21 January, 2016

Response to Dr Reinhard Well's comments on the manuscript '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.' (Manuscript ID = doi:10.5194/bgd-12-12653-2015)

We are grateful to Dr Well for the additional comments he supplied as part of the ongoing review of our manuscript. We believe that these additional comments/suggestions significantly improved the clarity of our results and discussion and the overall impact of our work. We have, therefore, attempted to accommodate all the suggestions where possible and amended the manuscript accordingly.

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

Major comments

1) Non-linearity of fluxes (N2 + N2O and N2O)

Following the reviewer's comments, we have amended the Supplementary Tables 4&5, where linearity is assessed on a per plot basis by calculating the ratio of evolved gas amount between the first and second hour (T2/T1) and first and last incubation interval (T3/T1). As described in the Tables' captions: 'If linear evolution of N_2 or N_2O in a constant headspace volume is assumed then T2/T1 = 2 and T3/T1 = 20'. From this analysis it becomes apparent that only in few cases (highlighted in bold font) the evolution of gases approached linearity. Subsequently we have amended Figure 2 in the manuscript by removing the linear regression and only showing the average increase of the evolved gases with time per land use type. Additionally, we have calculated the flux rate of N_2 and N_2O at each sampling interval and compared the means per land use type with additional statistical tests and included this comparison in Figure 3 in the manuscript. These additional results of N_2 and N_2O fluxes after 1, 2 and 20 hr incubation are described in section 3.2 (Lines: 434-456). Following this additional temporal analysis of N_2 and N_2O fluxes and prompted by the reviewer's suggestion we have re-structured the discussion section

4.2, first to reflect the order the results are presented but also accommodating additional discussion for the temporal analysis and provision of recommendations for further improvements of the pitfalls in the observed methodology (Lines: 543-731). Finally, the additional results from the temporal analysis are also summarised in the abstract and the conclusion as per the reviewer's request (Abstract Lines: 42-48; Conclusion Lines: 792-801).

2) Non-homogeneity of labelling

In the discussion (Lines: 569-606) we acknowledge the fact that our tracer distribution was sub-optimal when compared to the optimised protocol suggested by Wu et al. 2011, but probably a necessary compromise for our large scale intensive measurements. We also clearly state that by comparing the estimated total soil NO_3^- pool enrichment and the calculated $^{15}X_N$ it is shown that there has been non-homogeneous mixing of the tracer with the ambient soil nitrate and this may have led to the underestimation of the calculated fluxes. However, we also refer to the literature to show that under field conditions, it is unlikely to achieve complete mixing of the added tracer with the ambient nitrate pool and that relatively accurate measurements are still possible with a less-uniformly labelled denitrifying pool. Drawing from the reviewer's suggestions we have included in the discussion some hypotheses as to how the non-uniform distribution of the tracer may have affected the flux rates due to soil moisture but also substrate availability effects (Lines: 608-617 and 647-653).

3) Moisture effect

We have missed to describe in the methods section that the injection of the tracer in the organic soil sites (C-PB, C-UG and R-HL) was done from the surface to 15 cm depth rather than 10 cm, which was the injection depth in all the other land use types. The purpose of this was to increase the volume of the labelled soil in these low bulk density soils in order to increase the probability of detectable denitrification activity. This information has been added in line 256. It was also our oversight to report an increase of the soil moisture content equivalent or less than 2 mm precipitation and this sentence has now been removed from the manuscript. Following the above clarifications, the volumes of soil water in the OS plots reported in Supplementary Table 1 are correct and within the expected range for soils with very low bulk density (< 0.2 g/cm3).

Moreover, we have added a clarification in the methods section (Lines: 268-270) to explain 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. Subsequently, in the discussion we hypothesise how the moisture effect from the addition of the tracer solution without equilibration with the soil water may have affected the gas flux rates (Lines: 608-617).

Finally, we were not able to do repeated measurements after ^{15}N labelling (over several days) in this study due to time and budget constraints, but we do recognize the usefulness of such a validation in future research work.

Minor comments:

1. L 267: moisture effect < 2 mm equivalent is incorrect in view of 5 % vol water content change: 5 % of 100 mm = 5 mm

This sentence was incorrect and has been removed from the manuscript

2. L 271 there was immediate enclosure and sampling after labeling (see general comments).

We have added a clarification in the methods section (Lines: 268-270) to explain 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.

