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.

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Abstract

Soil denitrification is considered the most un-constrained 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 fertilised agro-ecosystems 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, 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 Pre-concentrator (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. 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 (Nr) 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 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_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 Nr sink (Kulkarni et al. 2008).

N_2 comprises ~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 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_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 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_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 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 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 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 Nr 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

138 formation of NO^+ ions that also have *m/z* 30 (Stevens et al. 1993). Commonly, this issue is
139 addressed in continuous flow isotope ratio mass spectrometers (CF-IRMS) with the inclusion
140 of a copper (Cu) oven for reducing O_2 in the gas sample (Russow et al. 1996). Recently, it has
141 been suggested that the interference at *m/z* 30 can be further reduced by including a molecular
142 sieve column in gas chromatograph IRMS (GC-IRMS) systems to not only separate N_2 and O_2
143 in the gas sample, but also to quantitatively remove O_2 and other trace gases such as carbon
144 monoxide (Lewicka-Szczebak et al. 2013, Yang et al. 2014). We hypothesise that the precision
145 for *m/z* 30 determination can be greatly improved by using a custom-built preparative unit for
146 the removal of H_2O, CO_2, N_2O, NO^+ and CO; a device which also permits the micro scale
147 injection of volumes of < 5 μL. These injection volumes are much smaller than have previously
148 been reported in the literature.

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150 Studies that have compared the ^{15}N Gas-Flux method with the AIT in the field are rare and
151 have exclusively focused on highly fertilised agro-ecosystems with moderate to low soil
152 moisture contents (Aulakh et al. 1991, Mosier et al. 1986, Rolston et al. 1982). These studies
153 have measured comparable denitrification rates by both field techniques, although the
154 relatively low soil moisture contents have probably allowed greater diffusion of C_2H_2 to the
155 anaerobic microsites where denitrification occurs (Malone et al. 1998), whilst the high nitrate
156 application rates have probably favoured nitrate reduction over N_2O reduction (Dendooven and
157 Anderson 1995) resulting in high denitrification rates from the AIT. Conversely, laboratory
158 studies have shown that the AIT significantly underestimates total denitrification compared to
159 the ^{15}N tracer approach (Yu et al. 2010) and the direct N_2 flux approach (Qin et al. 2012) due
160 to the incomplete inhibition of N_2O reduction to N_2 by C_2H_2 in wet soils (Yu et al. 2010) or in
161 soils with low nitrate content, where N_2O reduction is more energetically favourable (Qin et
162 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 $^{15}N\text{-}N_2O$ 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 (Figure 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 μAmps. 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 $\delta^{15}N$ better than 0.05 ‰ was achieved. Additionally, 10 consecutive injections (4 μL) of atmospheric air were analysed prior to the analysis of actual samples. Precision of the instrument was better than $\delta^{15}N$ 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

209 the instrument. The N_2O was further cryofocused in a second liquid nitrogen trap prior to
210 being introduced onto a 25 m x 0.32 mm Poraplot Q gas chromatography column
211 (Chrompack column, Varian, Surrey, U.K). The column separated N_2O from any residual
212 CO_2, and both entered the IRMS via an open split. The retention time between the first
213 eluting CO_2 (< 2^{E-10} amplitude) and second eluting N_2O peak typically fell in the range
214 between 60 - 70 seconds to avoid isobaric interference of the CO_2 with the calculated ^{15}N.
215 The N_2O was directed towards the triple collectors of the isotope ratio mass spectrometer
216 where *m/z* 44, *m/z* 45 and *m/z* 46 mass ions were measured and recorded. Instrument
217 stability checks were performed prior to each analysis by running a series of 10 reference
218 pulses of N_2O (BOC special gases) until a standard deviation of $\delta^{15}N$ better than 0.05 ‰
219 was achieved. Prior to each sample batch analysis, trace gas N_2O measurements were made
220 on three 100 mL flasks containing atmospheric air collected from outside the stable isotope
221 laboratory. $\delta^{15}N$ precisions using the Trace gas Preconcentrator and Isoprime IRMS were
222 better than 0.3 ‰ respectively at 600 μAmp trap current.

223

224 2.2. Field application of the ^{15}N Gas-Flux and AIT techniques

225 *In situ* measurements of N_2 and N_2O were made using static chambers according to the ^{15}N
226 Gas-Flux method (Mosier and Klemedtsson 1994). Five plots were randomly established
227 in June 2013 in each of four study sites in the Ribble - Wyre River catchments (area 1145
228 km^2; NW England, 53º59'99" N, 2º41'79" W). The study sites were a heathland (R-HL), a
229 deciduous woodland (R-DW), an unimproved grassland (R-UG) and an improved
230 grassland (R-IG). In August 2013, four more study sites were tested in the Conwy River
231 catchment (area 345 km^2; N. Wales, 52º59'82" N, 3º46'06" W) following a similar sampling
232 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 m^2; 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 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 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 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 minimising 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 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 ≈ 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).

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

293

294 2.3. Flux calculations

295 The ^{15}N content of the N_2 in each 12 mL vial was determined using the IRMS system
296 described above and the ratios R29 ($^{29}N_2/^{28}N_2$) and R30 ($^{30}N_2/^{28}N_2$) were measured in both
297 enriched (T=1, 2 and 20 hours) and reference samples (T=0 hours). The inclusion of air
298 reference standards between every 10 samples indicated an upward drift for R30 over time,
299 potentially due to the formation of NO^+ in the ion source despite the inclusion of the Cu
300 reduction step (Lewicka-Szczebak et al. 2013). Subsequently, every sample batch was drift
301 corrected by fitting a linear regression through the air reference standards and calculating
302 an offset correction for both R29 and R30 (Yang et al. 2014). The minimum detectable
303 change (MDC) in R29 and R30 was defined with repeated manual analyses of air reference
304 standards (n=10) and was calculated using the following equation (Matson et al. 2009):

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

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

$$Evolved\ N_2 = d[N_2]/(1-d) \quad (5)$$

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 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:

$$R29 = R45 - R17 \quad (6)$$

$$R30 = \big(R46 - (R29R17)\big) - R18 \quad (7)$$

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 x 10^{-5} and 2.9 x 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-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 hours. 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 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_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

415 average between 13 and 25 % (detailed data on the ^{15}N tracer application per field site are
416 shown in Supplementary Table 2).

