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Application of the ^{15}N gas-flux method for measuring in situ N_2 and N_2O fluxes due to denitrification in natural and semi-natural terrestrial ecosystems and comparison with the acetylene inhibition technique

Fotis Sgouridis[1,a], Andrew Stott[2], and Sami Ullah[1]

[1]School of Physical and Geographical Sciences, Keele University, Staffordshire, UK
[2]NERC Life Sciences Mass Spectrometry Facility, Centre for Ecology & Hydrology, Lancaster Environment Centre, Lancaster, UK
[a]now at: School of Geographical Sciences, University of Bristol, UK

Correspondence to: Fotis Sgouridis (f.sgouridis@bristol.ac.uk)

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Abstract. Soil denitrification is considered the most unconstrained process in the global N cycle due to uncertain in situ N_2 flux measurements, particularly in natural and semi-natural terrestrial ecosystems. ^{15}N tracer approaches can provide in situ measurements of both N_2 and N_2O simultaneously, but their use has been limited to fertilized agroecosystems due to the need for large ^{15}N additions in order to detect $^{15}N_2$ production against the high atmospheric N_2. For ^{15}N–N_2 analyses, we have used an "in-house" laboratory designed and manufactured N_2 preparation instrument which can be interfaced to any commercial continuous flow isotope ratio mass spectrometer (CF-IRMS). The N_2 prep unit has gas purification steps and a copper-based reduction furnace, and allows the analysis of small gas injection volumes (4 µL) for ^{15}N–N_2 analysis. For the analysis of N_2O, an automated Tracegas Preconcentrator (Isoprime Ltd) coupled to an IRMS was used to measure the ^{15}N–N_2O (4 mL gas injection volume). Consequently, the coefficient of variation for the determination of isotope ratios for N_2 in air and in standard N_2O (0.5 ppm) was better than 0.5 %. The ^{15}N gas-flux method was adapted for application in natural and semi-natural land use types (peatlands, forests, and grasslands) by lowering the ^{15}N tracer application rate to 0.04–0.5 kg ^{15}N ha^{-1}. The minimum detectable flux rates were 4 µg N m^{-2} h^{-1} and 0.2 ng N m^{-2} h^{-1} for the N_2 and N_2O fluxes respectively. Total denitrification rates measured by the acetylene inhibition technique in the same land use types correlated ($r = 0.58$) with the denitrification rates measured under the ^{15}N gas-flux method, but were underestimated by a factor of 4, and this was partially attributed to the incomplete inhibition of N_2O reduction to N_2, under a relatively high soil moisture content, and/or the catalytic NO decomposition in the presence of acetylene. Even though relatively robust for in situ denitrification measurements, methodological uncertainties still exist in the estimation of N_2 and N_2O fluxes with the ^{15}N gas-flux method due to issues related to non-homogenous distribution of the added tracer and subsoil gas diffusion using open-bottom chambers, particularly during longer incubation duration. Despite these uncertainties, the ^{15}N gas-flux method constitutes a more reliable field technique for large-scale quantification of N_2 and N_2O fluxes in natural terrestrial ecosystems, thus significantly improving our ability to constrain ecosystem N budgets.

1 Introduction

There has been a renewed interest recently in developing new or enhancing existing measurement approaches for improving our ability to constrain dinitrogen (N_2) fluxes due to denitrification in terrestrial ecosystems (Kulkarni et al., 2014; Lewicka-Szczebak et al., 2013; Wang et al., 2011; Yang et

al., 2014). Denitrification, the reduction within soils of nitrogen oxides (NO_3^- and NO_2^-) to NO, N_2O, and ultimately N_2 gas, constitutes the most important mechanism for the removal of reactive nitrogen (N_r) in terrestrial ecosystems (Galloway et al., 2008; Groffman, 2012). Despite its importance, denitrification is considered the most un-constrained process in the global N cycle (Groffman, 2012; Kulkarni et al., 2008) due to uncertainties in N_2 flux estimations that are likely leading to underestimations of denitrification rates at multiple scales (Butterbach-Bahl et al., 2013). Considering contemporary atmospheric N deposition rates globally including the UK (Dore et al., 2012; Galloway et al., 2008; Payne, 2014), the available N_r pool in soils may be greater than the capacity of denitrification for its removal, with important consequences of chronic N enrichment of natural terrestrial ecosystems (Galloway et al., 2008; Limpens et al., 2003). Moreover, nitrous oxide (N_2O), an obligate intermediate of denitrification, is a potent greenhouse gas involved in the breakdown of stratospheric ozone (Ravishankara et al., 2009). Therefore, a reliable estimation of the relative magnitude of the major denitrification end products ($N_2 + N_2O$) in soils is crucial in evaluating the role of denitrification as an N_r sink (Kulkarni et al., 2008).

N_2 comprises $\sim 78\,\%$ of the atmosphere and thus it is extremely difficult to measure small N_2 fluxes from soil against this high background, particularly in natural terrestrial ecosystems (Groffman et al., 2006). Available methods for measuring both N_2 and N_2O are limited and can be categorized into the direct flux and ^{15}N isotope tracer methods (Kulkarni et al., 2014), whilst micrometeorological approaches (Eddy covariance) are impossible in the N_2-rich atmosphere (Felber et al., 2012). The gas-flow soil core method (Burgin and Groffman, 2012; Butterbach-Bahl et al., 2002; Scholefield et al., 1997; Wang et al., 2011) allows the direct measurement of N_2 flux (without the addition of any substrate such as nitrate) from intact soil cores where the soil atmosphere is replaced by a mixture of He / O_2. However, despite the high precision of the technique, cores still need to be extracted from the field and conditioned over lengthy periods of time for the complete removal of N_2 from the soil atmosphere. This method is therefore time and resource intensive, which precludes its application to intensive temporal and large spatial scales (Kulkarni et al., 2014). Moreover, the gas-flow soil core method cannot discriminate between sources of N_2O, thus overestimating the denitrification product ratio $N_2O / (N_2 + N_2O$; Butterbach-Bahl et al., 2013; Morse et al., 2015). The acetylene inhibition technique (AIT) is also a direct flux method that exploits the ability of acetylene (C_2H_2) at high concentrations ($10\,\%\ v/v$) to inhibit the reduction of N_2O to N_2 (Tiedje et al., 1989); thus, total denitrification ($N_2 + N_2O$) is measured in C_2H_2 amended soil cores in situ, whilst N_2 flux is estimated indirectly by difference from un-amended soil cores. Despite its simplicity and cost-effectiveness, the AIT is becoming increasingly unpopular due its several limitations (Groffman et al., 2006), of which the catalytic decomposition of NO in the presence of C_2H_2 under oxic or suboxic conditions in the field (Bollmann and Conrad, 1997; Nadeem et al., 2013), in particular, precludes its use for reliable estimates of in situ denitrification rates (Felber et al., 2012).

The ^{15}N gas-flux method (Mosier and Klemedtsson, 1994) has the advantage of providing in situ measurements of both N_2 and N_2O simultaneously, thus allowing its application over large temporal and spatial scales. It requires the addition of a ^{15}N-labelled tracer in a soil enclosure in the field which is subsequently covered by a chamber while the chamber headspace is progressively enriched with $^{15}N–N_2$ and $^{15}N–N_2O$ produced by denitrification (Stevens and Laughlin, 1998). Assuming that both N_2 and N_2O originate from the same uniformly labelled soil NO_3^- pool (Stevens and Laughlin, 2001), the true denitrification product ratio can be more accurately estimated as opposed to the direct flux approaches (Bergsma et al., 2001). Field applications of the ^{15}N gas-flux method so far have been limited to fertilized agro-ecosystems (Baily et al., 2012; Cuhel et al., 2010; Graham et al., 2013) and more recently restored peatland soils (Tauchnitz et al., 2015) with high ^{15}N tracer application rates (between 10 and $200\,kg\,N\,ha^{-1}$), with the exception of Kulkarni et al. (2014), who have measured denitrification rates in northern hardwood forests of the USA by adding tracer amounts of ^{15}N-labelled nitrate, and Morse and Bernhardt (2013), who applied the same technique in intact soil cores collected from mature and restored forested wetlands in North Carolina, USA. These recent studies hold much promise that the ^{15}N gas-flux method can be applied to a range of natural and semi-natural terrestrial ecosystems, allowing the quantification of the relative magnitude of N_2 and N_2O fluxes due to denitrification from these under-represented ecosystems.

Natural and semi-natural terrestrial ecosystems in the UK (i.e. peatlands, heathlands, acid grasslands, deciduous and coniferous forests), where there is no fertilizer use and the impact from grazing and commercial forestry is minimal (Mills et al., 2013), along with improved and unimproved grasslands (grazed and/or fertilized) constitute approximately 49 and 85 % of rural land use cover in England and Wales respectively (Morton et al., 2011). Unlike arable agriculture, these land use types have been poorly investigated for their role in N_r loss through denitrification.