3. L 238 -250 only 10 injections for 0.05 m2 not enough (see general comments)

In the discussion (Lines: 569-606) we acknowledge the fact that our tracer distribution was sub-optimal when compared to the optimised protocol suggested by Wu et al. 2011, but probably a necessary compromise for our large scale intensive measurements.

4. Table 2: the fact that 15XN by far exceeded expected enrichment of total soil NO3 demonstrates huge non-homogeneity of labeling. The small number of injections apparently caused denitrifying hot spots in the injection area with 15XN (0.8 to 0.9 on average) close to the enrichment of the tracer solution (0.98) but far from the NO3 target enrichment (0.13 to 0.25). Note that due to imperfect distribution of tracer solution the local increase in water content was far more than the average of 5% (which is still quite a lot) (see also general comments). So the non-homogenity of the label is an indication that the moisture effect on 15N fluxes was much larger than expected from the increase in average water content in the entire soil.

Please see response for major comments 2&3 above

5. Table S1: Soil water numbers are questionable (up to 5 L) since the volume of labelled soil was 5 L only. Please check.

Please see response for major comment 3 above

6. Table S6: the fact that there were no clear time trends for the product ratio probably shows the overlap of several processes (see general comments)

An additional discussion has been added on the temporal variability of the N_2 and N_2O gas fluxes as well as the denitrification product ratio to attempt an explanation for the observed inconsistent patterns. Briefly, the lack of a consistent pattern of N_2 flux rate change particularly with incubation time among the different land use types suggested a more complex temporal variability of N_2 fluxes that apart from the duration of incubation could have also been affected by the distribution of the added nitrate tracer, with more details presented in Lines 644-676.

7. L 652-657 the conclusion with respect to hybrid N2 or N2o is incorrect (see Spott & Satnge 2007 and Spott et al., 2011): hybrid N2 and/or N2o would be proven by 15XN was lower than 15N atom fraction of NO3 but not from the deviation between 15XN of N2 and N2O. In fact the fraction of hybrid gas could be different in N2 and N2O fluxes which could lead to different values in 15XN. But this could not be determined due to missing 15NO3 analysis and the large non-homogeneity in labeling.

The above conclusion has been removed from the discussion as incorrect.

8. L 535 to 538 this statement is not well justified. Your precision for R29 and R30 is in the same order compared to previous studies including as early as Siegel et al., 1982 (see comparison of precision in Well ea 1998). So please formulate more cautious or give exact numbers in identical units (eg. Standard dev for R29 and R30) to show to which extent your analysis was better.

The above statement has been changed to: 'Therefore, the analytical precision achieved for both $^{15}N\text{-}N_2$ and $^{15}N\text{-}N_2O$ 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.' Moreover,

our achieved precision for R29 and R30 is presented in Table 1 and in the discussion (Lines: 512-517) it is stated that it was comparable to the recent studies by Lewicka-Szczebak et al. (2013) and Yang et al. (2014).

9. L 563 to 567 it is not well clarified what this means. Suggest: "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). But please check if the reference still fits to this modification.

The sentence has been adapted following the reviewer's suggestion (Lines: 624-627)

10. L 570 -572 this is indeed by no means the case (see first general comment). So you have to keep the possibility that increasing subsoil diffusion during extended chamber closure was a potential source of bias.

The above sentence has been removed and replaced by additional discussion to explain the temporal patterns of gas fluxes during the incubation period (Lines: 627-642).

11. L 681-684 this would not only result from subsoil diffusion of N2O but also from enhanced reduction in the topsoil due to increasing N2O concentration during extended cover periods.

The above suggestion has been added in the discussion (Lines: 633-637)

12. L 734 please cite also Bollman & Conrad 1996, who were the first to show the artefacts by catalytic NO decomposition and to clarify that this artefact is known since long.

The above citation was added in Lines 107 & 771.

