21 February, 2016

Response to Dr Reinhard Well’s comments on the manuscript ‘Application of the 15N-Gas Flux method for measuring in situ N₂ and N₂O fluxes due to denitrification in natural and semi-natural terrestrial ecosystems and comparison with the acetylene inhibition technique.’ (Manuscript ID = doi:10.5194/bgd-12-12653-2015)

We are grateful to Dr Well for his final comments before the acceptance of our manuscript. We have accommodated all the suggestions where possible and amended the manuscript accordingly.

Response (in bold-face) to each comment (in italics) of the Reviewers follows:

Minor comments

1) RW: ok, but I suggest to reword the caption:

“Evaluation of the linearity of the evolved N₂ during field incubation, per sampling plot in each field site. Only those samples that were above the MDC value are used. Linear evolution of N₂ in a constant headspace volume is proven when T2/T1 = 2 and T3/T1 = 20. T1 = 1 hour, T2 = 2 hours and T3 ~ 20 hours of incubation time. Ratios close to the ideal values are highlighted in bold font” please add a criterion here, e.g deviation from ideal ratio < x %.

We have amended the captions of Supplementary Tables 4&5 as suggested by the reviewer.

2) RW: when referring to nitrate enrichment level please replace % with atom % 15N

Atom % 15N inserted as per the reviewer’s instruction.

3) Line 594-596 “The non-significant change of 15XN with incubation time suggested only one
denitrifying pool for both N2 and N2O, assuming negligible N2 production from anammox and codenitrification (Spott and Stange 2007). Information on hybrid N2 or N2O can only be obtained from the comparison of 15XN and 15N atom fraction of extracted NO3. But since you did not analyse extracts, there is no evidence for or against hybrid fluxes. So I suggest to delete this sentence.

Sentence deleted.

4) L 600-6003: Not clear to me for two reason: 15XN was measured in each gas sample, so the decrease in 15XN was taken into account in the calculation, hence no bias from that. Also I don’t see why low enrichment would lead to less dilution effect, since the relative change in the difference between 15XN and natural abundance is always the same irrespective of the initial enrichment So I suggest to delete this phrase.

Sentence deleted.

5) L 626-627: “and/or reduction of gas exchanges at the soil-atmosphere interface due to positive pressure build up in the chamber headspace (Healy et al. 1996).”

Was this addressed in Healy et al? This could not occur in vented chambers. In unvented chambers pressure fluctuations might result in both, enhanced or inhibited emissions depending on increasing or decreasing atmospheric pressure during closure. But pressure differences would hardly affect diffusion. I remember that Healy mainly focused on diffusive fluxes, showing that decreasing fluxes are due to decreasing CONCENTRATION GRADIENTS. Suggest to double check this and eventually modify accordingly.

Sentence modified to reflect the reviewer’s comment for the appropriate use of the literature reference.

6) L 632 “enhanced N2O reduction due to both subsoil diffusion and the increasing concentration of the N2O in the topsoil”

N2O reduction to N2 in topsoil would not be enhanced by subsoil diffusion. Do you mean “extended enclosure time lead to lowering of N2O fluxes due to subsoil diffusion and enhanced N2O reduction to N2”?
Sentence corrected as per the reviewer’s instruction.

7) L 666 please refer to Table S6 here

Suggest to reformulate “could potentially be explained by a delay in the de novo synthesis of DENITRIFICATION ENZYMES AND THE FACT THAT THE N2O reductase enzyme is known to have a slower expression than the preceding reduction enzymes (Knowles, 1982), leading to N2O accumulation and lower N2 production after 2 hours of incubation.” since the product ratio first increases until T2 which could not be explained by a change in N2O reductase only.

Sentence modified according to the reviewer’s comment

8) L 697 do you mean 15XN value of 60 atom %? Please be consistent in these units

The unit 15N at % has been added

9) L 37-40

“The total denitrification rates measured by the acetylene inhibition technique in the same land use types correlated (r = 38.0.58) with the denitrification rates measured under the 15N Gas-Flux method but were underestimated by a factor of 4 and this was partially attributed to the incomplete inhibition of N2O reduction to N2 under a relatively high soil moisture content.”

RW: You did not prove whether incomplete inhibition or catalytic NO decomposition was more important. The latter has been convincingly demonstrated as a serious source of bias in several previous studies. Therefore I suggest that you mention both explanations in the abstract.

