Nitrogen turnover and N_2:N_2O partitioning from agricultural soils – a simplified incubation assay

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Abstract
Nitrogen turnover and related denitrification losses are a major uncertainty when estimating N loss and replacement from agro-ecosystems, due to methodological constraints quantifying N_2 and laborious analytical procedures. We present a novel, simplified incubation assay that combines the ^{15}N gas flux method with the ^{15}N pool dilution method, to quantify denitrification losses as a function of N turnover. This assay was tested using a pasture soil from sub-tropical Australia. N-fertiliser (35 µg g^{-1} soil) was applied either as a single ($NH_4{}^{15}NO_3^-$) or double ($^{15}NH_4{}^{15}NO_3$) labelled treatment at 10 atom %, with a third treatment ($NH_4{}^{15}NO_3^-$) at 60 atom % to quantify N_2 emissions. Gross rates of N mineralisation, nitrification and related N_2 and N_2O emissions were measured during 48 hours of incubation at 80% WFPS. Gross N production and gross N consumption was consistent with the directly measured N pool sizes, with denitrification losses (N_2+N_2O) at 7.0 $\pm$ 1.4 µg N g^{-1} soil accounting for 62% of the calculated NO_3^- consumption. N turnover was dominated by mineralisation and nitrification, increasing the NO_3^- pool by a factor of 3. High NO_3^- concentrations shifted the N_2:N_2O ratio towards N_2O, with 60 % of denitrification losses emitted as N_2O. More than 25% of the applied ^{15}N fertiliser was lost via denitrification, showing the significance of denitrification as a major pathway of N loss from agro-ecosystems. The simplified incubation assay proved to be an efficient tool to quantify N pools and emissions, and as such is an effective method to establish comprehensive datasets of denitrification losses linked to N turnover from agro-ecosystems.

Key Words Denitrification; Dinitrogen; Nitrous oxide; ^{15}N pool dilution; Mineralisation; Nitrification

Introduction
Denitrification losses in the form of dinitrogen (N_2) and nitrous oxide (N_2O) from agricultural soils are a major uncertainty when estimating nitrogen (N) loss and replacement, reflecting the difficulties of measuring N_2 against a high atmospheric background and laborious analytical procedures involved. Denitrification losses are tightly coupled to N-turnover in soil, where plant uptake, microbial N immobilisation, dissimilatory nitrate reduction (DNRA) and denitrification compete for the mineral N supplied into the NO_3^- pool. Net rates of N turnover show only the change of the mineral N pools over time, e.g. the increase or decrease of the ammonia (NH_4^+) and nitrate (NO_3^-) pool. In contrast, gross N production and consumption rates quantify the actual influx of N into and efflux from the respective mineral N pool, revealing the relation between production and consumption of NH_4^+ and NO_3^-. This is significant for both magnitude and N_2:N_2O partitioning of denitrification losses: N turnover controls the amount of NO_3^- available for denitrification; and high NO_3^- levels shift the N_2:N_2O ratio towards N_2O (Giles et al., 2012).
Denitrification is a major pathway of N-loss from upland soils, however the magnitude of N_2 and N_2O emissions and related gross rates of N turnover are still unknown for a wide range of agroecosystems (Butterbach-Bahl et al., 2013). The ^{15}N gas flux method enables the direct quantification of N_2 and N_2O emissions after ^{15}N fertiliser addition (Mulvaney, 1984). The ^{15}N pool dilution method (Kirkham and Bartholomew, 1954) is based on the isotopic dilution of the ^{15}N labelled N pool and quantifies gross rates of N turnover.
Combining both methods allows to address major uncertainties of the N-cycle: gross rates of mineralisation and nitrification, the fraction of N_2O derived from nitrification as a function of gross nitrification and the magnitude of denitrification including N_2:N_2O partitioning. The objective of this study is to develop and test an efficient method to obtain comprehensive datasets on N cycling, combining the ^{15}N gas flux method with the ^{15}N pool dilution method in a simplified soil incubation assay.