13. In the entire manuscript: use consistently the correct spelling of the product ratio: N2O/(N2+N2O), one or both brackets were often missing

Spelling consistency checked and corrected throughout the manuscript

Minor comments to the response file with marked changes of the text:

1. L 682: suggest: " to maintain natural drainage and root growth during the measurements" since natural drainage is also needed if the ground water table is far below

Sentence changed to the reviewer's suggestion (Lines: 240-241)

*2. L 699 delete "equal" since 4*6 is not equally spaced. A pattern with triangles of equal side length would be optimal. So the distance between your injections varied between 4, 6 and about 7.5 cm, isn't it?*

The word equal has been deleted

3. L 881-895 these statements are not justified, see general comments

The results section the above comment refers to has been completely re-written (Lines: 434-456) to reflect the additional temporal analysis of gas fluxes.

4. L 913-914 but this statement only applies for landuse average, whereas individual sites could have any pattern. Please be more detailed here and explain that there was no consistent pattern for all sites.

The sentence has been amended in response to the above comment (Lines: 470-472).

5. L 1070 to 1073 sentence not clear to me. Do you want to highlight that you could detect fluxes in view of low enrichment? But in fact your active pool was close to the enrichment of the tracer solution since 15XN was around 90 at%. So you can't state that your method worked at low enrichment.

The overall aim of this study and the larger scale one presented in Sgouridis & Ullah (2015) was to measure in situ N_2 and N_2O fluxes with the lowest possible addition of nitrate tracer. Before each campaign the strength of the tracer was adjusted between 10 and 15 % of the total soil nitrate pool, and this target was achieved when looking at the annual average application rate per site presented in Supplementary Table 2. However, the complications due to the non-homogeneous tracer distribution are also discussed further in this section in Lines 569-606.

6. Conclusions must be partly rewritten:
L 1257 to 1260 not clear to me why this is related to smaller sample size. In fact you improved analytical (IRMS) precision somewhat , but not greatly. Also your fluxes came from highly enriched pools
Pleas add some conclusions on the aspects raised in the general comments

The conclusions have been re-written to reflect the additional temporal analysis for the N_2 and N_2O fluxes and to also make recommendations for future method improvements.

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

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}. ~~For our chamber design (volume/ surface = 8:1 cm^3:cm^2) and up to 20 h incubation period, the~~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 ~~so far~~, methodological ~~U~~uncertainties still exist~~s~~ in the estimation of N_2 and N_2O fluxes with the ^{15}N Gas-Flux method ~~were associated with~~due to issues related to non-homogenous

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distribution of the added tracer~~the inhomogeneity of the tracer distribution,~~ and subsoil gas diffusion using open-bottom chambers,~~ and decreasing gas diffusion gradients due to extended incubation period (up to 20 hours)~~ particularly ~~,~~ during longer incubation duration.~~The N_2 flux ranged between 2.4 and 416.6 µg N m^{-2} h^{-1}, and the grassland soils showed on average 3 and 14 times higher denitrification rates than the woodland and organic soils respectively. The N_2O flux was on average 20 to 200 times lower than the N_2 flux, while the denitrification product ratio (N_2O/ N_2 + N_2O) was low, ranging between 0.03 and 13 %. 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 relatively high soil moisture content~~Despite these uncertainties, the ^{15}N Gas Flux method constitutes a more reliable field technique ~~. The results show that the ^{15}N Gas Flux method can be used~~ for large scale ~~quantifying~~ quantification of N_2 and N_2O ~~production rates~~fluxes in natural terrestrial ecosystems, thus significantly improving our ability to constrain ecosystem N budgets.

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305 **1. Introduction**

306 There has been a renewed interest recently in developing new or enhancing existing
307 measurement approaches for improving our ability to constrain dinitrogen (N_2) fluxes due to
308 denitrification in terrestrial ecosystems (Kulkarni et al. 2014, Lewicka-Szczebak et al. 2013,
309 Wang et al. 2011, Yang et al. 2014). Denitrification, the reduction within soils of nitrogen
310 oxides (NO_3^- and NO_2^-) to NO, N_2O and ultimately N_2 gas, constitutes the most important
311 mechanism for the removal of reactive nitrogen (Nr) in terrestrial ecosystems (Galloway et al.
312 2008, Groffman 2012). Despite its importance, denitrification is considered the most un-
313 constrained process in the global N cycle (Groffman 2012, Kulkarni et al. 2008) due to
314 uncertainties in N_2 flux estimations that are likely leading to underestimations of
315 denitrification rates at multiple scales (Butterbach-Bahl et al. 2013). Considering
316 contemporary atmospheric N deposition rates globally including UK (Dore et al. 2012,
317 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 ~~technique~~ method can be applied to a range of natural and semi-natural terrestrial ecosystems allowing

368 the quantification of the relative magnitude of N_2 and N_2O fluxes due to denitrification from
369 these under-represented ecosystems.

