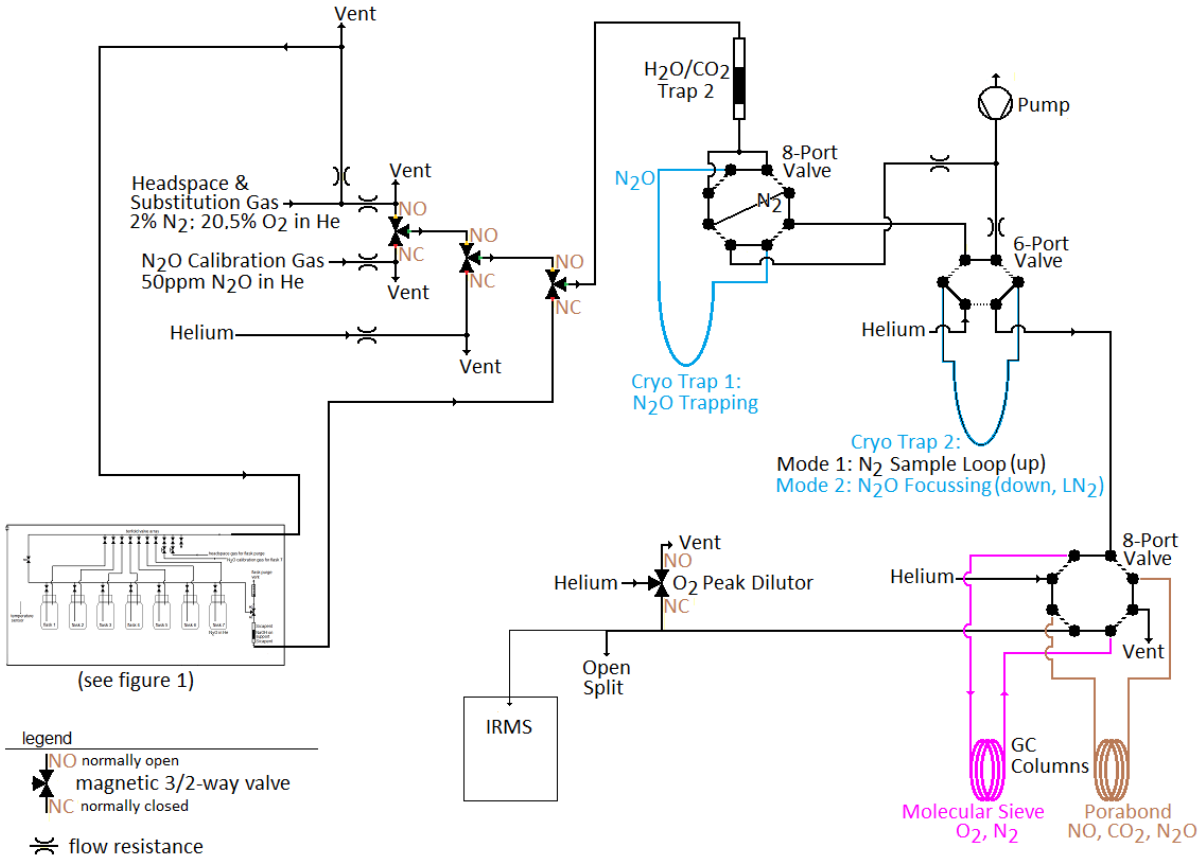

Figure 2. Schematic diagram of the automated gas sampling system.

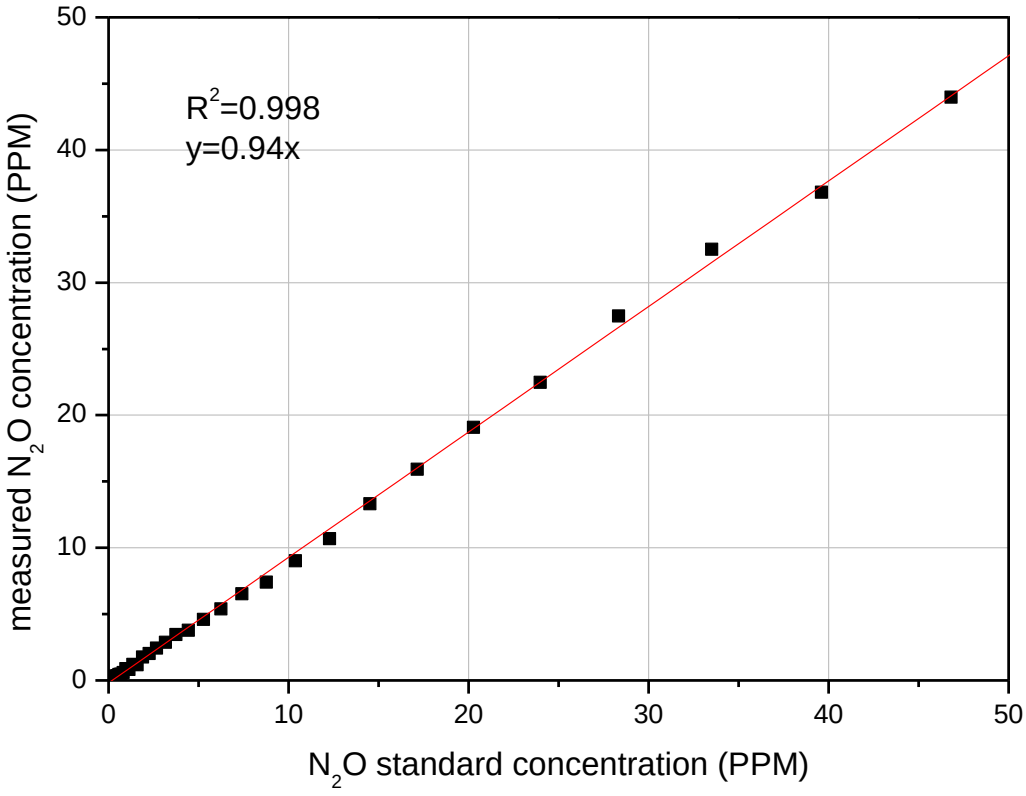

Figure 3: Linearity of the IRMS measurement of N_2O concentrations in the range of 0.064 to 50 ppm (R^2 = 0.998).