Material and Methods
Incubation
Bulk soil samples (0-10cm) were collected from an intensively managed pasture in subtropical Casino, New South Wales, Australia. The soil is a heavy clay Vertosol with a pH of 6.6. Soil samples were air dried, sieved to < 4 mm and stored in a cold room. Prior to incubation, soil water content was determined gravimetrically after drying the soil at 105°C for 24 hours. Soil microcosms were established in centrifuge

tubes (50 ml) using the exact equivalent of 8 g oven dry soil, with 4 replicates per fertiliser treatment. NH_4NO_3 equivalent to 35 µg g^{-1} soil was applied to each replicate (1ml solution $8g^{-1}$ soil). The NH_4NO_3 was either single ($NH_4{}^{15}NO_3^-$) labelled (a) or double ($^{15}NH_4{}^{15}NO_3$) labelled (b) at 10 atom %. A second single labelled treatment ($NH_4{}^{15}NO_3^-$) at 60 atom % (c) was used to quantify N_2 emissions. Additional water was applied to achieve the targeted water filled pore space (WFPS) of 80% at an adjusted soil bulk density of 1 (g cm^{-3}). Water and fertiliser solution were applied dropwise on two layers of 4 g of soil each to ensure homogenous ^{15}N labelling. After fertilisation, centrifuge tubes were closed with Suba-seals (Sigma Aldrich). Soil microcosms were kept closed in an incubator at a constant temperature of 25°C.

Gas samples
Ambient air samples (n=4) were taken daily before closing the centrifuge tubes, to quantify ambient N_2O concentrations. Specific background samples were taken above the respective soil microcosms treated with $NH_4{}^{15}NO_3$ at 60 atom % (c) for $^{15}N_2$ analysis before closing the tubes. The entire headspace atmosphere was sampled 24 and 48 h after closure using a gas-tight syringe. After gas sampling, the Suba-seals were removed for 10 minutes, allowing the headspace atmosphere to equilibrate. Gas samples were transferred into pre-evacuated 12 ml Exetainer tubes with a double wadded Teflon/silicon septa cap (Labco Ltd, Buckinghamshire, UK) and stored until N_2O analysis by gas chromatography (Shimadzu GC-2014). Gas samples were analysed for $^{15}N_2O$ and $^{15}N_2$ using an automated isotope ratio mass spectrometer (IRMS) (Sercon Limited, 20-20, UK).

Soil samples
Soil microcosms were extracted with 40 ml 2 M KCL, immediately and 48 h after fertiliser application. Four additional soil microcosms were extracted before fertiliser application to determine starting conditions. All soil extractions were conducted in the centrifuge tubes to avoid subsampling. The centrifuge tubes were shaken with a horizontal shaker (150 rpm/min) for one hour and extracts were filtered through Whatman no. 42 filter paper. Inorganic NH_4^+-N and NO_3^--N in the KCL extract was determined by $AQ2^+$ (SEAL Analytical WI, USA). The ^{15}N enrichments of the NH_4^+) and NO_3^- pool were determined by the diffusion method (Stark and Hart, 1996). To account for microbial ^{15}N assimilation and immobilization on clay surfaces, the extracted soil samples were oven-dried at 60°C, finely ground using a planetary mill and analysed for ^{15}N by IRMS.

Fluxes of N_2 and N_2O
The flux rates of N_2O were calculated from the slope of the linear increase in gas concentration during the closure period and were corrected for temperature and air pressure. Assuming that N_2 and N_2O originate from the same source pool undergoing denitrification, the ^{15}N enrichment of the NO_3^- pool is calculated based on ^{15}N-N_2O (Stevens and Laughlin, 2001). ^{15}N-N_2O gas analysis enables the calculation of the fraction of N_2O (*d*'D) derived from denitrification, and the ^{15}N enrichment of the NO_3^- pool undergoing denitrification (*a*D). Fluxes of N_2 were calculated using *a*D and the increase of ^{15}N-N_2 in the chamber headspace following denitrification (Mulvaney, 1984). Flux calculations are described in detail in Friedl et al. (2016).

^{15}N pool dilution and DNRA
Gross N mineralisation and gross NH_4^+ consumption were calculated based on the isotopic dilution of the $^{15}NH_4^+$ pool in treatment (b), with the ^{15}N enrichment of treatment (b) corrected for ^{15}N recovered in the NH_4^+ pool of treatment (a). Gross nitrification and gross NO_3^- consumption were calculated based on the isotopic dilution of the $^{15}NO_3^-$ pool in treatment (a), assuming nitrified NH_4^+ at mean $^{15}NH_4^+$ pool excess abundance (Barraclough, 1991). DNRA was calculated as described in Huygens et al. (2008).