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371 Natural and semi-natural terrestrial ecosystems in the UK (i.e. peatlands, heathlands, acid
372 grasslands, deciduous and coniferous forests), where there is no fertiliser use and the impact
373 from grazing and commercial forestry is minimal (Mills et al. 2013), along with improved
374 and unimproved grasslands (grazed and/or fertilised) constitute approximately 49 % and 85
375 % of rural land use cover in England and Wales, respectively (Morton et al. 2011). Unlike
376 arable agriculture, these land use types have been poorly investigated for their role in Nr loss
377 through denitrification.

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379 The major challenge in measuring ^{15}N-N_2 at near natural abundance levels is the possibility
380 of interference at *m/z* 30 ($^{30}N_2$) due to the reaction of oxygen in the ion source with N and the
381 formation of NO^+ ions that also have *m/z* 30 (Stevens et al. 1993). Commonly, this issue is
382 addressed in continuous flow isotope ratio mass spectrometers (CF-IRMS) with the inclusion
383 of a copper (Cu) oven for reducing O_2 in the gas sample (Russow et al. 1996). Recently, it
384 has been suggested that the interference at *m/z* 30 can be further reduced by including a
385 molecular sieve column in gas chromatograph IRMS (GC-IRMS) systems to not only
386 separate N_2 and O_2 in the gas sample, but also to quantitatively remove O_2 and other trace
387 gases such as carbon monoxide (Lewicka-Szczebak et al. 2013, Yang et al. 2014). We
388 hypothesise that the precision for *m/z* 30 determination can be greatly improved by using a
389 custom-built preparative unit for the removal of H_2O, CO_2, N_2O, NO^+ and CO; a device
390 which also permits the micro scale injection of volumes of < 5 µL. These injection volumes
391 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_2H_2 to the anaerobic microsites where denitrification occurs (Malone et al. 1998), whilst the high nitrate application rates have probably favoured nitrate reduction over N_2O reduction (Dendooven and Anderson 1995) resulting in high denitrification rates from the AIT. Conversely, laboratory studies have shown that the AIT significantly underestimates total denitrification compared to the ^{15}N tracer approach (Yu et al. 2010) and the direct N_2 flux approach (Qin et al. 2012) due to the incomplete inhibition of N_2O reduction to N_2 by C_2H_2 in wet soils (Yu et al. 2010) or in soils with low nitrate content, where N_2O reduction is more energetically favourable (Qin et al. 2013, 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-N_2 and ^{15}N-N_2O at low enrichment levels, (2) to adapt the ^{15}N Gas-Flux method for application across natural and semi-natural terrestrial ecosystems and (3) to compare the validity and applicability of the ^{15}N Gas-Flux method with the AIT for measuring *in situ* denitrification rates.

2. Materials and methods

2.1. IRMS system

For N_2 gas isotopic analysis we used an Isoprime isotope ratio mass spectrometer (Isoprime Ltd, UK, Wythenshawe) coupled to an in house built N_2 preparative interface (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 the instrument. The N_2O 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 N_2O from any residual CO_2, and both entered the IRMS via an open split. The retention time between the first eluting CO_2 (< 2^{E-10} amplitude) and second eluting N_2O peak typically fell in the range between 60 - 70 seconds to avoid isobaric interference of the CO_2 with the calculated ^{15}N. The N_2O was directed towards the triple collectors of the isotope ratio mass spectrometer where *m/z* 44, *m/z* 45 and *m/z* 46 mass ions were measured and recorded. Instrument stability checks were performed prior to each analysis by running a series of 10 reference pulses of N_2O (BOC special gases) until a standard deviation of $\delta^{15}N$ better than 0.05 ‰

was achieved. Prior to each sample batch analysis, trace gas N_2O measurements were made on three 100 mL flasks containing atmospheric air collected from outside the stable isotope laboratory. $\delta^{15}N$ 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 ^{15}N Gas-Flux and AIT techniques

In situ measurements of N_2 and N_2O were made using static chambers according to the ^{15}N Gas-Flux method (Mosier and Klemedtsson 1994). Five plots were randomly established in June 2013 in each of four study sites in the Ribble - Wyre River catchments (area 1145 km^2; 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 km^2; 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 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~~date. The collars were open at the bottom to ~~permit natural water table levels~~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~~n equally spaced~~ 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. ~~Since the volume of the added solution corresponded to a precipitation amount of ≤ 2 mm, the increase of the volumetric water content was considered minor (Tauchnitz et al. 2015).~~

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).