The major challenge in measuring $^{15}N–N_2$ at near-natural abundance levels is the possibility of interference at $m/z\,30$ ($^{30}N_2$) due to the reaction of oxygen in the ion source with N and the formation of NO^+ ions that also have $m/z\,30$ (Stevens et al., 1993). Commonly, this issue is addressed in a continuous flow isotope ratio mass spectrometer (CF-IRMS) with the inclusion of a copper (Cu) oven for reducing O_2 in the gas sample (Russow et al., 1996). Recently, it has been suggested that the interference at $m/z\,30$ can be further reduced by including a molecular sieve column in gas chromatograph IRMS (GC-IRMS) systems to not only separate N_2 and O_2 in the gas sample, but also to quantitatively

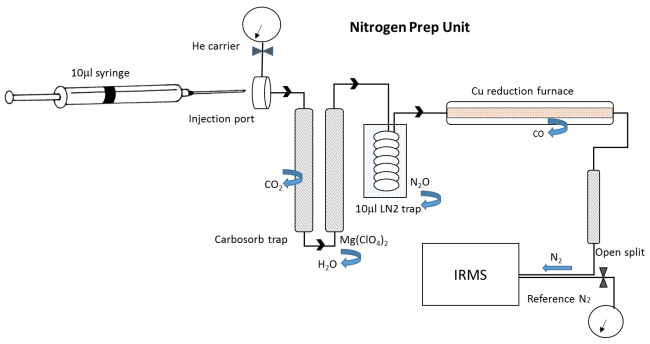

Figure 1. Schematic of the $^{15}N–N_2$ analysis system.

remove O_2 and other trace gases such as carbon monoxide (Lewicka-Szczebak et al., 2013; Yang et al., 2014). We hypothesize that the precision for m/z 30 determination can be greatly improved by using a custom-built preparative unit for the removal of H_2O, CO_2, N_2O, NO^+ and CO, a device which also permits the micro-scale injection of volumes of < 5 µL. These injection volumes are much smaller than have previously been reported in the literature.

Studies that have compared the ^{15}N gas-flux method with the AIT in the field are rare and have exclusively focused on highly fertilized agro-ecosystems with moderate to low soil moisture contents (Aulakh et al., 1991; Mosier et al., 1986; Rolston et al., 1982). These studies have measured comparable denitrification rates with both field techniques, although the relatively low soil moisture contents have probably allowed greater diffusion of C_2H_2 to the anaerobic microsites where denitrification occurs (Malone et al., 1998), whilst the high nitrate application rates have probably favoured nitrate reduction over N_2O reduction (Dendooven and Anderson, 1995), resulting in high denitrification rates from the AIT. Conversely, laboratory studies have shown that the AIT significantly underestimates total denitrification compared to the ^{15}N tracer approach (Yu et al., 2010) and the direct N_2 flux approach (Qin et al., 2012) due to the incomplete inhibition of N_2O reduction to N_2 by C_2H_2 in wet soils (Yu et al., 2010) or in soils with low nitrate content, where N_2O reduction is more energetically favourable (Qin et al., 2013, 2014). A comparison of the ^{15}N gas-flux method with the AIT under in situ conditions across a range of natural and semi-natural terrestrial ecosystems has not been attempted before. It can provide valuable insights in terms of the validity and applicability of the two field techniques for measuring denitrification rates across broad spatial and temporal scales.

The objectives of the present study were (1) to determine the precision and suitability of our preparative-IRMS instrumentation for measuring $^{15}N–N_2$ and $^{15}N–N_2O$ at low enrichment levels, (2) to adapt the ^{15}N gas-flux method for application across natural and semi-natural terrestrial ecosystems, and (3) to compare the validity and applicability of the ^{15}N gas-flux method with the AIT for measuring in situ denitrification rates.

2 Materials and methods

2.1 IRMS system

For N_2 gas isotopic analysis we used an Isoprime isotope ratio mass spectrometer (Isoprime Ltd, UK, Wythenshawe) coupled to an in-house built N_2 preparative interface (Fig. 1). Headspace gas (4 µL) was manually injected with a gas-tight syringe (SGE Analytical science) into the preparative interface via an open split. Prior to its introduction into the IRMS, the sample was treated as follows: (a) dried by passing through $Mg(ClO_4)_2$ (Elemental Microanalysis Ltd, Devon, UK), (b) CO_2 removed with 0.7–1.2 mm Carbosorb (Elemental Microanalysis Ltd, Devon, UK), (c) N_2O cryogenically trapped under liquid nitrogen, and (d) O_2 removed over a copper-packed reduction furnace heated at 600 °C. The N_2 was then directed towards the triple collectors of the isotope ratio mass spectrometer where m/z 28, m/z 29 and m/z 30 mass ions were measured. Mass / charge ratios for the m/z 28, m/z 29, and m/z 30 nitrogen ($^{28}N_2$, $^{29}N_2$, and $^{30}N_2$) were recorded for each sample at a trap current of 300 µA. Instrument stability checks were performed prior to each analysis by running a series of 10 reference pulses of N_2 (BOC special gases) until a standard deviation of δ^{15}N better than 0.05 ‰ was achieved. Additionally, 10 consecutive injections (4 µL) of atmospheric air were analysed prior to the analysis of actual samples. The precision of the instrument was better than δ^{15}N 0.08 ‰ in all quality control tests.

Nitrous oxide was analysed using modified headspace methods described for the analysis of nitrogen gas above. Headspace gas (ca. 4 mL) was injected into a TraceGas™ Preconcentrator coupled to an Isoprime™ IRMS (GV instruments Ltd, UK) whereupon the sample was directed through a series of chemical traps designed to remove H_2O and CO_2. The N_2O was cryogenically trapped under liquid nitrogen. The waste was flushed out of the instrument. The N_2O was further cryofocused in a second liquid nitrogen trap prior to being introduced onto a 25 m × 0.32 mm Poraplot Q gas chromatography column (Chrompack column, Varian, Surrey, UK). The column separated N_2O from any residual CO_2, and both entered the IRMS via an open split. The retention time between the first eluting CO_2 ($< 2 \times 10^{-10}$ amplitude) and second eluting N_2O peak typically fell in the range between 60 and 70 s to avoid isobaric interference of the CO_2 with the calculated ^{15}N. The N_2O was directed towards the triple collectors of the isotope ratio mass spectrometer where m/z 44, m/z 45, and m/z 46 mass ions were measured and recorded. Instrument stability checks were performed prior to each analysis by running a series of 10 reference pulses of N_2O (BOC special gases) until a standard deviation of δ^{15}N better than 0.05 ‰ was achieved. Prior to each sample batch

analysis, trace gas N_2O measurements were made on three 100 mL flasks containing atmospheric air collected from outside the stable isotope laboratory. δ^{15}N precisions using the TraceGas Preconcentrator and Isoprime IRMS were better than 0.3 ‰ respectively at the 600 µA trap current.

2.2 Field application of the ^{15}N gas-flux and AIT methods

In situ measurements of N_2 and N_2O were made using static chambers according to the ^{15}N gas-flux method (Mosier and Klemedtsson, 1994). Five plots were randomly established in June 2013 in each of four study sites in the Ribble–Wyre River catchments (area 1145 km^2; north-western England; 53°59′99″ N, 2°41′79″ W). The study sites were a heathland (R-HL), a deciduous woodland (R-DW), an unimproved grassland (R-UG), and an improved grassland (R-IG). In August 2013, four more study sites were tested in the Conwy River catchment (area 345 km^2; northern Wales; 52°59′82″ N, 3°46′06″ W) following a similar sampling design. These sites were an acid grassland (C-UG), an ombrotrophic peat bog (C-PB), a mixed deciduous and coniferous woodland (C-MW), and an improved grassland (C-IG). Further details on the location, land management status, and major soil properties for all study sites can be found in Sgouridis and Ullah (2014).