417

418 The ^{15}N fraction in the denitrifying pool ($^{15}X_N$), as calculated from the measured isotopic
419 ratios of the N_2O after 1 hour of incubation using Eq. (2), ranged between 65 and 93 ^{15}N
420 at%. The average change of the $^{15}X_N$ with incubation time, indicated by the slope shown in
421 Table 2, was not different from 0 in case of the organic (t-test; $t = 0.520$, $df = 18$, $p > 0.05$)
422 and grassland soils (t-test; $t = 0.047$, $df = 28$, $p > 0.05$), whilst it was significantly below 0
423 for the woodland soils (t-test; $t = 2.917$, $df = 18$, $p < 0.05$). Separating the woodland soils
424 to C-MW and R-DW sites, only the former displayed a significant negative slope of $^{15}X_N$
425 with incubation time (t-test; $t = 3.306$, $df = 8$, $p < 0.05$), suggesting N_2O production from a
426 second nitrate pool, possibly nitrate produced from the oxidation of NH_4^+ via nitrification,
427 in the C-MW. In cases where the $^{15}X_N$ could be calculated from the N_2 isotope ratio data
428 (woodland and grassland soils; data shown in Supplementary Table 3), this was not
429 significantly different from their respective $^{15}X_N$ calculated from the N_2O isotope ratio data
430 (t-test; $t\text{-}_{WL} = 0.929$, $df = 12$, $p > 0.05$; $t\text{-}_{GL} = 1.511$, $df = 20$, $p > 0.05$).

431

432 The mean evolved amount of N_2 and N_2O gases due to denitrification in each land use type
433 increased with increasing incubation time (Figure 2). The increase in the evolved N_2 was
434 statistically significant after 20 hours incubation in GL (ANOVA; $F = 19.8$, $p < 0.01$),
435 whilst due to the high variability among plots, shown by the large error bars at 20 hours
436 incubation in Figure 2a, it was not significant for the OS and WL soils. The amount of N_2O
437 accumulated after 20 hours (Figure 2b) was significantly higher than in the previous time
438 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_2O 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; χ^2=11.35, p=0.003), between 1 and 20 hours in the WL (Kruskal-Wallis; χ^2=10.78, p=0.005) and between 1 and 2 hours in the GL (Kruskal-Wallis; χ^2=10.10, p=0.006). Conversely, the N_2O 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; χ^2_{OS} = 3.58, χ^2_{WL} = 3.47, χ^2_{GL} = 3.01, $p > 0.05$).

The N_2 flux ranged between 2.4 and 416.6 μg 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_2O flux due to denitrification (range: 0.003 - 20.8 μg 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 (Figure 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 (Figure 4c). The change of 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 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_2O to N_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_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 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 (Figure 5a) with 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_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 (Figure 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 (Figure 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 (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 (3.81×10^{-5}) compared to the value (1.6×10^{-4}) reported by Lewicka-Szczebak et al. (2013). Consequently, correcting the R30 ratio for the NO^+ 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^{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 ($\delta^{15}N$), 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_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 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 %, 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 %, 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 %) 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

556 to current estimates of daily atmospheric N deposition (0.05 kg N ha^{-1} d^{-1}) in the UK (~ 15
557 - 20 kg N ha^{-1} y^{-1}) (Dore et al. 2012, Payne 2014), whilst for the grassland soils the tracer
558 application mimicked a daily fertiliser application rate of 0.5 kg N ha^{-1} d^{-1}. Due to the
559 inclusion of the NO_3^--rich C-MW site in the woodland soils, tracer application rates were
560 higher than the daily atmospheric N deposition rates, but also reflecting internal N cycling
561 processes (e.g. nitrification) as an additional source of nitrate in these well-drained forest
562 soils. Therefore, the application of the ^{15}N tracer at these low rates should not be expected
563 to enrich the soil nitrate pool significantly, and potentially enhance the denitrification
564 activity, in excess of the amount of nitrogen normally deposited via natural processes and
565 common management practices.

566

567 The major assumptions of the ^{15}N Gas-Flux method and the associated 'non-equilibrium
568 equations' are that the denitrifying soil NO_3^- pool is uniformly labelled with ^{15}N and that
569 the N_2 and N_2O originate from the same denitrifying pool (Stevens and Laughlin 1998).
570 The ^{15}N fraction in the denitrifying pool ($^{15}X_N$), calculated non-destructively from the
571 measured isotope ratios, ranged between 65 and 93 % and was well above the 10 %
572 threshold for the correct application of the 'non-equilibrium equations' (Lewicka-Szczebak
573 et al. 2013). However, the calculated $^{15}X_N$ was higher than the estimated total soil NO_3^-
574 pool enrichment (range: 2 - 40 %) suggesting non-homogeneous mixing of the added tracer
575 (98 ^{15}N at %) with the ambient soil nitrate at natural abundance despite our effort for
576 uniform tracer application with multiple injections across the investigated soil depth
577 (Ruetting et al. 2011). Wu et al. (2011) have optimised the number of injections and the
578 volume of tracer needed to achieve homogeneous labelling of a soil core (diameter 15 cm;
579 height 20 cm) and reported that 38 injections of 4 mL volume each were necessary. We
580 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_2O, 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.