Adjacent to each PVC collar in each plot, two intact soil cores (50 mm I.D., 15 cm long) were extracted from 10 cm depth leaving the top 5 cm void as a headspace volume. The cores were capped on both ends with the top cap fitted with a rubber septum for gas sampling. One set of cores was amended with pure C_2H_2 with 5 mL injected through the septum directly in the middle of the soil core before 10 % of the headspace being also replaced with pure C_2H_2. The second set of cores was not amended with C_2H_2 and both

cores were placed back in the ground where they came from. Gas samples (5 mL) were collected with a gas tight syringe (SGE Analytical science) through the septa of the cores at T = 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 ^{15}N content of the N_2 in each 12 mL vial was determined using the IRMS system described above and the ratios R29 ($^{29}N_2/^{28}N_2$) and R30 ($^{30}N_2/^{28}N_2$) were measured in both enriched (T=1, 2 and 20 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-Szczebak et al. 2013). Subsequently, every sample batch was drift corrected by fitting a linear regression through the air reference standards and calculating an offset correction for both R29 and R30 (Yang et al. 2014). The minimum detectable change (MDC) in R29 and R30 was defined with repeated manual analyses of air reference standards (n=10) and was calculated using the following equation (Matson et al. 2009):

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

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}

578 h^{-1} representing the total N_2 flux from the mixture of the ^{15}N-labelled tracer and the soil N
579 at natural abundance (Stevens and Laughlin 1998).

580

581 The ^{15}N content of the N_2O in the same 12 mL vials as well as the ratios R45 ($^{45}N_2O$
582 /$^{44}N_2O$) and R46 ($^{46}N_2O$ /$^{44}N_2O$) were measured in both enriched (T=1, 2 and 20 hours)
583 and reference samples (T=0 hours). The application of the 'non-equilibrium' equations to
584 N_2O is analogous to N_2 after correcting for the naturally occurring oxygen isotopes
585 (Bergsma et al. 2001). Therefore, the ratios R45 and R46 were converted to ratios of R29
586 and R30 respectively by applying the following equations:

587 $$R29 = R45 - R17 \quad (6)$$

588 $$R30 = \big(R46 - (R29R17)\big) - R18 \quad (7)$$

589 where for *R17* ($^{17}O/^{16}O$) the value 0.000373 was used and for *R18* ($^{18}O/^{16}O$) the value
590 0.0020052 was used (Bergsma et al. 2001). There was no significant instrumental drift for
591 the ratios R45 and R46 over time. The MDC was defined, for the converted R29 and R30,
592 with repeated automatic analyses of 0.5 ppm N_2O standards (n=15) as 3.4×10^{-5} and $2.9 \times$
593 10^{-5} respectively. The second set of gas samples collected at the same time in the field
594 were analysed for total N_2O on a GC-μECD (7890A GC Agilent Technologies Ltd.,
595 Cheshire, UK) and the concentration of [N_2O] (μg N) was used in Eq. (5) to calculate the
596 N_2O flux due to denitrification of the mixture of the ^{15}N-labelled tracer and the soil N and
597 expressed in μg $N\text{-}N_2O$ m^{-2} h^{-1}. Assuming that the N_2O originates from the same
598 uniformly labelled pool as N_2, the $^{15}X_N$ from N_2O was used to estimate d for N_2 using
599 either R30 (Eq. 3) or R29 (Eq. 4), thus lowering the limit of detection for N_2 (Stevens and

600 Laughlin 2001) and allowing measurement of N_2 gas flux from natural terrestrial
601 ecosystems at low ^{15}N-tracer application rates.