Abstract modified as per the reviewer’s suggestion
Application of the $^{15}$N-Gas Flux method for measuring in situ N$_2$ and N$_2$O fluxes due to denitrification in natural and semi-natural terrestrial ecosystems and comparison with the acetylene inhibition technique.

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Keywords: Organic soils, forest, grassland, $^{15}$N tracer, acetylene inhibition technique, nitrous oxide.
Abstract

Soil denitrification is considered the most unconstrained process in the global N cycle due to uncertain *in situ* N2 flux measurements, particularly in natural and semi-natural terrestrial ecosystems. 15N tracer approaches can provide *in situ* measurements of both N2 and N2O simultaneously, but their use has been limited to fertilised agro-ecosystems due to the need for large 15N additions in order to detect 15N2 production against the high atmospheric N2. For 15N-N2 analyses, we have used an ‘in house’ laboratory designed and manufactured N2 preparation instrument which can be interfaced to any commercial continuous flow isotope ratio mass spectrometer (CF-IRMS). The N2 prep unit has gas purification steps, a copper based reduction furnace, and allows the analysis of small gas injection volumes (4 μL) for 15N-N2 analysis. For the analysis of N2O, an automated Tracegas Pre-concentrator (Isoprime Ltd) coupled to an IRMS was used to measure the 15N-N2O (4 mL gas injection volume). Consequently, the coefficient of variation for the determination of isotope ratios for N2 in air and in standard N2O (0.5 ppm) was better than 0.5 %. The 15N Gas-Flux method was adapted for application in natural and semi-natural land use types (peatlands, forests and grasslands) by lowering the 15N tracer application rate to 0.04 - 0.5 kg 15N 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 N2 and N2O 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 15N Gas-Flux method but were underestimated by a factor of 4 and this was partially attributed to the incomplete inhibition of N2O reduction to N2 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 N2 and N2O fluxes with the 15N 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$_2$O 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\textsubscript{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\textsubscript{3} and NO\textsubscript{2}) to NO, N\textsubscript{2}O and ultimately N\textsubscript{2} gas, constitutes the most important mechanism for the removal of reactive nitrogen (Nr) in terrestrial ecosystems (Galloway et al. 2008, Groffman 2012). Despite its importance, denitrification is considered the most unconstrained process in the global N cycle (Groffman 2012, Kulkarni et al. 2008) due to uncertainties in N\textsubscript{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 UK (Dore et al. 2012, Galloway et al. 2008, Payne 2014), the available Nr 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\textsubscript{2}O), 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\textsubscript{2} + N\textsubscript{2}O) in soils is crucial in evaluating the role of denitrification as an Nr sink (Kulkarni et al. 2008).