Results
N_2 and N_2O emissions
Gaseous N emissions and net and gross rates of N turnover are shown in Figure 1. Over the 2-day incubation, 7.01 $\pm$ 1.39 µg N g^{-1} were emitted via denitrification (N_2+N_2O). The main product of denitrification was N_2O, with cumulative fluxes of 4.3 $\pm$ 0.9 µg N-N_2O g^{-1}, exceeding cumulative N_2 fluxes of 2.8$\pm$ 1.1 µg N-N_2 g^{-1} by a factor of 1.5. Nitrification contributed 1.3 $\pm$ 0.1 µg N-N_2O g^{-1} to overall N_2O fluxes. The method detection limit (DL) for N_2 ranged from 0.014 µg g^{-1} day^{-1} with *a*D assumed at 50 atom % to 0.127 µg g^{-1} day^{-1} with *a*D assumed at 20 atom %.

N-turnover
The NH_4^+ pool remained constant over the 2 day incubation, while the NO_3^- concentrations increased by a factor of 3 to 65.5$\pm$ 1.5 µg N-NO_3^- g^{-1}, reflected in the respective net rates. Gross rates of mineralisation and

nitrification averaged at 55.6 ± 11.5 and 54.5 ± 6.5 µg N- g^{-1} over two days respectively, and showed a high variation between soil microcosms. N_2 and N_2O emissions accounted for 67% of the calculated gross NO_3^- consumption.

Recovery
The fertiliser recovery for the single labelled treatment ($NH_4^{15}NO_3$) is shown in Table 1. Recoveries were calculated based on treatment (a) and (c). Immediately after fertiliser addition, 88% percent of the applied fertiliser was recovered in the NO_3^- pool, with less than 1 % recovered in the soil after extraction. At the end of the incubation, more than 50% of the fertiliser remained in the NO_3^- pool, with only minor amounts found in the NH_4^+ pool. N_2 and N_2O emissions accounted for more than 25% of N fertiliser applied.

Table 1: ^{15}N fertiliser recovery in % for the single labelled treatment ($NH_4^{15}NO_3$). Values are mean ±1 standard error of the mean (n=4).

N -fertiliser recovery in %	$NH_4^{15}NO_3$
after fertiliser addition	
NH_4^+ pool	0.01 ± 0.00
NO_3^- pool	88.64 ± 0.96
Immobilised	0.90 ± 0.06
Sum	**89.54 ± 1.00**
at the end of the incubation	
NH_4^+ pool	0.23 ± 0.04
NO_3^- pool	51.15 ± 4.53
Immobilised	6.35 ± 0.31
N_2O emission	16.47 ± 1.19
N_2 emissions	10.34 ± 4.10
Sum	**84.5 ± 6.2**

Discussion

The simplified soil incubation assay directly quantified denitrification losses and N_2 and N_2O partitioning as a function of gross rates of N turnover. This novel method minimizes the workload in terms of the experimental setup, sampling and soil extraction, requiring only inexpensive equipment and small amounts of ^{15}N labelled fertiliser. As such, it allows for large numbers of samples to be processed, recommending its use to establish comprehensive datasets of denitrification losses linked to N turnover.
At 80% WFPS, N turnover was dominated by mineralisation and nitrification (Figure 1), with gross rates of >45 µg g^{-1} over 2 days for both processes. The gross mineralisation rate reveals the high N supply from the organic N pool in subtropical pasture soils, and the difference between gross nitrification and gross NH_4^+ consumption indicates a potential contribution of heterotrophic nitrification to NO_3^- production. The relationship between these specific processes is not reflected in the respective net rates, emphasising the importance of gross rates of mineralisation and nitrification to accurately capture N cycling in the soil.
Gross nitrification exceeded NO_3^- consumption by a factor of 5, which led to a sharp increase of the NO_3^- concentration in the soil. Denitrification was the main NO_3^- consuming process, and only minor amounts of N were recycled into the NH_4^+ pool via DNRA, with both processes accounting for 74% of gross NO_3^- consumption. Significantly, the aerobic process of nitrification dominated at 80% WFPS over denitrification, which is typically favored by low O_2 availability. This can be explained by the occurrence of these processes at different microsites in the soil matrix, which is further evidenced by the substantial amount of N_2O emitted via nitrification.
Denitrification losses were dominated by N_2O emissions, exceeding N_2 emissions by a factor of 1.5. This is consistent with an incubation study using a subtropical pasture soil, reporting low product ratios of denitrification ($N_2/(N_2+N_2O)$) in the first 3 days at 80% WFPS (Friedl et al., 2016). At 80% WFPS, the remaining oxygen (O_2) in the soil matrix is likely to inhibit the N_2O reductase, shifting the $N_2/(N_2+N_2O)$ ratio towards N_2O. High NO_3^- concentrations as observed at the end of the incubation may have also inhibited the reduction of N_2O to N_2 by decreasing the C/NO_3 ratio and/or as a consequence of preferential NO_3^- reduction (Giles et al., 2012).
The ^{15}N recovery in the different N pools from the single labelled treatments of 85 % of the applied fertiliser shows that the simplified incubation assay can provide reliable results for N_2 and N_2O emissions (Table 1). The high ^{15}N enrichment of the fertiliser and a closure time of 24 hours resulted in a DL for N_2 between 0.014 and 0.127 µg g^{-1} day^{-1}, depending on ^{15}N enrichment of the NO_3^- pool. This low DL enables the detection of significantly lower N_2 fluxes than observed in this study, an important trait when obtaining datasets on denitrification over a range of agricultural soils with a different denitrification potential.