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

615

616 Most studies using ^{15}N tracers and static chambers in highly fertilised systems typically
617 deploy their chambers between 1 and 2 hours (Baily et al. 2012, Cuhel et al. 2010,
618 Tauchnitz et al. 2015), but it has been shown that longer incubation periods (up to 24 or 48
619 hours) may be needed in case of low ^{15}N enrichment applications in intact soil cores (Morse
620 and Bernhardt 2013) and laboratory incubations (Yang et al. 2014) for a more precise and
621 accurate detectable ^{15}N-N_2 signal. However, it should be noted that in these cases the soil
622 cores or slurries were incubated in fully enclosed systems and were thus not affected by
623 potential bias from diffusion of evolved N_2 and N_2O to the subsoil (Clough et al. 2005).
624 The open-bottom, un-vented static chamber design used in this study in combination with
625 the extended incubation period up to 20 hours may have potentially allowed some loss of
626 the evolved N_2 and N_2O through downward subsoil diffusion and/or reduction of gas
627 exchanges at the soil-atmosphere interface due to positive pressure build up in the chamber
628 headspace (Healy et al. 1996). This could partly explain the non-linear increase of the
629 evolved N_2 and N_2O in the chamber headspace (Figures 2a & b) and also the decrease of

630 the N_2 flux rate with increasing incubation time (Figure 3a). The N_2O flux rate increased
631 up to 2 hours incubation followed by a decrease after 20 hours consistently across land use
632 types (Figure 3b) and this was an indication of potentially enhanced N_2O reduction due to
633 both subsoil diffusion and the increasing concentration of the N_2O in the topsoil. However,
634 due to the high spatial heterogeneity within each land use type, the mean N_2O flux rate was
635 not significantly different between the different incubation intervals. In other words, the
636 non-linearity of N_2O evolution had less effect on the flux rate estimation than the inherent
637 spatial variability within each land use type, which is in agreement with the findings of
638 Chadwick et al. (2014), who suggested that the spatial variability of N_2O fluxes far exceeds
639 the bias due to assumed linearity of fluxes.

640

641 The lack of a consistent pattern of N_2 flux rate change with incubation time among the
642 different land use types suggested a more complex temporal variability of N_2 fluxes that
643 apart from the duration of incubation could have also been affected by the distribution of
644 the added nitrate tracer. In the OS sites with the lowest average nitrate content (Table 2)
645 and the highest water filled pore space (Mean WFPS: C-PB = 70 ± SE 3.21 %; C-UG = 66
646 ± SE 1.58 %; R-HL = 69 ± SE 2.00 %), non-homogeneous tracer distribution ($^{15}X_N$ = 90%)
647 could have led to the creation of hotspots of denitrification activity due to substrate
648 availability resulting in potentially overestimated flux rates in the first or even the second
649 hour of incubation. However, analytical uncertainty due to fluxes being close to the limit
650 of detection could not be ruled out. Conversely, in the soil moisture limited forest site (C-
651 MW), the injection of even 50 mL of tracer solution could have led to an increased moisture
652 induced denitrification activity within the first 1 – 2 hours of incubation, until the added
653 water started to equilibrate with the ambient soil moisture. Therefore the N_2 flux rate in C-
654 MW may be significantly overestimated after 1 hour of incubation. In the grassland sites

655 and the R-DW forest site with intermediate soil moistures (Mean WFPS: R-DW = 65 ± SE
656 1.79 %; R-UG = 64 ± SE 1.41 %; C-IG = 60 ± SE 1.45 %; R-IG = 61± SE 2.46 %) and
657 nitrate content, the tracer injection is unlikely to have significantly affected the
658 denitrification rate when all the conditions (i.e. soil moisture and substrate availability)
659 were favourable, and therefore flux rates estimated after one hour of incubation should be
660 more reliable as long as the bias from analytical uncertainty was low. In these sites
661 denitrification rates estimated after one or 20 hours of incubation were not significantly
662 different (Figure 3a), suggesting a quasi-linear N_2 evolution throughout the incubation
663 period (at least in 37.5% of the sampling plots, see Supplementary Table 4). However, the
664 N_2 flux rates were significantly lower after 2 hours of incubation, whereas the N_2O flux
665 rates were maximum at 2 hours of incubation consequently leading to an increased product
666 ratio $N_2O/ (N_2 + N_2O)$. This observation could potentially be explained by a delay in the
667 *de novo* synthesis of the N_2O reductase enzyme, known to have a slower expression than
668 the preceding reduction enzymes (Knowles, 1982), leading to N_2O accumulation and lower
669 N_2 production after 2 hours of incubation. After 20 hours incubation, the decrease in the
670 product ratio could be explained by a higher reduction rate of N_2O to N_2 due to probably
671 higher N_2O reductase activity but also slower soil-atmosphere exchange of N_2O due to the
672 decreasing concentration gradient (Healy et al. 1996).