In each plot a round PVC collar (basal area 0.05 m^2; chamber volume 4 L) was inserted into the soil at ca. 10 cm depth (15 cm for the R-HL and C-PB plots) 2–4 weeks before the measurement date. The collars were open at the bottom to maintain natural drainage and root growth during the measurements. The natural vegetation cover at the soil surface of each installed collar remained unchanged. The PVC collars were fitted with a circular groove of 25 mm depth to fit in an acrylic cylindrical cover (chamber) providing a gas-tight seal when filled with water (Ullah and Moore, 2011). The gas leak rate from the chamber was determined in the laboratory by placing the sealed collar and chamber over a tray of water, injecting CH_4 (10 ppm) and determining the change in CH_4 concentration within the chamber headspace over time (Yang et al., 2011). The CH_4 concentration change within 24 h was negligible, with the relative standard deviation (RSD) being < 5 %. We did not use a vent tube for pressure equilibration, as suggested by Hutchinson and Mosier (1981), in our chamber design, which could have diluted the chamber headspace with atmospheric N_2, as part of our effort to increase the probability of a detectable ^{15}N–N_2 signal in the chamber headspace. Instead chambers were covered with reflective foil for minimizing temperature increase within the chamber headspace during the incubation period (Ullah and Moore, 2011). Labelled $K^{15}NO_3^-$ (98 at % ^{15}N, Sigma-Aldrich) was applied in each plot via 10 injections of equal volume through a grid (4 × 6 cm) using custom-made 10 cm long lumber needles (15 cm for the R-HL and C-PB plots) attached to a plastic syringe (Ruetting et al., 2011). The ^{15}N tracer was delivered as the needle was pushed into the soil from the surface up to 10 or 15 cm depth, aiming to achieve as uniform as possible labelling of the soil volume enclosed by the collar, as required by the ^{15}N gas-flux method (Mosier and Klemedtsson, 1994). The volume and concentration of the labelled $K^{15}NO_3^-$ tracer solution was determined from measurements of soil nitrate and moisture content, as well as bulk density adjacent to each plot made during the installation of the collars (Morse and Bernhardt, 2013). Lower application rates (< 0.1 kg N ha^{-1}) were administered to natural study sites (e.g. peat bog, heathland) and higher rates (< 1 kg N ha^{-1}) administered to semi-natural ones (e.g. unimproved and improved grasslands). The tracer solution (50–200 mL) was adjusted between 3 and 5 % of the ambient volumetric water content (see Supplement Table S1 for detailed data from each sampling plot). It should be noted that no time was allowed for the equilibration of the added tracer solution in the soil enclosure to avoid significant loss of the low amount of added nitrate via plant uptake.

Following the ^{15}N tracer application, the collars were covered with the acrylic chamber fitted with a rubber septum for gas sampling. Two sets of gas samples (20 mL each) were collected with a gas-tight syringe (SGE Analytical Science) through the septum of the chamber cover at $T = 1$ h, $T = 2$ h, and $T \approx 20$ h after the tracer injection, while a $T = 0$ h sample was collected immediately after tracer injection above the plot surface before fitting the chamber cover. The gas samples were transferred into pre-evacuated (< 100 Pa) 12 mL borosilicate glass vials with butyl rubber septa (Exetainer vial; Labco Ltd., High Wycombe, United Kingdom) for storage under positive pressure and were analysed within 8 weeks from collection without any significant change in the gas concentration (Laughlin and Stevens, 2003).

Adjacent to each PVC collar in each plot, two intact soil cores (50 mm I.D., 15 cm long) were extracted from 10 cm depth, leaving the top 5 cm void as a headspace volume. The cores were capped on both ends with the top cap fitted with a rubber septum for gas sampling. One set of cores was amended with pure C_2H_2 with 5 mL injected through the septum directly in the middle of the soil core before 10 % of the headspace was also replaced with pure C_2H_2. The second set of cores was not amended with C_2H_2 and both cores were placed back in the ground where they came from. Gas samples (5 mL) were collected with a gas-tight syringe (SGE Analytical Science) through the septa of the cores at $T = 1$ h and $T = 2$ h after amendment with acetylene. The gas samples were transferred into pre-evacuated (< 100 Pa) 3 mL borosilicate glass vials with butyl rubber septa (Exetainer vial; Labco Ltd., High Wycombe, UK) for storage under positive pressure.

2.3 Flux calculations

The ^{15}N content of the N_2 in each 12 mL vial was determined using the IRMS system described above and the ra-

Table 1. Measured ratios of R29 and R30 for N_2 in ambient air ($n = 10$), ratios of R45 and R46 in standard N_2O gas (0.5 ppm concentration, $n = 15$), and ^{15}N at % abundance calculated from the respective ratios for both gases. SD: standard deviation; CV: coefficient of variation.

	R29 (N_2)	R30 (N_2)	R45 (N_2O)	R46 (N_2O)	^{15}N at % (N_2)	^{15}N at % (N_2O)
Mean	7.38×10^{-3}	5.16×10^{-5}	8.00×10^{-3}	2.21×10^{-3}	3.71×10^{-1}	3.88×10^{-1}
SD	2.77×10^{-7}	2.26×10^{-7}	1.25×10^{-5}	1.04×10^{-5}	2.09×10^{-5}	1.01×10^{-3}
CV (%)	0.00	0.44	0.16	0.47	0.01	0.26

tios R29 ($^{29}N_2$ / $^{28}N_2$) and R30 ($^{30}N_2$ / $^{28}N_2$) were measured in both enriched ($T = 1$, 2, and 20 h) and reference samples ($T = 0$ h). The inclusion of air reference standards between every 10 samples indicated an upward drift for R30 over time, potentially due to the formation of NO^+ in the ion source despite the inclusion of the Cu reduction step (Lewicka-Szczebak et al., 2013). Subsequently, every sample batch was drift corrected by fitting a linear regression through the air reference standards and calculating an offset correction for both R29 and R30 (Yang et al., 2014). The minimum detectable change (MDC) in R29 and R30 was defined with repeated manual analyses of air reference standards ($n = 10$) and was calculated using the following equation (Matson et al., 2009):

$$\mathrm{MDC} = \mu_{\text{pair diff}} + (2\sigma_{\text{pair diff}}), \quad (1)$$

where μ is the mean difference of all possible unique pairs of air reference standards ($n = 45$) and σ is the standard deviation between sample pairs. The MDC for R29 was 7.7×10^{-7} and for R30 it was 6.1×10^{-7}, and these values were used to determine whether each time step sample was significantly different from ambient reference samples ($T = 0$ h), and, if not, they were excluded from the flux calculations.

For calculating the total N_2 flux from a uniformly labelled soil nitrate pool when both R29 and R30 are measured, the "non-equilibrium" equations were applied as described by Mulvaney (1984) for estimating first the ^{15}N fraction in the soil NO_3^- denitrifying pool ($^{15}X_N$) as

$$^{15}X_N = 2(\Delta R30/\Delta R29)/(1 + 2(\Delta R30/\Delta R29)), \quad (2)$$

where ΔR29 and ΔR30 are the difference between R29 and R30 respectively between enriched ($T = 1$, 2, and 20 h) and reference samples ($T = 0$ h). Subsequently, the $^{15}X_N$ allows the quantification of the fraction of the N_2 evolved from the ^{15}N-labelled pool (d) using either ΔR30 or ΔR29:

$$d = \frac{\Delta R30}{\left(^{15}X_N\right)^2}, \quad (3)$$

$$d = \frac{\Delta R29}{2\left(^{15}X_N\right)\left(1 - ^{15}X_N\right)^2}. \quad (4)$$

Using d and the concentration of [N_2] (µg N) in the chamber headspace, the evolved N_2 from the soil pool was calculated:

$$\text{Evolved}\,N_2 = \mathrm{d}[N_2]/(1 - d), \quad (5)$$

The N_2 flux was then calculated using linear regression between the maximum evolved N_2 and the respective incubation time per plot surface area, and was expressed in µg N m^{-2} h^{-1} representing the total N_2 flux from the mixture of the ^{15}N-labelled tracer and the soil N at natural abundance (Stevens and Laughlin, 1998).

The ^{15}N content of the N_2O in the same 12 mL vials as well as the ratios R45 ($^{45}N_2O$ / $^{44}N_2O$) and R46 ($^{46}N_2O$ / $^{44}N_2O$) were measured in both enriched ($T = 1$, 2, and 20 h) and reference samples ($T = 0$ h). The application of the "non-equilibrium" equations to N_2O is analogous to N_2 after correcting for the naturally occurring oxygen isotopes (Bergsma et al., 2001). Therefore, the ratios R45 and R46 were converted to ratios of R29 and R30 respectively by applying the following equations:

$$\mathrm{R29} = \mathrm{R45} - \mathrm{R17}, \quad (6)$$

$$\mathrm{R30} = (\mathrm{R46} - (\mathrm{R29}R17)) - \mathrm{R18}, \quad (7)$$

where for R17 (^{17}O / ^{16}O) the value 0.000373 was used and for R18 (^{18}O / ^{16}O) the value 0.0020052 was used (Bergsma et al., 2001). There was no significant instrumental drift for the ratios R45 and R46 over time. The MDC was defined, for the converted R29 and R30, with repeated automatic analyses of 0.5 ppm N_2O standards ($n = 15$) as 3.4×10^{-5} and 2.9×10^{-5} respectively. The second set of gas samples collected at the same time in the field was analysed for total N_2O on a GC-μECD (7890A GC Agilent Technologies Ltd., Cheshire, UK) and the concentration of [N_2O] (µg N) was used in Eq. (5) to calculate the N_2O flux due to denitrification of the mixture of the ^{15}N-labelled tracer and the soil N and expressed in µg N–N_2O m^{-2} h^{-1}. Assuming that the N_2O originates from the same uniformly labelled pool as N_2, the $^{15}X_N$ from N_2O was used to estimate d for N_2 using either R30 (Eq. 3) or R29 (Eq. 4), thus lowering the limit of detection for N_2 (Stevens and Laughlin, 2001) and allowing measurement of N_2 gas flux from natural terrestrial ecosystems at low ^{15}N-tracer application rates.