N\textsubscript{2} comprises ~78 % of the atmosphere and thus it is extremely difficult to measure small N\textsubscript{2} fluxes from soil against this high background, particularly in natural terrestrial ecosystems (Groffman et al. 2006). Available methods for measuring both N\textsubscript{2} and N\textsubscript{2}O are limited and can
be categorised 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 limits 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$_2$O thus overestimating the denitrification product ratio N$_2$O/ (N$_2$ + N$_2$O) (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$_2$H$_2$) at high concentrations (10% v/v) to inhibit the reduction of N$_2$O to N$_2$ (Tiedje et al. 1989), thus total denitrification (N$_2$ + N$_2$O) is measured in C$_2$H$_2$ 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 to its several limitations (Groffman et al. 2006), of which the catalytic decomposition of NO in the presence of C$_2$H$_2$ under oxic or suboxic conditions in the field (Bollmann and Conrad 1996, 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$_2$O 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-$\text{N}_2$ and $^{15}$N-$\text{N}_2\text{O}$ produced by denitrification (Stevens and Laughlin 1998). Assuming that both $\text{N}_2$ and $\text{N}_2\text{O}$ 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 fertilised 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 - 200 kg N ha$^{-1}$), with the exception of Kulkarni et al. (2014) who have measured denitrification rates in Northern hardwood forests of the US 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 $\text{N}_2$ and $\text{N}_2\text{O}$ 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 fertiliser use and the impact from grazing and commercial forestry is minimal (Mills et al. 2013), along with improved and unimproved grasslands (grazed and/or fertilised) 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 $\text{Nr}$ loss through denitrification.
The major challenge in measuring $^{15}$N-$\text{N}_2$ at near natural abundance levels is the possibility of interference at m/z 30 ($^{15}$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 continuous flow isotope ratio mass spectrometers (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 remove O$_2$ and other trace gases such as carbon monoxide (Lewicka-Szczebak et al. 2013, Yang et al. 2014). We hypothesise that the precision for m/z 30 determination can be greatly improved by using a custom-built preparative unit for the removal of H$_2$O, CO$_2$, N$_2$O, 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 fertilised 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 by both field techniques, although the relatively low soil moisture contents have probably allowed greater diffusion of C$_2$H$_2$ to the anaerobic microsites where denitrification occurs (Malone et al. 1998), whilst the high nitrate application rates have probably favoured nitrate reduction over N$_2$O 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$_2$O reduction to N$_2$ by C$_2$H$_2$ in wet soils (Yu et al. 2010) or in
soils with low nitrate content, where N$_2$O reduction is more energetically favourable (Qin et al. 2013, Qin et al. 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-$\text{N}_2$ and $^{15}$N-N$_2$O 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\textsubscript{2} gas isotopic analysis we used an Isoprime isotope ratio mass spectrometer (Isoprime Ltd, UK, Wythenshawe) coupled to an in house built N\textsubscript{2} preparative interface (Figure 1). Headspace gas (4 \(\mu\)L) 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\textsubscript{4})\textsubscript{2} (Elemental Microanalysis Ltd, Devon, UK), b) CO\textsubscript{2} removed with 0.7 - 1.2 mm Carbosorb (Elemental Microanalysis Ltd, Devon, UK), c) N\textsubscript{2}O cryogenically trapped under liquid nitrogen, and d) O\textsubscript{2} removed over a copper-packed reduction furnace heated at 600°C. The N\textsubscript{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 (\textsuperscript{28}N\textsubscript{2}, \textsuperscript{29}N\textsubscript{2} and \textsuperscript{30}N\textsubscript{2}) were recorded for each sample at a trap current of 300 \(\mu\)Amps. Instrument stability checks were performed prior to each analysis by running a series of 10 reference pulses of N\textsubscript{2} (BOC special gases) until a standard deviation of \(\delta^{15}\text{N}\) better than 0.05 \(\%\) was achieved. Additionally, 10 consecutive injections (4 \(\mu\)L) of atmospheric air were analysed prior to the analysis of actual samples. Precision of the instrument was better than \(\delta^{15}\text{N} 0.08 \%\) 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\textsuperscript{TM}
Preconcentrator coupled to an Isoprime™ IRMS (GV instruments Ltd, UK) whereupon the sample was directed through a series of chemical traps designed to remove H₂O and CO₂. The N₂O was cryogenically trapped under liquid nitrogen. The waste was flushed out of the instrument. The N₂O was further cryofocused in a second liquid nitrogen trap prior to being introduced onto a 25 m x 0.32 mm Poraplot Q gas chromatography column (Chrompack column, Varian, Surrey, U.K). The column separated N₂O from any residual CO₂, and both entered the IRMS via an open split. The retention time between the first eluting CO₂ (< 2⁻¹⁰ amplitude) and second eluting N₂O peak typically fell in the range between 60 - 70 seconds to avoid isobaric interference of the CO₂ with the calculated ¹⁵N. The N₂O 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 N₂O (BOC special gases) until a standard deviation of δ¹⁵N better than 0.05 ‰ was achieved. Prior to each sample batch analysis, trace gas N₂O measurements were made on three 100 mL flasks containing atmospheric air collected from outside the stable isotope laboratory. δ¹⁵N precisions using the Trace gas Preconcentrator and Isoprime IRMS were better than 0.3 ‰ respectively at 600 µAmp trap current.

2.2. Field application of the ¹⁵N Gas-Flux and AIT techniques

In situ measurements of N₂ and N₂O were made using static chambers according to the ¹⁵N 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 km²; NW 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 km²; N. 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 & Ullah (2014).