673

674 It has been shown that the N_2 flux estimation with the ^{15}N Gas Flux method is sensitive to
675 the incubation time interval and the homogeneity of the tracer distribution due to the
676 combination of several antagonistic effects such as decreasing gas diffusion gradients and
677 soil moisture and substrate availability effects due to the added tracer solution. The
678 uncertainty in the estimated in situ N_2 fluxes can be significantly reduced if additional effort
679 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_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 hours (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 0.6, 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

705 soils compare well with the rates reported in the literature for restored forested wetlands in
706 North America (Morse and Bernhardt 2013) and with the rates from northern hardwood
707 forests in US (Kulkarni et al. 2014), using ^{15}N tracers at similar or lower application rates
708 to ours. Our results are also comparable to the rates reported from central European forests,
709 under similar atmospheric N deposition rates, using the gas-flow soil core method
710 (Butterbach-Bahl et al. 2002). For the grassland soils, the N_2 fluxes measured in the present
711 study were significantly lower than previous applications of the ^{15}N Gas-Flux method at
712 high fertiliser application rates (Baily et al. 2012, Cuhel et al. 2010, Graham et al. 2013),
713 whilst for the organic soils our rates were significantly lower than the ones reported by
714 Tauchnitz et al. (2015) since their ^{15}N tracer application rate (30 kg N ha^{-1}) was 300 times
715 higher than ours. The N_2O fluxes were up to 200 times lower than the N_2 fluxes leading to
716 low denitrification product ratios in all land use types, a result which is in line with the N_2O
717 yields reported from ^{15}N tracer studies in forest (Kulkarni et al. 2014, Morse and Bernhardt
718 2013) and grassland soils (Baily et al. 2012, Bergsma et al. 2001). In the present study we
719 have compared the in situ denitrification rates between three major land use types using an
720 extended field incubation period to increase the probability of detecting a reliable ^{15}N-N_2
721 signal, particularly under conditions of low denitrifier activity due to seasonality of
722 denitrification and/or inherent capacity of soils (for example organic and deciduous forest
723 soils). However, these rates should be considered conservative since confounding issues
724 such as subsoil diffusion and non-homogeneous labelling of the soil nitrate pool may in
725 some cases have led to underestimations of the in situ denitrification rates.

726

727 4.3. Comparison with the AIT

728 The total denitrification rates measured with the C_2H_2 amended intact soil cores followed
729 the same trend as the total denitrification (N_2 and N_2O fluxes combined) from the ^{15}N Gas-
730 Flux measurements, while they were on average 168 times lower than the denitrification
731 potential measured in the same land use types in anaerobic soil slurries amended with
732 acetylene and nitrate in a previous study (Sgouridis and Ullah 2014), thus reflecting lower
733 in situ rates. The AIT denitrification rates were between 3 and 5 times lower than the ^{15}N
734 Gas-Flux rates despite the fact that the AIT intact soil cores were capped at the bottom,
735 thus not allowing any subsoil diffusion of the evolved gases due to denitrification.
736 Therefore, the AIT rates should have been higher than the ^{15}N Gas-Flux rates if serious
737 underestimation was occurring due to subsoil diffusion in the open-bottom static chambers,
738 which was not the case. Adding nitrate to the C_2H_2 amended cores would have been
739 desirable for directly evaluating the priming effect of the added substrate on denitrification
740 rates. The ^{15}N tracer addition to the static chambers corresponded to the amounts of N
741 naturally deposited in these land use types either via management practices and/or
742 atmospheric deposition, thus avoiding excessive N fertilisation of the sampling plots.
743 However, it cannot be conclusively argued that the same amount of applied nitrate would
744 not have led to similar denitrification rates between the AIT and the ^{15}N Gas-Flux methods.
745 Previous comparisons between the AIT and the ^{15}N tracer method in field studies showed
746 no significant difference between the two methods in measuring *in situ* total denitrification
747 rates when tracer is applied at high fertilisation rates (50 - 200 kg N ha^{-1}) and relatively low
748 soil moisture contents (WFPS: 40 - 60 %) (Aulakh et al. 1991, Mosier et al. 1986).
749 Conversely, in laboratory incubations it was shown that the AIT significantly
750 underestimated total denitrification compared to the ^{15}N tracer approach (Yu et al. 2010)
751 and the direct N_2 flux approach (Qin et al. 2012) due to the incomplete inhibition of N_2O
752 reduction to N_2 by C_2H_2 in wet soils (Yu et al. 2010) or in soils with low nitrate content

753 (Qin et al. 2013, Qin et al. 2014). In our study, the soil WFPS ranged between 60 and 70
754 % in all land use types, with the exception of the C-MW site (mean WFPS 42 %), whilst
755 the $^{15}N\text{-}NO_3^-$ tracer application rate was low (< 1 kg N ha^{-1}). Moreover, the disturbance of
756 the soil structure during the extraction of the soil cores and the effect of the acetylene
757 addition to microbial activity were not significant as it was suggested by the similar CO_2
758 production rates (Aulakh et al. 1991), representing soil respiration (Felber et al. 2012), in
759 the static chambers and the C_2H_2 amended and un-amended intact soil cores. Therefore, we
760 could argue that it is possible that the AIT underestimated total denitrification rates
761 compared to the ^{15}N Gas-Flux method due to the likely incomplete inhibition of N_2O
762 reduction to N_2 under relatively high soil moisture contents, although the shorter incubation
763 time (2h for the intact cores) may have limited the ability of C_2H_2 to fully equilibrate within
764 soil pore spaces. Other confounding factors such as the catalytic decomposition of NO in
765 the presence of C_2H_2 (Bollmann and Conrad 1996, Nadeem et al. 2013) may have also
766 contributed to the lower denitrification rates measured by the AIT. This study has
767 confirmed some of the drawbacks of the AIT as a quantification method of in situ
768 denitrification rates compared to the ^{15}N Gas-Flux.

769

770 The estimation of the denitrification product ratio using the AIT method, from the un-
771 amended cores (N_2O only) and the C_2H_2 amended cores (N_2 + N_2O), is usually
772 overestimated since the source of N_2O cannot be discriminated with the AIT, whilst the N_2
773 flux is underestimated due to the incomplete inhibition of N_2O reduction (Butterbach-Bahl
774 et al. 2013). This was confirmed in the present study for all the land use types and even the
775 maximum denitrification product ratio after 2 hours incubation in the case of the grassland
776 soils (23 %), was still significantly lower than the respective ratio from the AIT (50 %).
777 Therefore, the much lower denitrification product ratio estimated from the ^{15}N Gas-Flux

778 measurements is significantly more reliable and the wider application of this field technique
779 across a range of land use types can have important implications for evaluating the role of
780 denitrification as a reactive nitrogen sink and as a source of N_2O emissions (Butterbach-
781 Bahl et al. 2013, Kulkarni et al. 2008).