Gas samples collected from the intact soil cores with or without acetylene amendment were analysed for N_2O on a GC-μECD (7890A GC Agilent Technologies Ltd., Cheshire, UK) and for CO_2 on a GC-FID (7890A GC Agilent Technologies Ltd., Cheshire, UK) and flux rates were determined by linear regression between 0 and 2 h. The instrument precision was determined from repeated analyses of 6 ppm N_2O

and 200 ppm CO_2 standards respectively ($n = 8$), and the RSD was < 1%.

2.4 Statistical analysis

Using factor analysis on selected soil physico-chemical properties, the samples from the eight field sites were grouped into three broad land use types: organic soils (C-PB, C-UG, R-HL), forest soils (C-MW, R-DW), and grassland soils (C-IG, R-UG, R-IG) according to Sgouridis and Ullah (2014). All subsequent statistical analyses were performed on the broad land use types rather than individual field sites. The data were analysed for normality and homogeneity of variance with the Kolmogorov–Smirnov test and the Levene statistic respectively and logarithmic transformations were applied as necessary. One-way ANOVA combined with Hochberg's GT2 post hoc test for unequal sample sizes or the Games–Howell post hoc test for unequal variances was performed for comparing the variance of the means between land use types for all gas fluxes. The non-parametric Kruskal–Wallis test was used to compare mean flux rates between incubation time intervals. Pearson correlation was used between log-transformed flux rates. Comparisons between the ^{15}N gas-flux and AIT techniques were made with an independent samples t test. All statistical analyses were performed using SPSS® 21.0 for Windows (IBM Corp., 2012, Armonk, NY).

3 Results

3.1 IRMS system evaluation

The precision of the IRMS systems was evaluated using repeated analyses of ambient air samples for N_2 ($n = 10$) injected manually in one batch and repeated analyses of N_2O gas standard at natural abundance and 0.5 ppm concentration ($n = 15$) using automated injections. The mean measured ratios of R29 and R30 for N_2 and of R45 and R46 for N_2O are shown in Table 1. Measurement precision was defined as the coefficient of variation (%), and it was lower for R29 compared to R30 and lower for R45 compared to R46, but still less than 0.5 % for all four measured ratios. We estimated the ^{15}N atom% abundance for both gases as per Yang et al. (2014), and the precision was less than 0.01 % for N_2 in air and 0.26 % for standard N_2O at natural abundance. The mean measured R30 (5.16×10^{-5}) was higher than the theoretical value of 1.35×10^{-5} for N_2 in ambient air, suggesting some interference at m/z 30 potentially due to the formation of NO^+ ions in the ion source of the mass spectrometer despite the inclusion of the Cu reduction oven. The contribution of NO^+ ions (R30 measured–R30 theoretical) was 3.81×10^{-5}, whilst the ratio of R30 theoretical / R30 measured was 0.26. Correcting the R30 ratio for the contribution of NO^+ ions results in a lower "true" precision for the R30 (CV = 1.67 %).

3.2 Field application of the ^{15}N gas-flux method

The ^{15}N tracer application rate was variable between land use types and ranged between 0.03 and 1 kg ^{15}N ha^{-1}, while it was lower in the case of the organic soils and higher for the woodland and grassland soils (Table 2). Based on the soil nitrate content on the day of the tracer amendments (Table 2), the estimated enrichment of the total soil nitrate pool was on average between 13 and 25 ^{15}N at % (detailed data on the ^{15}N tracer application per field site are shown in Table S2).

The ^{15}N fraction in the denitrifying pool ($^{15}X_N$), as calculated from the measured isotopic ratios of the N_2O after 1 h of incubation using Eq. (2), ranged between 65 and 93 ^{15}N at %. The average change in the $^{15}X_N$ with incubation time, indicated by the slope shown in Table 2, was not different from 0 in the case of the organic (t test; $t = 0.520$, $df = 18$, $p > 0.05$) and grassland soils (t test; $t = 0.047$, $df = 28$, $p > 0.05$), whilst it was significantly below 0 for the woodland soils (t test; $t = 2.917$, $df = 18$, $p < 0.05$). Separating the woodland soils into C-MW and R-DW sites, only the former displayed a significant negative slope of $^{15}X_N$ with incubation time (t test; $t = 3.306$, $df = 8$, $p < 0.05$), suggesting N_2O production from a second nitrate pool, possibly nitrate produced from the oxidation of NH_4^+ via nitrification, in the C-MW. In cases where the $^{15}X_N$ could be calculated from the N_2 isotope ratio data (woodland and grassland soils; data shown in Table S3), this was not significantly different from their respective $^{15}X_N$ calculated from the N_2O isotope ratio data (t test; t-$_{WL} = 0.929$, $df = 12$, $p > 0.05$; t-$_{GL} = 1.511$, $df = 20$, $p > 0.05$).

The mean evolved amount of N_2 and N_2O gases due to denitrification in each land use type increased with increasing incubation time (Fig. 2). The increase in the evolved N_2 was statistically significant after 20 h incubation in GL (ANOVA; $F = 19.8$, $p < 0.01$), whilst due to the high variability among plots, shown by the large error bars at 20 h incubation in Fig. 2a, it was not significant for the OS and WL soils. The amount of N_2O accumulated after 20 h (Fig. 2b) was significantly higher than at the previous time points for all land use types (ANOVA; $F_{OS} = 4.6$, $F_{WL} = 5.1$, $F_{GL} = 14.7$, $p < 0.05$). However, this pattern was not consistent in every sampling plot (data presented in Tables S4 and S5); for example, in C-MW, the highest N_2 accumulations were observed after the first or second hour of incubation, whilst in most cases the increase in N_2 and N_2O concentrations was not linear throughout the incubation period (Tables S4 and S5). This suggested a complex temporal sequence of events, which was not consistent between plots among the different land use types, probably as a result of complex interactions between environmental controls of denitrification and the length of the incubation period (details below). Consequently, the N_2 flux rate decreased with increasing incubation time (Fig. 3a), and this decrease was significant between each time interval in the OS (Kruskal–Wallis; $\chi^2 = 11.35$, $p = 0.003$), between 1 and 20 h in the WL (Kruskal–Wallis; $\chi^2 = 10.78$,

Table 2. The ambient soil nitrate pool, the ^{15}N tracer application rate, the estimated enrichment of the total soil nitrate pool, the calculated $^{15}X_N$ value from N_2O, and the slope of the $^{15}X_N$ change with incubation time in the three land use types. Data are means with standard errors in parentheses.

Land use type	Ambient NO_3^- ($kg\,N\,ha^{-1}$)	Tracer application rate ($kg^{15}N\,ha^{-1}$)	Enrichment of total soil NO_3^- pool (^{15}N at %)	$^{15}X_N$ (%)	$^{15}X_N$ slope
Organic soil ($n=3$)	0.53 (0.44)	0.04 (0.02)	25 (11.8)	90 (1.5)	0.003 (0.0054)
Woodland ($n=2$)	3.86 (2.42)	0.62 (0.41)	13 (0.7)	79 (8.3)	−0.007 (0.0025)
Grassland ($n=3$)	1.81 (0.96)	0.51 (0.19)	24 (5.1)	81 (8.4)	0.000 (0.0037)

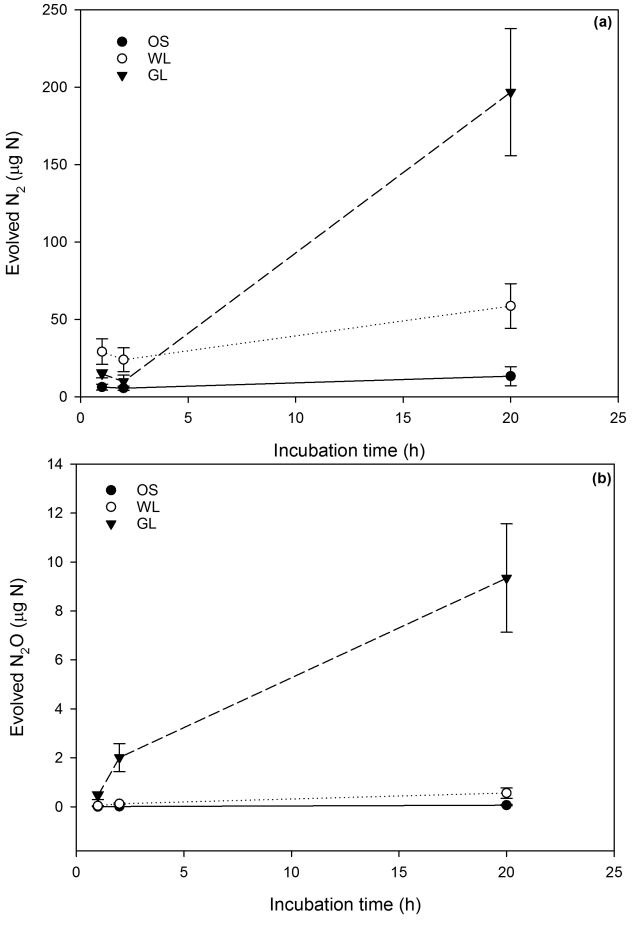

Figure 2. Evolved **(a)** N_2 and **(b)** N_2O gas measured between 1, 2, and 20 h incubation time intervals using the ^{15}N gas-flux method in the organic soil (OS), woodland (WL), and grassland (GL) land use types. Data points are means and the error bars represent standard errors.