In each plot a round PVC collar (basal area 0.05 m²; chamber volume 4 L) was inserted into the soil at c. 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 CH₄ (10 ppm), and determining the change in CH₄ concentration within the chamber headspace over time (Yang et al. 2011). The CH₄ concentration change within 24 hours 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 N₂, as part of our effort to increase the probability of a detectable ¹⁵N-N₂ signal in the chamber headspace. Instead chambers were covered with reflective foil for minimising temperature increase within the chamber headspace during the incubation period (Ullah and Moore 2011). Labelled K¹⁵NO₃ (98 at. % ¹⁵N, Sigma-Aldrich) was applied in each plot via ten injections of equal volume through a grid (4 x 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 (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 Supplementary Table 1 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 = 1h, T = 2h and T $\approx$ 20h after the tracer injection, while a T = 0h 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 of 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 C₂H₂ with 5 mL injected through the septum directly in the middle of the soil core before 10% of the headspace being also replaced with pure C₂H₂. The second set of cores was not amended with C₂H₂ 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 = 1h and T = 2h 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, United Kingdom) for storage under positive pressure.

2.3. Flux calculations

The ¹⁵N content of the N₂ in each 12 mL vial was determined using the IRMS system described above and the ratios R₂⁹ (²⁹N₂/²⁸N₂) and R₃₀ (³⁰N₂/²⁸N₂) were measured in both enriched (T=1, 2 and 20 hours) and reference samples (T=0 hours). 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-Szczepak 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):

\[ MDC = \mu_{\text{pair diff}} + (2\sigma_{\text{pair diff}}) \]  

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

For calculating the total N\textsubscript{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 \(^{15}\text{N} \) fraction in the soil NO\textsubscript{3} \textsuperscript{-} denitrifying pool
\((^{15}X_0)\) as:

\[ ^{15}X_0 = \frac{2(\Delta R30/\Delta R29)}{1 + 2(\Delta R30/\Delta R29)} \]  

where \( \Delta R29 \) and \( \Delta R30 \) is the difference between R29 and R30 respectively between
enriched (T=1, 2 and 20 hours) and reference samples (T=0 hours). Subsequently, the
\(^{15}X_0 \) allows the quantification of the fraction of the N\textsubscript{2} evolved from the \(^{15}\text{N} \)-labelled pool
\((d) \) using either the \( \Delta R30 \) or the \( \Delta R29 \):

\[ d = \frac{\Delta R30}{(^{15}x_0)^2} \]  

\[ d = \frac{\Delta R29}{2(^{15}x_0)(1-^{15}x_0)^2} \]
Using \( d \) and the concentration of \([N_2]\) (\( \mu g \) N) in the chamber headspace, the evolved \( N_2 \) from the soil pool was calculated:

\[
Evolved \ N_2 = d[N_2]/(1 - d)
\]  

(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 \( \mu g \) 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 \( R45 \) (\(^{15}N_2O/^{14}N_2O\)) and \( R46 \) (\(^{16}N_2O/^{16}N_2O\)) were measured in both enriched (\( T=1, 2 \) and 20 hours) and reference samples (\( T=0 \) hours). 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:

\[
R29 = R45 - R17
\]  

(6)

\[
R30 = (R46 - (R29R17)) - R18
\]  

(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 \( 3.4 \times 10^{-5} \) and \( 2.9 \times 10^{-5} \) respectively. The second set of gas samples collected at the same time in the field were analysed for total \( N_2O \) on a GC-\( \mu \)ECD (7890A GC Agilent Technologies Ltd., Cheshire, UK) and the concentration of \([N_2O]\) (\( \mu g \) 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 $\mu g$ N-N$_2$O m$^{-2}$ h$^{-1}$. Assuming that the N$_2$O originates from the same uniformly labelled pool as N$_2$, the $^{15}$X$_N$ from N$_2$O 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$_2$O on a GC-$\mu$ECD (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 hours. The instrument precision was determined from repeated analyses of 6 ppm N$_2$O 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 8 field sites were ordinated in 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 the 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 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\(_2\)O 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\(_2\)O 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\(_2\)O at natural abundance. The mean measured R30 (5.16 x 10\(^{-5}\)) was higher than the theoretical value of 1.35 x 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 x 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 Supplementary Table 2).