782

783 **5. Conclusion**

784 The improved analytical precision for both ^{15}N-N_2 and ^{15}N-N_2O analyses allowed us to
785 quantify in situ N_2 and N_2O fluxes with low ^{15}N tracer addition under field conditions in
786 natural and semi-natural land use types for the first time. The estimation of N_2 fluxes was
787 sensitive to the incubation time interval and the homogeneity of the tracer distribution due
788 to the combination of several antagonistic effects such as decreasing gas diffusion gradients
789 over time and soil moisture and substrate priming effects due to the added nitrate tracer
790 solution. The spatial variability of N_2O fluxes superseded any bias associated with non-
791 linear fluxes due to the extended incubation period. The uncertainty in the estimated N_2 and
792 N_2O fluxes can be significantly reduced by increasing the homogeneity of the tracer
793 application and by closely monitoring the linear evolution of the produced gases with more
794 frequent gas sampling at shorter equal incubation intervals to avoid under or over estimation
795 of denitrification. Comparing the ^{15}N Gas-Flux method with the AIT confirmed the
796 drawbacks of the AIT as a reliable quantification method of in situ denitrification rates.
797 Moreover, the AIT method overestimated the denitrification product ratio compared to the
798 ^{15}N Gas-Flux method. The ^{15}N Gas-Flux method holds much promise as a more reliable
799 field technique for measuring in situ denitrification rates and its wider application across a
800 range of terrestrial ecosystems can lead to its refinement and improvement and in the long
801 term can significantly contribute to our understanding of the role of denitrification as a
802 reactive nitrogen sink.

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Tables

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

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 (%)	$^{15}X_N$ (%)	$^{15}X_N$ 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)