$p = 0.005$), and between 1 and 2 h in the GL (Kruskal–Wallis; $\chi^2 = 10.10$, $p = 0.006$). Conversely, the N_2O flux rates increased between the first and second hour of incubation (Fig. 3b), followed by a decrease after 20 h, albeit the mean differences between time intervals were not statistically significant in any land use type (Kruskal–Wallis; $\chi^2_{OS} = 3.58$, $\chi^2_{WL} = 3.47$, $\chi^2_{GL} = 3.01$, $p > 0.05$).

Table 3. Comparison of mean flux rates and ratios between land use types for the two field methods using one-way ANOVA. All variables are log-transformed. F: F statistic; P: probability level.

^{15}N gas-flux	F	P
Denitrification	19.4	< 0.001
N_2O emission	31.1	< 0.001
N_2O / (N_2 + N_2O)	7.4	< 0.01
Total bulk N_2O	19.4	< 0.001
CO_2 production	19.8	< 0.001
AIT		
Denitrification	12.7	< 0.001
Total bulk N_2O	9.4	< 0.01
N_2O / (N_2 + N_2O)	0.3	> 0.05
CO_2 production (un-amended cores)	11.2	< 0.001
CO_2 production (C_2H_2 amended cores)	11.7	< 0.001

The N_2 flux ranged between 2.4 and 416.6 $\mu g\,N\,m^{-2}\,h^{-1}$ and was significantly different among land use types based on 20 h incubation duration for comparison purposes (Table 3). The grassland soils showed on average 3 and 14 times higher denitrification rates than the woodland and organic soils respectively (Fig. 4a). A similar pattern was observed for the N_2O flux due to denitrification (range: 0.003–20.8 $\mu g\,N\,m^{-2}\,h^{-1}$), with the grassland soils emitting on average 14 and 120 times more N_2O than the woodland and organic soils respectively (Fig. 4b), whilst the N_2O flux was on average 20 to 200 times lower than the N_2 flux among land use types. Consequently, the denitrification product ratio N_2O / (N_2 + N_2O) was low, ranging between 0.03 and 13 %, and was highest in the GL and similar between the WL and OS (Fig. 4c). The change in the denitrification product ratio with incubation time was evaluated in each sampling plot where both N_2 and N_2O fluxes were available (data shown in Table S6). Generally, there was no consistent pattern between individual sampling plots, with the exception of the grassland soils, where the maximum product ratio was observed after 2 h of incubation (ANOVA; $F = 6.11$, $p < 0.05$). This was

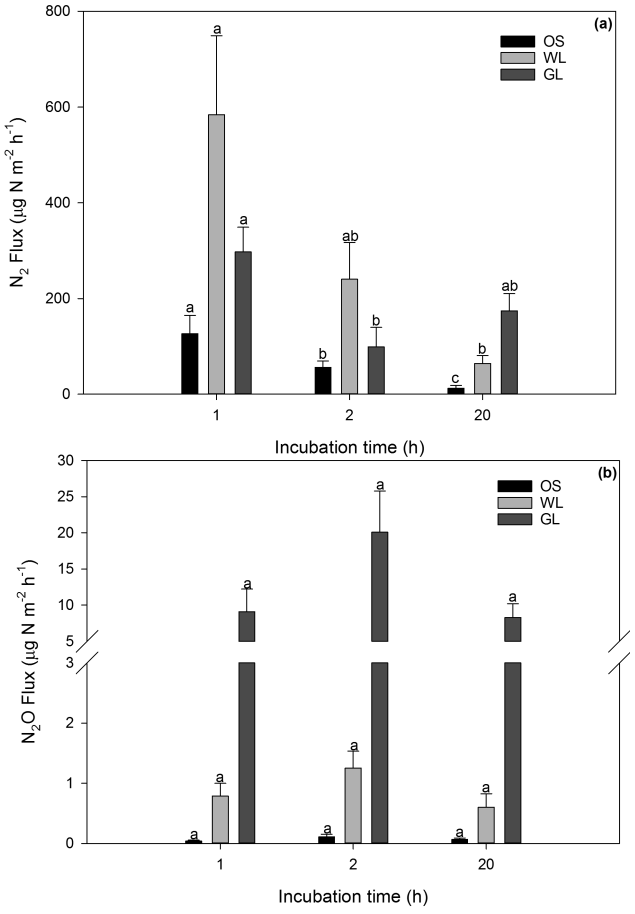

Figure 3. Mean rates of **(a)** N_2 flux and **(b)** N_2O flux due to denitrification at the three incubation time intervals in the three land use types (OS: organic soils; WL: woodland; and GL: grassland). Same lower-case letters indicate no significant differences ($p > 0.05$) between incubation time intervals according to the non-parametric Kruskal–Wallis test. Error bars represent standard errors.

an indication of some reduction of the denitrification-derived N_2O to N_2 during the extended closure period (up to 20 h) in the grassland soils.

3.3 Comparison with the AIT

The total denitrification rate measured from the C_2H_2 amended intact soil cores in the same land use types ranged between 0.5 and 325.2 μg N m^{-2} h^{-1} and correlated positively with the total denitrification rate (N_2 and N_2O fluxes combined) measured with the ^{15}N gas-flux method (Pearson; $r = 0.581$, $n = 25$, $p < 0.01$) following a similar trend among land use types, albeit with only the OS being significantly lower than the grassland and woodland soils (Table 3). The AIT denitrification rates were between 3 and 5 times lower than the total denitrification from the ^{15}N gas-flux (Fig. 5a), with the difference being significant in woodland

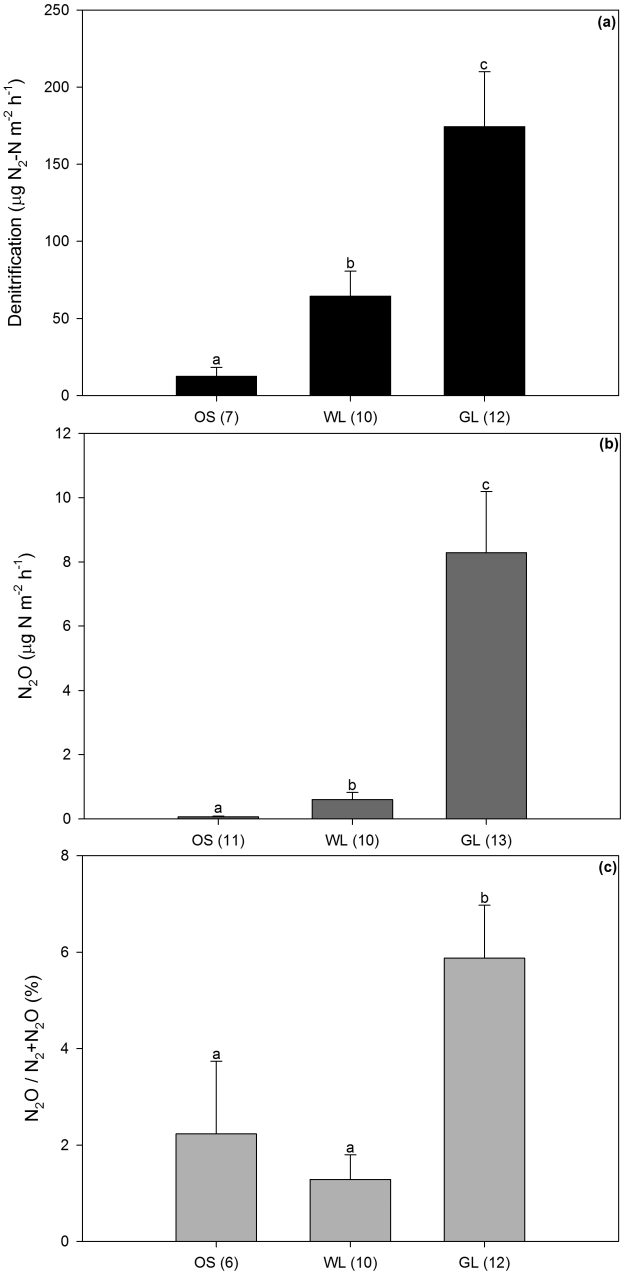

Figure 4. Mean rates of **(a)** N_2 flux, **(b)** N_2O emission due to denitrification, and **(c)** the denitrification product ratio N_2O / (N_2 + N_2O) in the three land use types (OS: organic soils; WL: woodland; and GL: grassland). Same lower-case letters indicate no significant differences ($p > 0.05$) between land use types according to one-way ANOVA and the Games–Howell post hoc test. The sample size (n) is given in parentheses for each land use type on the x axis. Error bars represent standard errors.

(t test; $t = 3.914$, $df = 18$, $p < 0.01$) and grassland (t test; $t = 3.521$, $df = 25$, $p < 0.01$) soils.