602

603 Gas samples collected from the intact soil cores with or without acetylene amendment
604 were analysed for N_2O on a GC-μECD (7890A GC Agilent Technologies Ltd., Cheshire,
605 UK) and for CO_2 on a GC-FID (7890A GC Agilent Technologies Ltd., Cheshire, UK)
606 and flux rates were determined by linear regression between 0 and 2 hours. The
607 instrument precision was determined from repeated analyses of 6 ppm N_2O and 200 ppm
608 CO_2 standards respectively (n = 8) and the RSD was <1%.

609

610 2.4. Statistical analysis

611 Using factor analysis on selected soil physico-chemical properties, the samples from the 8
612 field sites were ordinated in three broad land use types: organic soils (C-PB, C-UG, R-
613 HL); forest soils (C-MW, R-DW) and grassland soils (C-IG, R-UG, R-IG) according to
614 Sgouridis and Ullah (2014). All subsequent statistical analyses were performed on the
615 broad land use types rather than individual field sites. The data were analysed for
616 normality and homogeneity of variance with the Kolmogorov-Smirnov test and the
617 Levene statistic respectively and logarithmic transformations were applied as necessary.
618 One-Way ANOVA combined with the Hochberg's GT2 *post hoc* test for unequal sample
619 sizes or the Games-Howell *post hoc* test for unequal variances was performed for
620 comparing the variance of the means between land use types for all gas fluxes. The non-
621 parametric Kruskal-Wallis test was used to compare mean flux rates between incubation
622 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 %).

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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

665 the woodland and grassland soils (Table 2). Based on the soil nitrate content on the day of
666 the tracer amendments (Table 2), the estimated enrichment of the total soil nitrate pool
667 was on average between 13 and 25 % (detailed data on the ^{15}N tracer application per field
668 site are shown in Supplementary Table 2).

669

670 The ^{15}N fraction in the denitrifying pool ($^{15}X_N$), as calculated from the measured isotopic
671 ratios of the N_2O after 1 hour of incubation using Eq. (2), ranged between 65 and 93 ^{15}N
672 at%. The average change of the $^{15}X_N$ with incubation time, indicated by the slope shown
673 in Table 2, was not different from 0 in case of the organic (t-test; $t = 0.520$, $df = 18$, $p >$
674 0.05) and grassland soils (t-test; $t = 0.047$, $df = 28$, $p > 0.05$), whilst it was significantly
675 below 0 for the woodland soils (t-test; $t = 2.917$, $df = 18$, $p < 0.05$). Separating the
676 woodland soils to C-MW and R-DW sites, only the former displayed a significant
677 negative slope of $^{15}X_N$ with incubation time (t-test; $t = 3.306$, $df = 8$, $p < 0.05$), suggesting
678 N_2O production from a second nitrate pool, possibly nitrate produced from the oxidation
679 of NH_4^+ via nitrification, in the C-MW. In cases where the $^{15}X_N$ could be calculated from
680 the N_2 isotope ratio data (woodland and grassland soils; data shown in Supplementary
681 Table 3), this was not significantly different from their respective $^{15}X_N$ calculated from
682 the N_2O isotope ratio data (t-test; $t\text{-}_{WL} = 0.929$, $df = 12$, $p > 0.05$; $t\text{-}_{GL} = 1.511$, $df = 20$, p
683 > 0.05).

684

685 The mean evolved amount of N_2 and N_2O gases due to denitrification in each land use
686 type increased with increasing incubation time (Figure 2). The increase in the evolved N_2
687 was statistically significant after 20 hours incubation in GL (ANOVA; $F = 19.8$, $p <$
688 0.01), whilst due to the high variability among plots, shown by the large error bars at 20

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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_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 ~~replicate~~ plots among the different land use types, probably as a result of ~~-~~complex interactions between ~~-of the combination of several antagonistic controlling factors~~environmental controls ~~-~~of denitrification~~-effects~~ 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; $\chi^2_{OS} = 3.58$, $\chi^2_{WL} = 3.47$, $\chi^2_{GL} = 3.01$, $p > 0.05$).