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

Figures

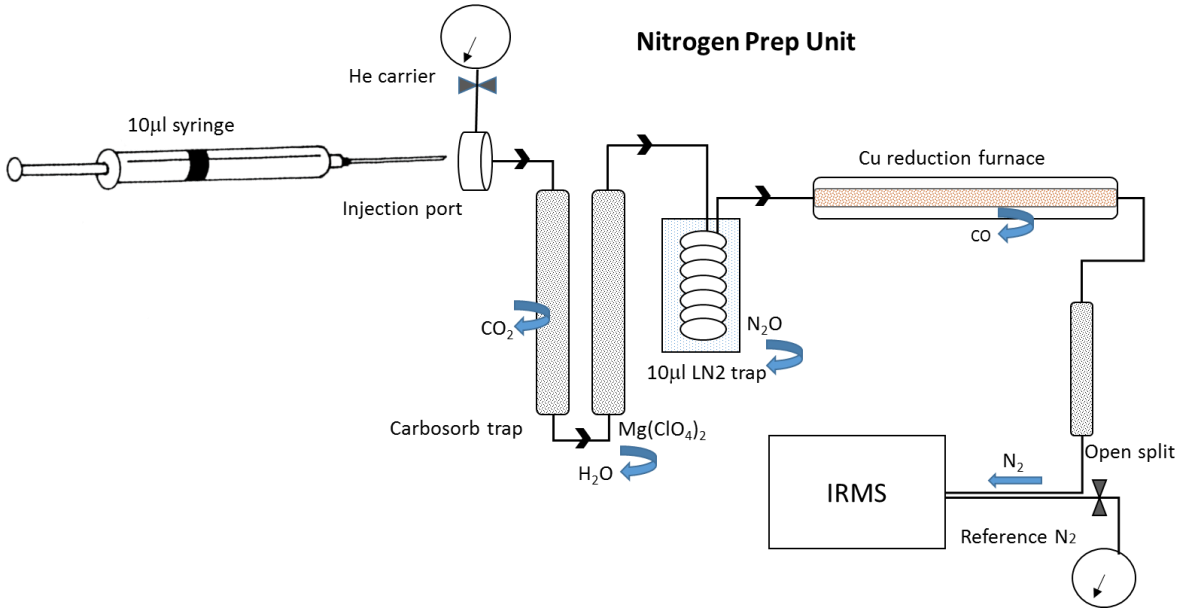


Figure 1: Schematic of the ^{15}N-N_2 analysis system

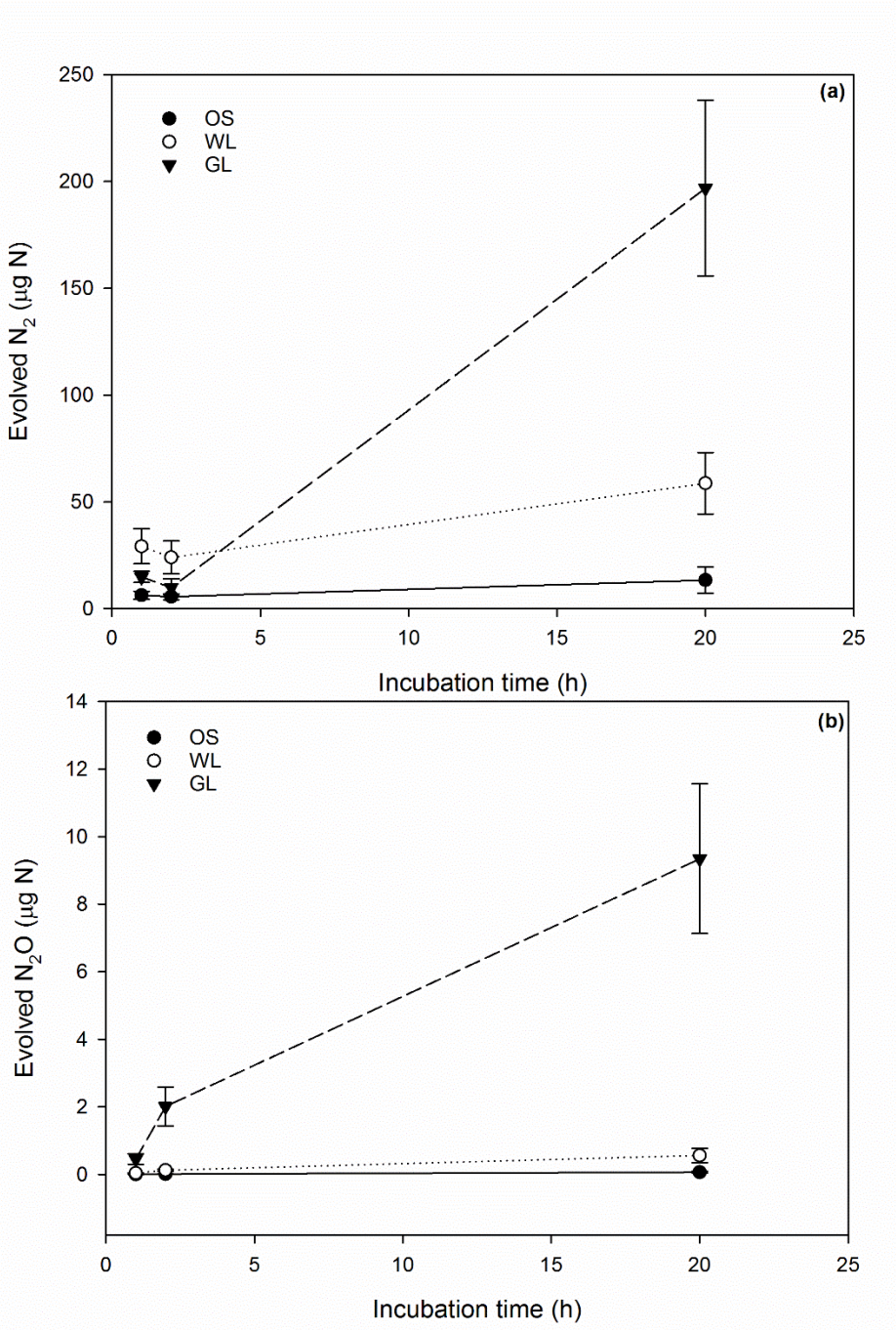
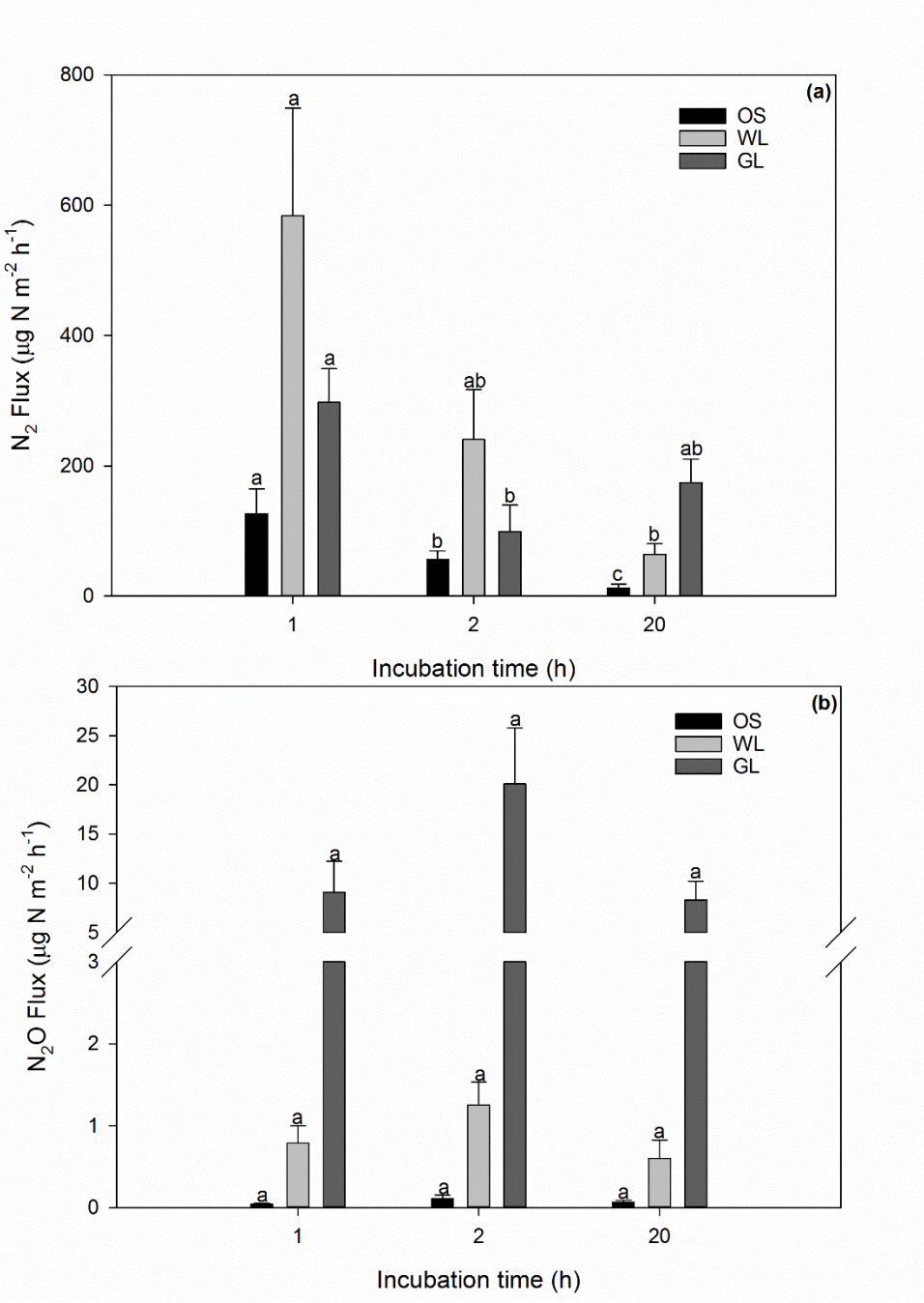