The total N_2O flux measured from the un-amended intact soil cores ranged between 0.15 and 86.6 µg N m^{-2} h^{-1} and was between 1 and 3 times lower than the total denitrification rate from the C_2H_2 amended cores. There were no significant differences between bulk N_2O fluxes measured with the static chambers and the un-amended intact soil cores (Fig. 5b), which indicated that total N_2O emissions were comparable between the two field techniques. Consequently, estimating the denitrification product ratio from the un-amended and C_2H_2 amended intact soil cores resulted in significantly higher ratios compared to the ^{15}N gas-flux approach (Fig. 5c), which were on average between 50 and 60 % and not significantly different among land use types (Table 3).

The mean CO_2 production rate was similar irrespective of whether it was measured in static chambers, in C_2H_2 amended or un-amended intact soil cores (Fig. 6), indicating that soil respiration (including both microbial and plant respiration) was not affected by the measurement technique.

4 Discussion

4.1 IRMS system evaluation

The precision of our trace gas isotope ratio mass spectrometer (TG-IRMS) for manual analysis of ^{15}N–N_2 in gas samples was comparable for both the R29 and R30 ratios to the recently developed gas chromatograph-IRMS (GC-IRMS) systems that included a combination of a copper reduction oven and a molecular sieve (Lewicka-Szczebak et al., 2013) or only a molecular sieve (Yang et al., 2014) for the removal of O_2 from the samples. This was achieved while injecting a trace amount of headspace gas sample (4 µL), which is less than half of what is used by Lewicka-Szczebak et al. (2013) and 10 times less than the required sample volume by Yang et al. (2014). Furthermore, the interference at m/z 30 by NO^+ ions was reduced by an order of magnitude (3.81×10^{-5}) compared to the value (1.6×10^{-4}) reported by Lewicka-Szczebak et al. (2013). Consequently, correcting the R30 ratio for the NO^+ ion interference led to a CV value of < 2 %, which was significantly lower than the precision reported for natural abundance samples in previous studies (Lewicka-Szczebak et al., 2013; Russow et al., 1996; Stevens et al., 1993), thus constituting a significant improvement in m/z 30 determination in N_2 gas samples with low ^{15}N enrichment. However, the correction of the R30 ratio is only useful for estimating the "true" instrument precision for m/z 30 and is not necessary for calculating N_2 fluxes as shown by Lewicka-Szczebak et al. (2013), unless using the mathematical formulations of Spott and Stange (2007).

The TraceGas™ Preconcentrator IRMS system used for ^{15}N–N_2O analysis displayed similar precision for the deter-

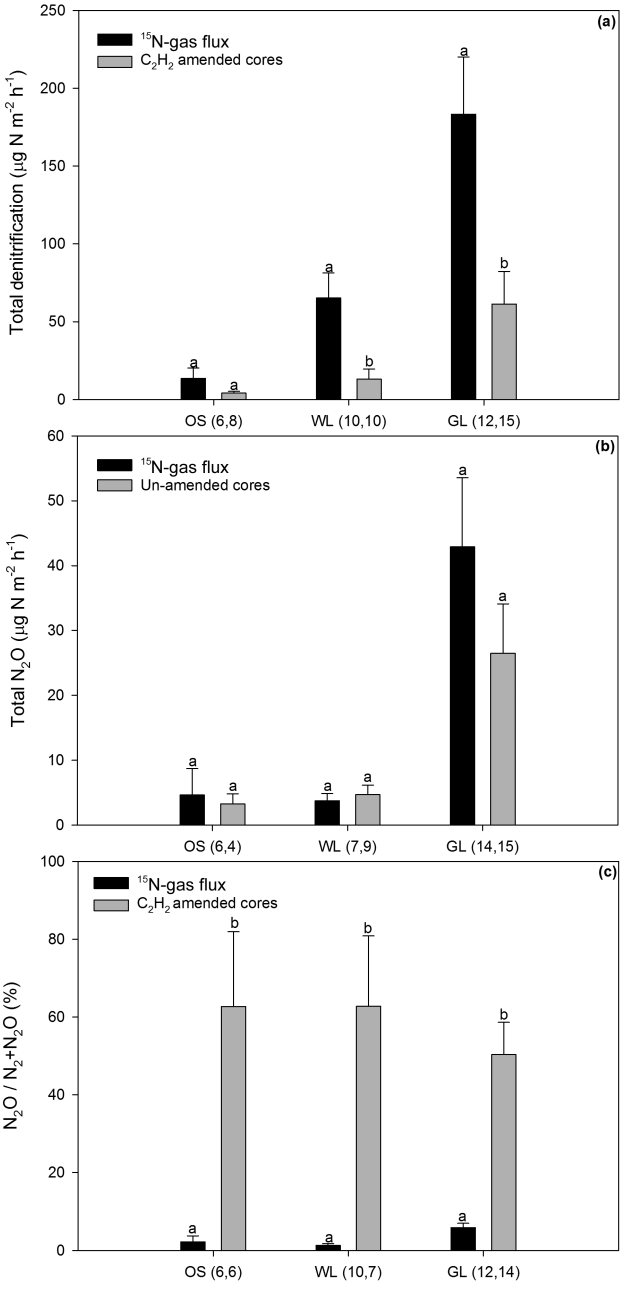

Figure 5. (a) Mean total denitrification measured with the ^{15}N gas-flux method and the AIT, **(b)** mean bulk N_2O emission measured in the static chambers of the ^{15}N gas-flux method and in un-amended intact soil cores, and **(c)** the denitrification product ratio $N_2O / (N_2 + N_2O)$ with the ^{15}N gas-flux method and the AIT in the three land use types (OS: organic soils; WL: woodland; and GL: grassland). Same lower-case letters indicate no significant differences ($p > 0.05$) between measurement methods according to an independent samples t test. The sample size (n) is given in parentheses for each land use type and each method on the x axis. Error bars represent standard errors.

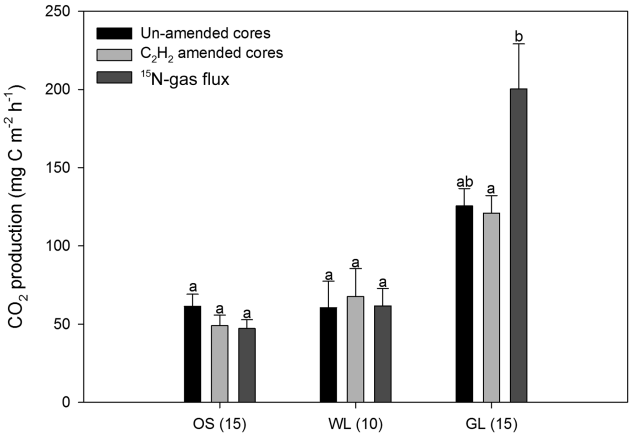

Figure 6. Mean CO_2 production measured in the static chambers of the ^{15}N gas-flux method, in un-amended and C_2H_2 amended intact soil cores in the three land use types (OS: organic soils; WL: woodland; and GL: grassland). Same lower-case letters indicate no significant differences ($p > 0.05$) between measurement methods according to an independent samples t test. The sample size (n) is given in parentheses for each land use type on the x axis. Error bars represent standard errors.

mination of R45 and R46 in standard N_2O gas at circa ambient concentration to a similar system used by Bergsma et al. (2001) while injecting only 4 mL of gas sample as opposed to 0.5 L used by Bergsma et al. (2001). When expressed in delta values (δ^{15}N), the precision of our system was better than 0.05 ‰, which is significantly better than the respective precisions reported in Lewicka-Szczebak et al. (2013) and Yang et al. (2014), but comparable to Well et al. (1998). Therefore, the analytical precision achieved for both the ^{15}N–N_2 and ^{15}N–N_2O analyses, using smaller gas sample volumes than previously reported, allowed us to quantify in situ N_2 and N_2O fluxes with low tracer addition under field conditions.

4.2 Field application of the ^{15}N gas-flux method

The average ^{15}N tracer application rate (0.04–0.5 kg ^{15}N ha^{-1} or 0.4–1.2 mg^{15}N kg^{-1} dry soil) across land use types was 1 to 2 orders of magnitude lower than previous applications of the ^{15}N gas-flux method in highly fertilized agricultural systems (Baily et al., 2012; Bergsma et al., 2001; Cuhel et al., 2010; Graham et al., 2013) and in restored peatland soils (Tauchnitz et al., 2015). The estimated enrichment of the total soil NO_3^- pool was variable (2–40 ^{15}N at %, Table S2) and this wide range was due to the fact that the tracer concentration was calculated based on the previous campaign's soil nitrate data, which in some cases did not reflect the soil nitrate content on the day of the tracer application a month later. It should be noted that the soil nitrate enrichment levels reported in this study correspond to the high end of the average soil NO_3^- pool enrichment (10–15 ^{15}N at %, Table S2) for the period April 2013 to October 2014, which is presented in a separate publication (Sgouridis and Ullah, 2015). To our knowledge, only Kulkarni et al. (2014) have applied the ^{15}N gas-flux method in the field with soil nitrate enrichment levels (5 ^{15}N at %) lower than in our study, but this had as a consequence poorly detected ^{15}N–N_2 fluxes. Nevertheless, for the organic soils the average tracer application rate corresponded to current estimates of daily atmospheric N deposition (0.05 kg N ha^{-1} d^{-1}) in the UK ($\sim$ 15–20 kg N ha^{-1} yr^{-1}; Dore et al., 2012; Payne, 2014), whilst for the grassland soils the tracer application mimicked a daily fertilizer application rate of 0.5 kg N ha^{-1} d^{-1}. Due to the inclusion of the NO_3^--rich C-MW site in the woodland soils, tracer application rates were higher than the daily atmospheric N deposition rates, but also reflected internal N cycling processes (e.g. nitrification) as an additional source of nitrate in these well-drained forest soils. Therefore, the application of the ^{15}N tracer at these low rates should not be expected to enrich the soil nitrate pool significantly, and potentially enhance the denitrification activity, in excess of the amount of nitrogen normally deposited via natural processes and common management practices.