The \(^{15}\)N fraction in the denitrifying pool (\(^{15}\)X\(_N\)), as calculated from the measured isotopic ratios of the \(N_2O\) after 1 hour of incubation using Eq. (2), ranged between 65 and 93 \(^{15}\)N at\%. The average change of the \(^{15}\)X\(_N\) with incubation time, indicated by the slope shown in Table 2, was not different from 0 in 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 to 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 Supplementary Table 3), 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 (Figure 2). The increase in the evolved \(N_2\) was statistically significant after 20 hours 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 hours incubation in Figure 2a, it was not significant for the OS and WL soils. The amount of \(N_2O\)
accumulated after 20 hours (Figure 2b) was significantly higher than in 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 Supplementary Tables 4 & 5), for example in C-MW 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$_2$O concentrations was not linear throughout the incubation period (Supplementary Tables 4 & 5). 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 (Figure 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 hours in the WL (Kruskal-Wallis; $\chi^2 = 10.78$, $p = 0.005$) and between 1 and 2 hours in the GL (Kruskal-Wallis; $\chi^2 = 10.10$, $p = 0.006$). Conversely, the N$_2$O flux rates increased between the first and second hour of incubation (Figure 3b), followed by a decrease after 20 hours, albeit the mean differences between time intervals were not statistically significant in any land use type (Kruskal-Wallis; $\chi^2_{OS} = 3.58$, $\chi^2_{WL} = 3.47$, $\chi^2_{GL} = 3.01$, $p > 0.05$).

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 hour 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 (Figure 4a). A similar pattern was observed for the N$_2$O 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$_2$O than the woodland and organic soils respectively (Figure 4b), whilst the N$_2$O flux was on average 20 to 200 times lower than
the N\textsubscript{2} flux among land use types. Consequently, the denitrification product ratio N\textsubscript{2}O/ (N\textsubscript{2} + N\textsubscript{2}O) was low, ranging between 0.03 and 13 % and was highest in the GL and similar between the WL and OS (Figure 4c). The change of the denitrification product ratio with incubation time was evaluated in each sampling plot where both N\textsubscript{2} and N\textsubscript{2}O fluxes were available (data shown in Supplementary Table 6). 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 hours of incubation (ANOVA; \( F = 6.11 \), \( p < 0.05 \)). This was an indication of some reduction of the denitrification derived N\textsubscript{2}O to N\textsubscript{2} during the extended closure period (up to 20 hours) in the grassland soils.

3.3. Comparison with the AIT

The total denitrification rate measured from the C\textsubscript{2}H\textsubscript{2} amended intact soil cores in the same land use types ranged between 0.5 and 325.2 \( \mu \text{g N m}^{-2} \text{h}^{-1} \) and correlated positively with the total denitrification rate (N\textsubscript{2} and N\textsubscript{2}O fluxes combined) measured with the \(^{15}\text{N Gas-Flux method} \) (Pearson; \( r = 0.581 \), \( n = 25 \), \( p < 0.01 \)) following a similar trend among land use types, albeit 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 C\textsubscript{2}H\textsubscript{2} amended cores. The difference being significant in woodland (t-test; \( t = 3.914 \), \( df = 18 \), \( p < 0.01 \)) and grassland soils (t-test; \( t = 3.521 \), \( df = 25 \), \( p < 0.01 \)).

The total N\textsubscript{2}O flux measured from the un-amended intact soil cores ranged between 0.15 and 86.6 \( \mu \text{g N m}^{-2} \text{h}^{-1} \) and was between 1 and 3 times lower than the total denitrification rate from the C\textsubscript{2}H\textsubscript{2} amended cores. There were no significant differences between bulk N\textsubscript{2}O
fluxes measured with the static chambers and the un-amended intact soil cores (Figure 5b), which indicated that total \(\text{N}_2\text{O}\) emissions were comparable between the two field techniques. Consequently, estimating the denitrification product ratio from the un-amended and \(\text{C}_2\text{H}_2\) amended intact soil cores resulted in significantly higher ratios compared to the \(^{15}\text{N}\) Gas-Flux approach (Figure 5c), which were on average between 50 and 60% and not significantly different among land use types (Table 3).