Figure 2: Evolved (a) N_2 and (b) N_2O gas measured between 1, 2 and 20 hours incubation time intervals using the ^{15}N Gas-Flux method in the organic soil (OS), woodland (WL) and grassland (GL) land use types. Data points are means and the error bars represent standard errors.



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1048 **Figure 3**: Mean rates of: (a) N_2 flux and (b) N_2O flux due to denitrification at the three
1049 incubation time intervals in the three land use types (OS; organic soils, WL; woodland and GL;
1050 grassland). Same lower case letters indicate no significant differences ($p > 0.05$) between
1051 incubation time intervals according to the non-parametric Kruskal-Wallis test. Error bars
1052 represent standard errors.

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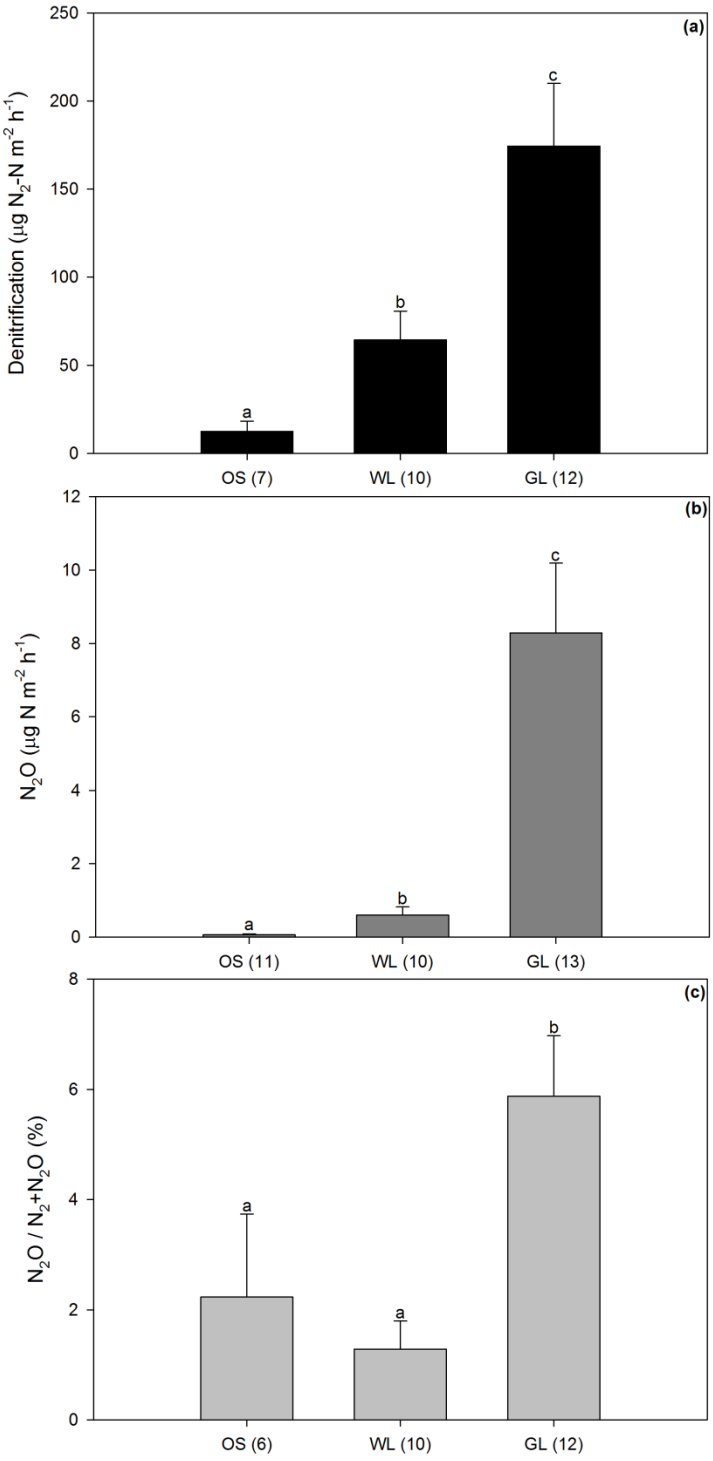
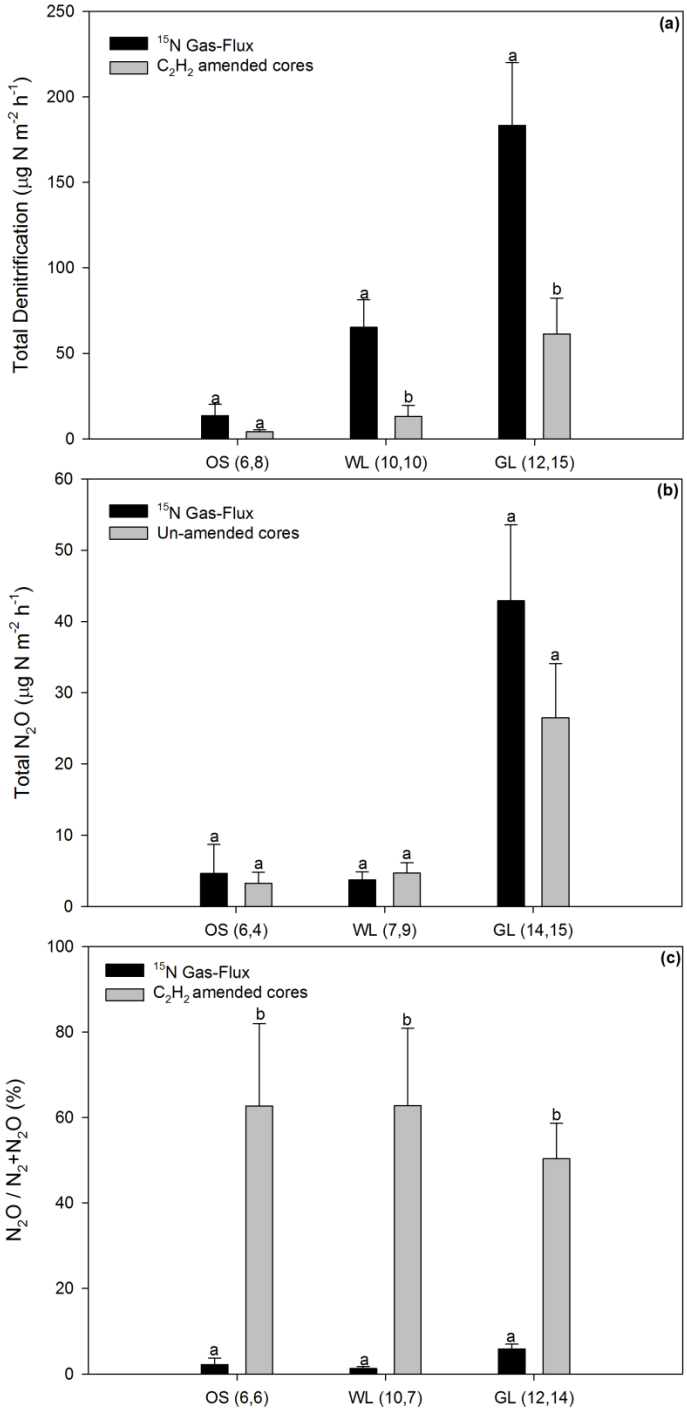