The major assumptions of the ^{15}N gas-flux method and the associated "non-equilibrium equations" are that the denitrifying soil NO_3^- pool is uniformly labelled with ^{15}N and that the N_2 and N_2O originate from the same denitrifying pool (Stevens and Laughlin, 1998). The ^{15}N fraction in the denitrifying pool ($^{15}X_N$), calculated non-destructively from the measured isotope ratios, ranged between 65 and 93 % and was well above the 10 % threshold for the correct application of the "non-equilibrium equations" (Lewicka-Szczebak et al., 2013). However, the calculated $^{15}X_N$ was higher than the estimated total soil NO_3^- pool enrichment (range: 2–40 ^{15}N at %), suggesting non-homogeneous mixing of the added tracer (98 ^{15}N at %) with the ambient soil nitrate at natural abundance despite our effort for uniform tracer application with multiple injections across the investigated soil depth (Ruetting et al., 2011). Wu et al. (2011) have optimized the number of injections and the volume of tracer needed to achieve homogeneous labelling of a soil core (diameter 15 cm; height 20 cm) and reported that 38 injections of 4 mL volume each were necessary. We have used only 10 injections of 5–20 mL volume (depending on the soil water content of each land use type) to minimize the disturbance of the soil matrix, particularly in the highly porous media such as peatland soils, and this was clearly sub-optimal for the homogenous labelling of the soil enclosure but probably a necessary compromise for large-scale intensive measurements. We were not able to sample the soil within the chamber collars to directly estimate the $^{15}NO_3^-$ content of the soil pool due to time and budget constraints. However, in cases where destructive soil sampling was used to measure the soil nitrate pool enrichment (Kulkarni et al., 2014), the results were

significantly different from the estimated enrichment due to sampling bias of the volume of soil affected by the tracer application. Non-uniform mixing of the ^{15}N label may lead to overestimation of the $^{15}X_N$ and underestimation of the denitrification flux rates (Boast et al., 1988). However, under field conditions, it is unlikely to achieve complete mixing of the added tracer with the ambient nitrate pool, and experimental studies (Mulvaney, 1988; Mulvaney and Van den Heuvel, 1988) have shown that the associated error is well constrained and that accurate measurements can be made even with a less-uniformly labelled denitrifying pool.

The larger volume of tracer per injection (> 4 mL) in combination with the lower number of injections compared to Wu et al. (2011) may have created localized saturation effects (saturated soil cylinders around the injection holes), even if the total soil moisture content of the enclosure was not increased by more than 5 %, which would require several hours to equilibrate with the ambient soil moisture. We did not allow time for this soil moisture equilibration to occur following the tracer injection to avoid significant loss of the added nitrate via plant uptake (measurements occurring during the growth season). Therefore, it is likely that in plots where denitrification activity may have been limited by soil moisture (e.g. C-MW with mean WFPS 42 ± SE 0.76 %), the flux rates after 1 and 2 h of incubation may be overestimated due to moisture-induced denitrification events.

Most studies using ^{15}N tracers and static chambers in highly fertilized systems typically deploy their chambers between 1 and 2 h (Baily et al., 2012; Cuhel et al., 2010; Tauchnitz et al., 2015), but it has been shown that longer incubation periods (up to 24 or 48 h) may be needed in case of low ^{15}N enrichment applications in intact soil cores (Morse and Bernhardt, 2013) and laboratory incubations (Yang et al., 2014) for a more precise and accurate detectable ^{15}N–N_2 signal. However, it should be noted that in these cases the soil cores or slurries were incubated in fully enclosed systems and were thus not affected by potential bias from diffusion of evolved N_2 and N_2O to the subsoil (Clough et al., 2005). The open-bottom, un-vented static chamber design used in this study in combination with the extended incubation period up to 20 h may have potentially allowed some loss of the evolved N_2 and N_2O through downward subsoil diffusion and/or reduction of gas exchanges at the soil–atmosphere interface due to decreasing concentration gradients (Healy et al., 1996). This could partly explain the non-linear increase in the evolved N_2 and N_2O in the chamber headspace (Fig. 2a and b) and also the decrease in the N_2 flux rate with increasing incubation time (Fig. 3a). The N_2O flux rate increased up to 2 h incubation followed by a decrease after 20 h consistently across land use types (Fig. 3b), indicating that the extended enclosure period lowered N_2O fluxes due to subsoil diffusion and enhanced N_2O reduction to N_2. However, due to the high spatial heterogeneity within each land use type, the mean N_2O flux rate was not significantly different between the different incubation intervals. In other words, the non-linearity of N_2O evolution had less effect on the flux rate estimation than the inherent spatial variability within each land use type, which is in agreement with the findings of Chadwick et al. (2014), who suggested that the spatial variability of N_2O fluxes far exceeds the bias due to assumed linearity of fluxes.

The lack of a consistent pattern of N_2 flux rate change with incubation time among the different land use types suggested a more complex temporal variability of N_2 fluxes that apart from the duration of incubation could have also been affected by the distribution of the added nitrate tracer. In the OS sites with the lowest average nitrate content (Table 2) and the highest water filled pore space (mean WFPS: C-PB = 70 ± SE 3.21 %; C-UG = 66 ± SE 1.58 %; R-HL = 69 ± SE 2.00 %), non-homogeneous tracer distribution ($^{15}X_N = 90$ ^{15}N at %) could have led to the creation of hotspots of denitrification activity due to substrate availability resulting in potentially overestimated flux rates in the first or even the second hour of incubation. However, analytical uncertainty due to fluxes being close to the limit of detection could not be ruled out. Conversely, in the soil moisture-limited forest site (C-MW), the injection of even 50 mL of tracer solution could have led to an increased moisture-induced denitrification activity within the first 1–2 h of incubation, until the added water started to equilibrate with the ambient soil moisture. Therefore the N_2 flux rate in C-MW may be significantly overestimated after 1 h of incubation. In the grassland sites and the R-DW forest site with intermediate soil moisture (mean WFPS: R-DW = 65 ± SE 1.79 %; R-UG = 64 ± SE 1.41 %; C-IG = 60 ± SE 1.45 %; R-IG = 61 ± SE 2.46 %) and nitrate content, the tracer injection is unlikely to have significantly affected the denitrification rate when all the conditions (i.e. soil moisture and substrate availability) were favourable, and therefore flux rates estimated after 1 h of incubation should be more reliable as long as the bias from analytical uncertainty was low. In these sites denitrification rates estimated after 1 or 20 h of incubation were not significantly different (Fig. 3a), suggesting a quasi-linear N_2 evolution throughout the incubation period (at least in 37.5 % of the sampling plots; see Table S4). However, the N_2 flux rates were significantly lower after 2 h of incubation, whereas the N_2O flux rates were maximum at 2 h of incubation, consequently leading to an increased product ratio N_2O / (N_2 + N_2O) (Table S6). This observation could potentially be explained by a delay in the de novo synthesis of denitrification enzymes and the fact that the N_2O reductase is known to have a slower expression than the preceding reduction enzymes (Knowles, 1982), leading to N_2O accumulation and lower N_2 production after 2 h of incubation. After 20 h incubation, the decrease in the product ratio could be explained by a higher reduction rate of N_2O to N_2 due to probably higher N_2O reductase activity but also slower soil–atmosphere exchange of N_2O due to the decreasing concentration gradient (Healy et al., 1996).

It has been shown that the N_2 flux estimation with the ^{15}N gas-flux method is sensitive to the incubation time interval and the homogeneity of the tracer distribution due to the combination of several antagonistic effects such as decreasing gas diffusion gradients and soil moisture and substrate availability effects due to the added tracer solution. The uncertainty in the estimated in situ N_2 fluxes can be significantly reduced if additional effort is made to increase the homogeneity of the tracer application by increasing the number of injections while reducing the volume of the applied tracer (particularly in soils where denitrification is limited by moisture). Moreover, allowing the equilibration of the added tracer solution with the ambient soil water before gas sampling commences and by closely monitoring the linear evolution of the produced gases with more frequent gas sampling at shorter equal incubation intervals could help in identifying the appropriate length of incubation, thus avoiding potential overestimation of denitrification in nitrate and moisture-limited ecosystems and potential underestimation due to subsoil diffusion of evolved gases. The detailed uncertainty analysis of the N_2 and N_2O flux estimation presented in this study complements the large-scale application of the ^{15}N gas-flux method in the same land use types between April 2013 and October 2014 for estimating annual rates of denitrification and N_2O emission, which is presented in Sgouridis and Ullah (2015).