The mean \(\text{CO}_2\) production rate was similar irrespective of whether it was measured in static chambers, in \(\text{C}_2\text{H}_2\) amended or un-amended intact soil cores (Figure 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 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 ten 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 x 10$^{-5}$) compared to the value (1.6 x 10$^{-4}$) reported by Lewicka-Szczebak et al. (2013). Consequently, correcting the R30 ratio for the NO$^+$ ions 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$^\text{TM}$ Preconcentrator IRMS system used for $^{15}$N-$N_2O$ analysis displayed similar precision for the determination 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 \(^{15}\)N-N\(_2\) and \(^{15}\)N-N\(_2\)O analyses, using smaller gas sample volumes than previously reported, allowed us to quantify in situ N\(_2\) and N\(_2\)O 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 one to two orders of magnitude lower than previous applications of the \(^{15}\)N Gas-Flux method in highly fertilised 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\%, Supplementary Table 2) 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\%, Supplementary Table 2) 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 (~15 - 20 kg N ha$^{-1}$ y$^{-1}$) (Dore et al. 2012, Payne 2014), whilst for the grassland soils the tracer application mimicked a daily fertiliser 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 reflecting 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$_2$O 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 optimised 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 minimise 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 for directly estimating 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 non-significant change of $^{15}$X$_N$ with incubation time suggested only one denitrifying pool for both N$_2$ and N$_2$O, assuming negligible N$_2$-production from anammox and co-denitrification (Spott and Stange 2007). Only in the case of the C-MW well-drained forest site, shown to exhibit the highest nitrification potential (Sgouridis and Ullah 2014), the slope of $^{15}$X$_N$ with time was negative suggesting dilution of the $^{15}$N-labelled soil NO$_3^-$ pool by the oxidation of the ambient ammonium (nitrification). It is therefore possible that N$_2$-flux rates may be overestimated in C-MW, due to the underestimation of the $^{15}$X$_N$, but Bergsma et al. (1999) showed that temporal changes of the soil NO$_3^-$ pool enrichment are negligible at $^{15}$N enrichment levels similar to ours.
The larger volume of tracer per injection (>4 mL) in combination with the fewer number of injections compared to Wu et al. (2011) may have created localised 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 hours of incubation may be overestimated due to moisture induced denitrification events.

Most studies using 15N tracers and static chambers in highly fertilised systems typically deploy their chambers between 1 and 2 hours (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 hours) may be needed in case of low 15N enrichment applications in intact soil cores (Morse and Bernhardt 2013) and laboratory incubations (Yang et al. 2014) for a more precise and accurate detectable 15N-N2 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 N2 and N2O 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 hours may have potentially allowed some loss of the evolved N2 and N2O through downward subsoil diffusion and/or reduction of gas exchanges at the soil-atmosphere interface due to positive pressure build up in the chamber headspace (Healy et al. 1996). This could partly explain the non-linear increase of the evolved N2 and N2O in the chamber headspace (Figures 2a &
b) and also the decrease of the N\textsubscript{2} flux rate with increasing incubation time (Figure 3a). The N\textsubscript{2}O flux rate increased up to 2 hours incubation followed by a decrease after 20 hours consistently across land use types (Figure 3b), indicating that the extended enclosure period lowered N\textsubscript{2}O fluxes due to subsoil diffusion and enhanced N\textsubscript{2}O reduction to N\textsubscript{2} and this was an indication of potentially enhanced N\textsubscript{2}O reduction due to both subsoil diffusion and the increasing concentration of the N\textsubscript{2}O in the topsoil. However, due to the high spatial heterogeneity within each land use type, the mean N\textsubscript{2}O flux rate was not significantly different between the different incubation intervals. In other words, the non-linearity of N\textsubscript{2}O 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\textsubscript{2}O fluxes far exceeds the bias due to assumed linearity of fluxes.

The lack of a consistent pattern of N\textsubscript{2} flux rate change with incubation time among the different land use types suggested a more complex temporal variability of N\textsubscript{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 (\textsuperscript{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 hours of incubation, until the added
water started to equilibrate with the ambient soil moisture. Therefore the \(N_2\) flux rate in C-750
MW may be significantly overestimated after 1 hour of incubation. In the grassland sites
and the R-DW forest site with intermediate soil moistures (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 one hour of incubation should be
more reliable as long as the bias from analytical uncertainty was low. In these sites
denitrification rates estimated after one or 20 hours of incubation were not significantly
different (Figure 3a), suggesting a quasi-linear \(N_2\) evolution throughout the incubation
period (at least in 37.5% of the sampling plots, see Supplementary Table 4). However, the
\(N_2\) flux rates were significantly lower after 2 hours of incubation, whereas the \(N_2O\) flux
rates were maximum at 2 hours of incubation consequently leading to an increased product
ratio \(N_2O/ (N_2 + N_2O)\) (Supplementary Table 6). 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 enzyme 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 hours of incubation. After 20 hours 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 \(^{15}\text{N}\) 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\textsubscript{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 over-estimation of denitrification in nitrate and moisture limited ecosystems and potential under-estimation due to subsoil diffusion of evolved gases. The detailed uncertainty analysis of the N\textsubscript{2} and N\textsubscript{2}O flux estimation presented in this study complements the large scale application of the 15N Gas Flux method in the same land use types between April 2013 and October 2014 for estimating annual rates of denitrification and N\textsubscript{2}O emission, which is presented in Sgouridis and Ullah (2015).