Figure 4: Mean rates of: (a) N_2 flux, (b) N_2O emission due to denitrification and (c) the denitrification product ratio N_2O/ (N_2 + N_2O) in the three land use types (OS; organic soils, WL; woodland and GL; grassland). Same lower case letters indicate no significant differences ($p > 0.05$) between 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.



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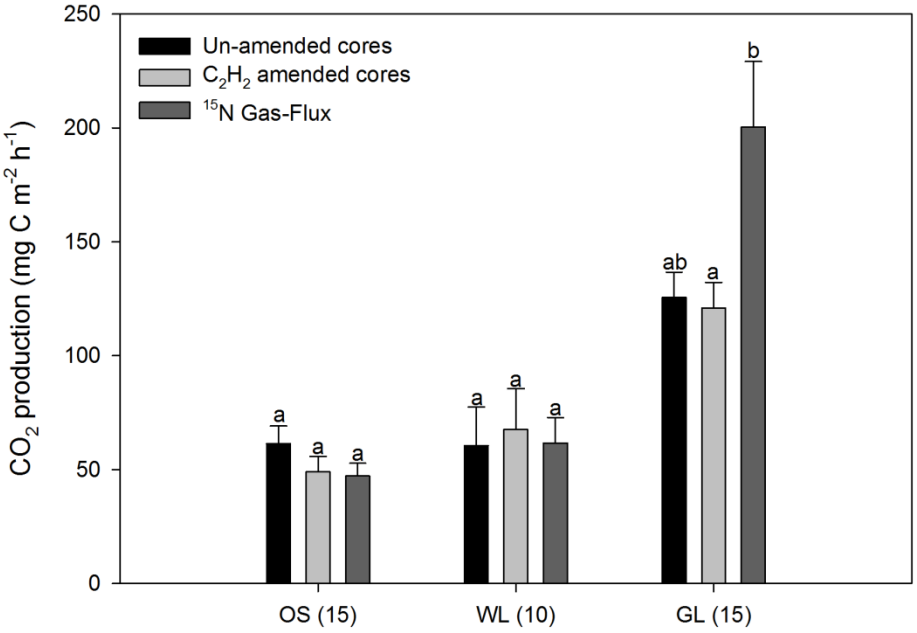
1062 **Figure 5**: (a) Mean total denitrification measured with the ^{15}N Gas-Flux method and the AIT,
1063 (b) Mean bulk N_2O emission measured in the static chambers of the ^{15}N Gas-Flux method and
1064 in un-amended intact soil cores and (c) the denitrification product ratio $N_2O/ (N_2 + N_2O)$ with
1065 the ^{15}N Gas-Flux method and the AIT in the three land use types (OS; organic soils, WL;
1066 woodland and GL; grassland). Same lower case letters indicate no significant differences ($p >$
1067 0.05) between measurement methods according to independent samples t-test. The sample size
1068 (n) is given in parenthesis for each land use type and each method on the x-axis. Error bars
1069 represent standard errors.



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1071 **Figure 6**: Mean CO_2 production measured in the static chambers of the ^{15}N Gas-Flux method,
1072 in un-amended and C_2H_2 amended intact soil cores in the three land use types (OS; organic
1073 soils, WL; woodland and GL; grassland). Same lower case letters indicate no significant
1074 differences ($p > 0.05$) between measurement methods according to independent samples t-test.
1075 The sample size (n) is given in parenthesis for each land use type on the x-axis. Error bars
1076 represent standard errors.

1077