The minimum detectable N_2 and N_2O fluxes depend on the precision of the IRMS systems, the soil NO_3^- pool enrichment, and the incubation parameters, such as the dimensions of the static chamber and the incubation time (Bergsma et al., 2001; Stevens and Laughlin 2001). For our chamber design, an incubation time of up to 20 h (which integrates the equilibration of the added tracer solution within the soil enclosure), and using the estimated MDC values (for both N_2 and N_2O) for calculating a $^{15}X_N$ value of 60 ^{15}N at %, the minimum detectable flux rates were 4 µg N m^{-2} h^{-1} and 0.2 ng N m^{-2} h^{-1} for the N_2 and N_2O fluxes respectively. These were significantly better than the minimum rates (175–900 µg N_2–N m^{-2} h^{-1} and 0.04–0.21 µg N_2O–N m^{-2} h^{-1}) reported by Bergsma et al. (2001), Kulkarni et al. (2014), and Tauchnitz et al. (2015), using similar field ^{15}N tracer approaches, and comparable to the minimum rates measured by a high-precision ^{15}N gas flux approach in a laboratory soil incubation (Yang et al., 2014) and the gas-flow soil core method (8 µg N_2–N m^{-2} h^{-1} and < 1 µg N_2O–N m^{-2} h^{-1}) by Wang et al. (2011). Our N_2 fluxes from woodland soils compare well with the rates reported in the literature for restored forested wetlands in North America (Morse and Bernhardt, 2013) and with the rates from northern hardwood forests in the USA (Kulkarni et al., 2014), using ^{15}N tracers at application rates similar to or lower than ours. Our results are also comparable to the rates reported from central European forests, under similar atmospheric N deposition rates, using the gas-flow soil core method (Butterbach-Bahl et al., 2002). For the grassland soils, the N_2 fluxes measured in the present study were significantly lower than previous applications of the ^{15}N gas-flux method at high fertilizer application rates (Baily et al., 2012; Cuhel et al., 2010; Graham et al., 2013), whilst for the organic soils our rates were significantly lower than the ones reported by Tauchnitz et al. (2015) since their ^{15}N tracer application rate (30 kg N ha^{-1}) was 300 times higher than ours. The N_2O fluxes were up to 200 times lower than the N_2 fluxes leading to low denitrification product ratios in all land use types, a result which is in line with the N_2O yields reported from ^{15}N tracer studies in forest (Kulkarni et al., 2014; Morse and Bernhardt 2013) and grassland soils (Baily et al., 2012; Bergsma et al., 2001). In the present study we have compared the in situ denitrification rates between three major land use types using an extended field incubation period to increase the probability of detecting a reliable ^{15}N–N_2 signal, particularly under conditions of low denitrifier activity due to seasonality of denitrification and/or inherent capacity of soils (for example, organic and deciduous forest soils). However, these rates should be considered conservative since confounding issues such as subsoil diffusion and non-homogeneous labelling of the soil nitrate pool may in some cases have led to underestimations of the in situ denitrification rates.

4.3 Comparison with the AIT

The total denitrification rates measured with the C_2H_2 amended intact soil cores followed the same trend as the total denitrification (N_2 and N_2O fluxes combined) from the ^{15}N gas-flux measurements, while they were on average 168 times lower than the denitrification potential measured in the same land use types in anaerobic soil slurries amended with acetylene and nitrate in a previous study (Sgouridis and Ullah, 2014), thus reflecting lower in situ rates. The AIT denitrification rates were between 3 and 5 times lower than the ^{15}N gas-flux rates despite the fact that the AIT intact soil cores were capped at the bottom, thus not allowing any subsoil diffusion of the evolved gases due to denitrification. Therefore, the AIT rates should have been higher than the ^{15}N gas-flux rates if serious underestimation was occurring due to subsoil diffusion in the open-bottom static chambers, which was not the case. Adding nitrate to the C_2H_2 amended cores would have been desirable for directly evaluating the priming effect of the added substrate on denitrification rates. The ^{15}N tracer addition to the static chambers corresponded to the amounts of N naturally deposited in these land use types either via management practices and/or atmospheric deposition, thus avoiding excessive N fertilization of the sampling plots. However, it cannot be conclusively argued that the same amount of applied nitrate would not have led to similar denitrification rates between the AIT and the ^{15}N gas-flux method. Previous comparisons between the AIT and the ^{15}N tracer method in field studies showed no significant difference between the two methods in measuring in situ total denitrification rates when the tracer is applied at high fertiliza-

tion rates (50–200 kg N ha^{-1}) and relatively low soil moisture contents (WFPS: 40–60 %; Aulakh et al., 1991; Mosier et al., 1986). Conversely, in laboratory incubations it was shown that the AIT significantly underestimated total denitrification compared to the ^{15}N tracer approach (Yu et al., 2010) and the direct N_2 flux approach (Qin et al., 2012) due to the incomplete inhibition of N_2O reduction to N_2 by C_2H_2 in wet soils (Yu et al., 2010) or in soils with low nitrate content (Qin et al., 2013, 2014). In our study, the soil WFPS ranged between 60 and 70 % in all land use types, with the exception of the C-MW site (mean WFPS 42 %), whilst the ^{15}N–NO_3^- tracer application rate was low (< 1 kg N ha^{-1}). Moreover, the disturbance of the soil structure during the extraction of the soil cores and the effect of the acetylene addition to microbial activity were not significant, as was suggested by the similar CO_2 production rates (Aulakh et al., 1991), representing soil respiration (Felber et al., 2012), in the static chambers and the C_2H_2 amended and un-amended intact soil cores. Therefore, we could argue that it is possible that the AIT underestimated total denitrification rates compared to the ^{15}N gas-flux method due to the likely incomplete inhibition of N_2O reduction to N_2 under relatively high soil moisture contents, although the shorter incubation time (2 h for the intact cores) may have limited the ability of C_2H_2 to fully equilibrate within soil pore spaces. Other confounding factors such as the catalytic decomposition of NO in the presence of C_2H_2 (Bollmann and Conrad, 1997; Nadeem et al., 2013) may have also contributed to the lower denitrification rates measured by the AIT. This study has confirmed some of the drawbacks of the AIT as a quantification method of in situ denitrification rates compared to the ^{15}N gas-flux.

The estimation of the denitrification product ratio using the AIT method, from the un-amended cores (N_2O only) and the C_2H_2 amended cores ($N_2 + N_2O$), is usually overestimated since the source of N_2O cannot be discriminated with the AIT, whilst the N_2 flux is underestimated due to the incomplete inhibition of N_2O reduction (Butterbach-Bahl et al., 2013). This was confirmed in the present study for all the land use types, and even the maximum denitrification product ratio after 2 h incubation in the case of the grassland soils (23 %) was still significantly lower than the respective ratio from the AIT (50 %). Therefore, the much lower denitrification product ratio estimated from the ^{15}N gas-flux measurements is significantly more reliable, and the wider application of this field technique across a range of land use types can have important implications for evaluating the role of denitrification as a reactive nitrogen sink and as a source of N_2O emissions (Butterbach-Bahl et al., 2013; Kulkarni et al., 2008).

5 Conclusions

The improved analytical precision for both ^{15}N–N_2 and ^{15}N–N_2O analyses allowed us to quantify in situ N_2 and N_2O fluxes with low ^{15}N tracer addition under field conditions in natural and semi-natural land use types for the first time. The estimation of N_2 fluxes was sensitive to the incubation time interval and the homogeneity of the tracer distribution due to the combination of several antagonistic effects such as decreasing gas diffusion gradients over time and soil moisture and substrate priming effects due to the added nitrate tracer solution. The spatial variability of N_2O fluxes superseded any bias associated with non-linear fluxes due to the extended incubation period. The uncertainty in the estimated N_2 and N_2O fluxes can be significantly reduced by increasing the homogeneity of the tracer application and by closely monitoring the linear evolution of the produced gases with more frequent gas sampling at shorter equal incubation intervals to avoid underestimation or overestimation of denitrification. Comparing the ^{15}N gas-flux method with the AIT confirmed the drawbacks of the AIT as a reliable quantification method of in situ denitrification rates. Moreover, the AIT method overestimated the denitrification product ratio compared to the ^{15}N gas-flux method. The ^{15}N gas-flux method holds much promise as a more reliable field technique for measuring in situ denitrification rates and its wider application across a range of terrestrial ecosystems can lead to its refinement and improvement and in the long term can significantly contribute to our understanding of the role of denitrification as a reactive nitrogen sink.

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