The minimum detectable N\textsubscript{2} and N\textsubscript{2}O fluxes depend on the precision of the IRMS systems, the soil NO\textsubscript{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 hours (which integrates the equilibration of the added tracer solution within the soil enclosure), and using the estimated MDC values (for both N\textsubscript{2} and N\textsubscript{2}O) for calculating a \textsuperscript{15}X\textsubscript{N} value of \(0.660\ \text{at\%}\), the minimum detectable flux rates were 4 \(\mu\text{g N m}^{-2} \text{h}^{-1}\) and 0.2 ng N m\textsuperscript{-2} h\textsuperscript{-1} for the N\textsubscript{2} and N\textsubscript{2}O fluxes respectively. These were significantly better than the minimum rates (175 - 900 \(\mu\)g N\textsubscript{2}-N m\textsuperscript{-2} h\textsuperscript{-1} and 0.04 - 0.21 \(\mu\)g N\textsubscript{2}O-N m\textsuperscript{-2} h\textsuperscript{-1}) reported by Bergsma et al. (2001), Kulkarni et al (2014) and Tauchnitz et al (2015), using similar field \textsuperscript{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 N₂-m⁻²-h⁻¹ and < 1 μg N₂O-N m⁻²-h⁻¹) by Wang et al. (2011). Our N₂ 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 US (Kulkarni et al. 2014), using $^{15}$N tracers at similar or lower application rates to 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 N₂ fluxes measured in the present study were significantly lower than previous applications of the $^{15}$N Gas-Flux method at high fertiliser 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 N₂O fluxes were up to 200 times lower than the N₂ fluxes leading to low denitrification product ratios in all land use types, a result which is in line with the N₂O 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-N₂ 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$_2$H$_2$ amended intact soil cores followed the same trend as the total denitrification (N$_2$ and N$_2$O 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$_2$H$_2$ 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 fertilisation 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 methods. 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 tracer is applied at high fertilisation 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$_2$O.
reduction to N\textsubscript{2} by C\textsubscript{2}H\textsubscript{2} in wet soils (Yu et al. 2010) or in soils with low nitrate content (Qin et al. 2013, Qin et al. 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 \textsuperscript{15}N-NO\textsubscript{3} tracer application rate was low (< 1 kg N ha\textsuperscript{-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 it was suggested by the similar CO\textsubscript{2} production rates (Aulakh et al. 1991), representing soil respiration (Felber et al. 2012), in the static chambers and the C\textsubscript{2}H\textsubscript{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 \textsuperscript{15}N Gas-Flux method due to the likely incomplete inhibition of N\textsubscript{2}O reduction to N\textsubscript{2} under relatively high soil moisture contents, although the shorter incubation time (2h for the intact cores) may have limited the ability of C\textsubscript{2}H\textsubscript{2} to fully equilibrate within soil pore spaces. Other confounding factors such as the catalytic decomposition of NO in the presence of C\textsubscript{2}H\textsubscript{2} (Bollmann and Conrad 1996, 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 \textsuperscript{15}N Gas-Flux.

The estimation of the denitrification product ratio using the AIT method, from the un-amended cores (N\textsubscript{2}O only) and the C\textsubscript{2}H\textsubscript{2} amended cores (N\textsubscript{2} + N\textsubscript{2}O), is usually overestimated since the source of N\textsubscript{2}O cannot be discriminated with the AIT, whilst the N\textsubscript{2} flux is underestimated due to the incomplete inhibition of N\textsubscript{2}O 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 hours 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$_2$O emissions (Butterbach-Bahl et al. 2013, Kulkarni et al. 2008).

5. Conclusion

The improved analytical precision for both $^{15}$N-N$_2$ and $^{15}$N-N$_2$O analyses allowed us to quantify in situ N$_2$ and N$_2$O 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$_2$O fluxes superseded any bias associated with non-linear fluxes due to the extended incubation period. The uncertainty in the estimated N$_2$ and N$_2$O 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 under or over estimation 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.