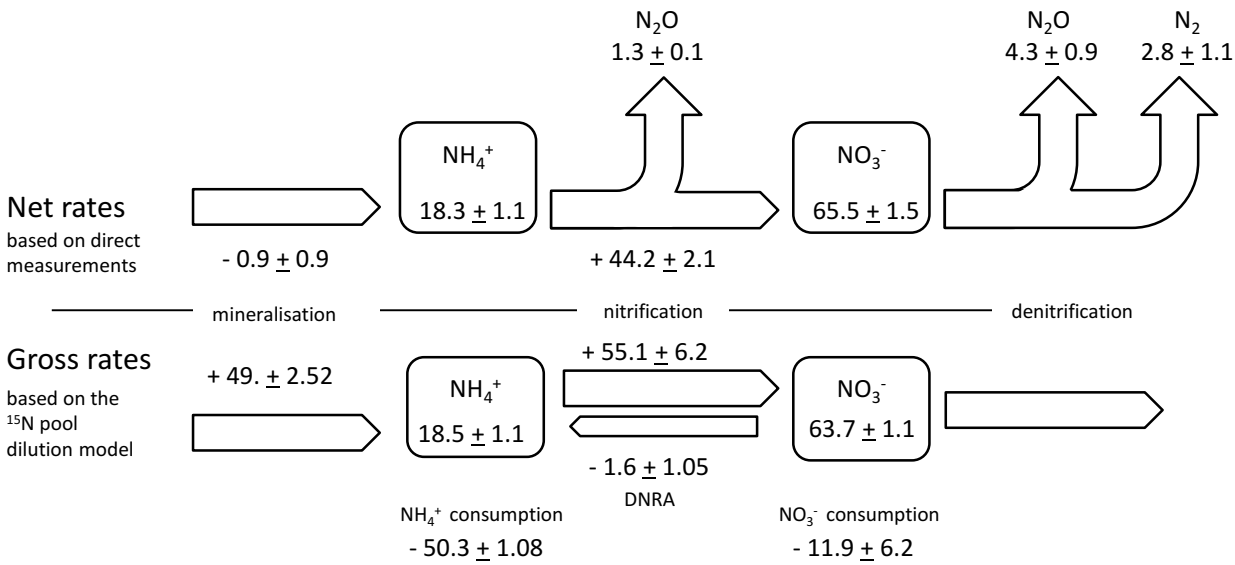

Figure 1: Nitrogen turnover rates in the soil atmosphere - system over two days of incubation (µg N g^{-1} soil) at 80% WFPS. Net rates show the change of the respective N pool over time. Gross rates quantify the influx into or the efflux from the respective N pool. Values are mean ± 1 standard error of the mean (n=4).

Conclusion

Combining the ^{15}N gas flux method and the ^{15}N pool dilution method, this novel incubation assay accurately quantified gross rates of N turnover in the soil-atmosphere system. This study confirms denitrification as a major pathway of N loss from subtropical pasture systems, and reveals the rapid N turnover in these agroecosystems. The simplified incubation assay allows large numbers of samples to be processed, and as such is an effective method to establish comprehensive datasets on N cycling in agro-ecosystems. These datasets, while improving a quantitative process understanding for denitrification, will enable biochemical models to accurately simulate the N cycle, revealing both short and long term effects of improved management practice on N use efficiency for fertilised agro-ecosystems.

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