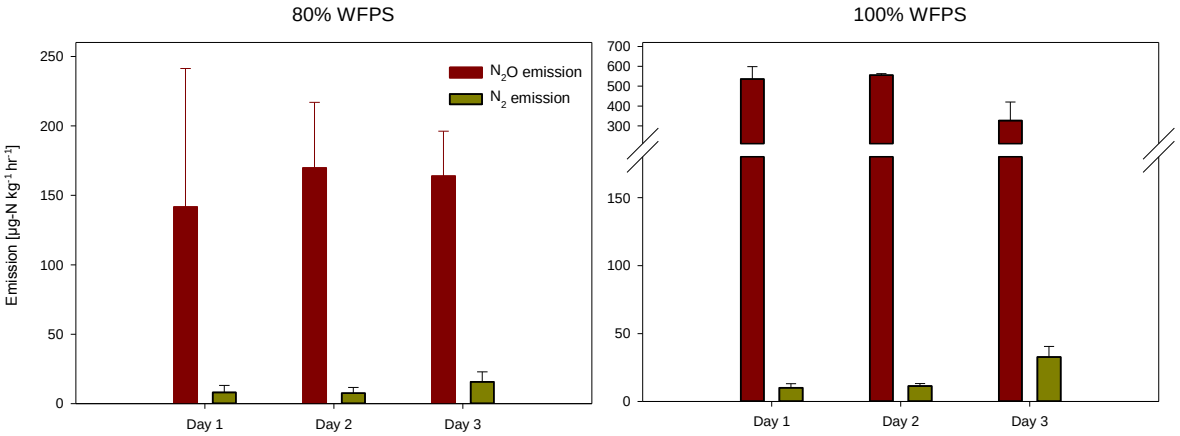

Figure 4: N_2 and N_2O production at 80 and 100% WFPS.

Table 1: Selected soil characteristics for the pasture site at Gympie

Soil type (ASC)	Dermosol
Texture (USDA)	Loam
Bulk density (g cm^{-3}), 0-10 cm	1.1
pH, 0-10 cm	6.1
Total nitrogen (%), 0-10 cm	0.5
C:N ratio, soil	10
Total organic carbon (%),0-10 cm	5

Table 2: Extractable soil C and N concentrations at the beginning and end of the incubation period (±SE).

[µg g^{-1} ds]	Start (no label)	Start (labelled)	End 80%WFPS	End 100%WFPS
Date	27/05/14	27/05/14	30/05/14	30/05/14
NO_3-N	76.2 ± 2.8	125.9 ± 12.5	133.7 ± 13.5	97.8 ± 3.6
NH_4-N	33.0 ± 1.8	26.3 ± 5.4	29.8 ± 7.9	50.3 ± 3.4
AT% ^{15}N (NO_3)	0.37 ± 0.002	1.74 ± 0.001	1.49 ± 0.005	1.55 ± 0.003
AT% ^{15}N (NH_4)	0.37 ± 0.003	0.37 ± 0.13	0.42 ± 0.02	0.42 ± 0.02
DOC	88.9 ± 2.2	82.4 ± 0.5	105.2 ± 2.6	114.9 ± 1.0
MBC	-	-	1971.2 ± 60.1	1839.4 ± 44.7
MBN	-	-	209.0 ± 41.2	260.1 ± 80.0

Table 3: Recovery of the added ^{15}N isotopic excess as NH_4^+, NO_3^- and gaseous N losses (N_2 and N_2O) from denitrification at the start and the end of the incubation.

^{15}N-excess recovery [µg ^{15}N/g soil]					
Start (27/05/2014)		NH_4	NO_3	SUM	Total Recovery [%]
80% WFPS		0.0	1.73 ± 0.17	1.73 ± 0.17	96%
100% WFPS		0.0	1.73 ± 0.17	1.73 ± 0.17	96%
End (30/05/2014)	N_2+N_2O lost (denitrification)	NH_4	NO_3	SUM	
80% WFPS	0.12 ± 0.05	0.02 ± 0.005	1.51 ± 0.15	1.64 ± 0.20	94.7
100% WFPS	0.36 ± 0.04	0.03 ± 0.002	1.16 ± 0.04	1.54 ± 0.08	89.0

Table 4: Average daily soil N turnover rates (± SE) over the three-day incubation.

Soil N turnover		
[µg/g ds/day]	80%WFPS	100%WFPS
Denitrification (calculated from N_2 and N_2O emissions)	3.4 ± 1.7	9.9 ± 0.9
Nitrification (calculated from pool dilution)	8.6 ± 0.5	5.7 ± 0.6
DNRA	0.4	0.6
Nitrification minus denitrification and DNRA	4.8	-4.8

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