6. Acknowledgements

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7. References


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Qin, S., Hu, C. and Oenema, O.: Quantifying the underestimation of soil denitrification potential as determined by the acetylene inhibition method, Soil Biology and Biochemistry, 47, 14-17, 2012.


Table 1: Measured ratios of R29 and R30 for N\textsubscript{2} in ambient air (n=10), ratios of R45 and R46 in standard N\textsubscript{2}O gas (0.5 ppm concentration, n=15) and \(^{15}\text{N}\) at\% abundance calculated from the respective ratios for both gases. SD; standard deviation, CV; coefficient of variation.

<table>
<thead>
<tr>
<th></th>
<th>R29 (N\textsubscript{2})</th>
<th>R30 (N\textsubscript{2})</th>
<th>R45 (N\textsubscript{2}O)</th>
<th>R46 (N\textsubscript{2}O)</th>
<th>(^{15}\text{N}) at% (N\textsubscript{2})</th>
<th>(^{15}\text{N}) at% (N\textsubscript{2}O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>7.38 (?10^{-3})</td>
<td>5.16 (?10^{-5})</td>
<td>8.00 (?10^{-3})</td>
<td>2.21 (?10^{-3})</td>
<td>3.71 (?10^{-1})</td>
<td>3.88 (?10^{-1})</td>
</tr>
<tr>
<td>SD</td>
<td>2.77 (?10^{-7})</td>
<td>2.26 (?10^{-7})</td>
<td>1.25 (?10^{-5})</td>
<td>1.04 (?10^{-5})</td>
<td>2.09 (?10^{-5})</td>
<td>1.01 (?10^{-3})</td>
</tr>
<tr>
<td>CV (%)</td>
<td>0.00</td>
<td>0.44</td>
<td>0.16</td>
<td>0.47</td>
<td>0.01</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Table 2: The ambient soil nitrate pool, the \(^{15}\text{N}\) tracer application rate, the estimated enrichment of the total soil nitrate pool, the calculated \(^{15}\text{X}_N\) value from N\textsubscript{2}O and the slope of the \(^{15}\text{X}_N\) change with incubation time in the three land use types. Data are means with standard errors in parentheses.

<table>
<thead>
<tr>
<th>Land Use Type</th>
<th>Ambient \text{NO}_\text{3} (kg N ha\textsuperscript{-1})</th>
<th>Tracer application rate (kg (^{15}\text{N}) ha\textsuperscript{-1})</th>
<th>Enrichment of total soil \text{NO}_\text{3} pool ((^{15}\text{N}) at%)</th>
<th>(^{15}\text{X}_N) (%)</th>
<th>(^{15}\text{X}_N) slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic Soil</td>
<td>0.53 (0.44)</td>
<td>0.04 (0.02)</td>
<td>25 (11.8)</td>
<td>90 (1.5)</td>
<td>0.003 (0.0054)</td>
</tr>
<tr>
<td>Woodland</td>
<td>3.86 (2.42)</td>
<td>0.62 (0.41)</td>
<td>13 (0.7)</td>
<td>79 (8.3)</td>
<td>-0.007 (0.0025)</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>15N Gas-Flux</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denitrification</td>
<td>19.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>N₂O emission</td>
<td>31.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>N₂O/ (N₂ + N₂O)</td>
<td>7.4</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Total bulk N₂O</td>
<td>19.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CO₂ production</td>
<td>19.8</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AIT</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denitrification</td>
<td>12.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Total bulk N₂O</td>
<td>9.4</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>N₂O/ (N₂ + N₂O)</td>
<td>0.3</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td>CO₂ production (un-amended cores)</td>
<td>11.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CO₂ production (C₂H₂ amended cores)</td>
<td>11.7</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
Figures

Figure 1: Schematic of the $^{15}$N-$N_2$ analysis system
Figure 2: Evolved (a) N$_2$ and (b) N$_2$O gas measured between 1, 2 and 20 hours 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.
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.
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 parenthesis for each land use type on the x-axis. Error bars represent standard errors.
Figure 5: (a) Mean total denitrification measured with the $^{15}$N Gas-Flux method and the AIT, (b) Mean bulk N$_2$O 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$_2$O/(N$_2$ + N$_2$O) 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 independent samples t-test. The sample size (n) is given in parenthesis 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$_2$H$_2$ 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 independent samples t-test. The sample size (n) is given in parenthesis for each land use type on the x-axis. Error bars represent standard errors.