~~The linearity of the evolved N_2 and N_2O fluxes in the chamber headspace between 1 and 20 hours of incubation time was evaluated in each sampling plot when all three time steps were above the MDC values (data presented in Supplementary Tables 4 & 5). With respect to the N_2 flux, significant deviation from linearity was observed only in C-MW (mean r^2 = 0.59, n = 5), whilst in C-PB, C-UG, R-HL and R-IG the per site analysis was~~

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~~not possible due to missing flux data between time steps. When the data were pooled per land use type (Figure 2a), the linear 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. Regarding the N_2O flux, this was found to increase linearly with time in all the field sites (Supplementary Table 5), with the exception of the R_IC (mean $r^2 = 0.49$, n = 4). When data were pooled per land use type (Figure 2b), the amount of N_2O accumulated after 20 hours was significantly higher than in the previous time points for all land use types (ANOVA; $F_{OS} = 4.6$, $F_{WL} = 5.1$, $F_{GL} = 14.7$, $p < 0.05$). Therefore, N_2 and N_2O flux rates were estimated using linear regression (when $r^2 > 0.95$) between 1 and 20 hours incubation using only those time points that were above the MDC values estimated for each gas.~~

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). ~~with t~~The grassland soils showed~~ing~~ on average 3 and 14 times higher denitrification rates than the woodland and organic soils respectively (Figure ~~3a~~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 ~~3b~~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 ~~3c~~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, ~~the product ratio increased with increasing incubation time~~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 ~~4a~~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 ~~4b~~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 ~~4c~~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 ~~5~~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 ~~improved~~ 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 ~~15N enrichment~~tracer addition under field conditions~~, which was previously not possible~~.

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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 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 ~~thus 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.

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The major assumptions of the ^{15}N Gas-Flux method and the associated 'non-equilibrium equations' are that the denitrifying soil NO_3^- pool is uniformly labelled with ^{15}N and that the N_2 and N_2O originate from the same denitrifying pool (Stevens and Laughlin 1998). The ^{15}N fraction in the denitrifying pool ($^{15}X_N$), calculated non-destructively from the measured isotope ratios, ranged between 65 and 93 % and was well above the 10 % threshold for the correct application of the 'non-equilibrium equations' (Lewicka-Szczebak et al. 2013). However, the calculated $^{15}X_N$ was higher than the estimated total soil NO_3^- pool enrichment (range: 2 - 40 %) suggesting non-homogeneous mixing of the

854 added tracer (98 ^{15}N at %) with the ambient soil nitrate at natural abundance despite our
855 effort for uniform tracer application with multiple injections across the investigated soil
856 depth (Ruetting et al. 2011). Wu et al. (2011) have optimised the number of injections and
857 the volume of tracer needed to achieve homogeneous labelling of a soil core (diameter 15
858 cm; height 20 cm) and reported that 38 injections of 4 mL volume each were necessary.
859 We have used only 10 injections of 5- 20 mL volume (depending on the soil water
860 content of each land use type) to minimise the disturbance of the soil matrix, particularly
861 in the highly porous media such as peatland soils, and this was clearly sub-optimal for the
862 homogenous labelling of the soil enclosure but probably a necessary compromise for
863 large scale intensive measurements. We were not able to sample the soil within the
864 chamber collars for directly estimating the $^{15}NO_3^-$ content of the soil pool due to time and
865 budget constraints. However, in cases where destructive soil sampling was used to
866 measure the soil nitrate pool enrichment (Kulkarni et al. 2014), the results were
867 significantly different from the estimated enrichment due to sampling bias of the volume
868 of soil affected by the tracer application. Non-uniform mixing of the ^{15}N label may lead to
869 overestimation of the $^{15}X_N$ and underestimation of the denitrification flux rates (Boast et
870 al. 1988). However, under field conditions, it is unlikely ~~under field conditions~~to achieve
871 complete mixing of the added tracer with the ambient nitrate pool; and experimental
872 studies (Mulvaney 1988, Mulvaney and Van den Heuvel 1988) have shown that the
873 associated error is well-constrained and that accurate measurements can be made even
874 with a less-uniformly labelled denitrifying pool. The non-significant change of $^{15}X_N$ with
875 incubation time suggested only one denitrifying pool for both N_2 and N_2O, assuming
876 negligible N_2 production from anammox and co-denitrification (Spott and Stange 2007).
877 Only in the case of the C-MW well-drained forest site, shown to exhibit the highest
878 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.

~~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, 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). We have managed to further lower the limit of detection for N_2 and N_2O fluxes due to the high precision of our preparative devices coupled to the IRMS systems, but also by lowering the volume to surface area ratio of our chambers from 16:1 to 8:1 (cm^3/cm^2) and by extending the incubation time to approximately 20 hours, for the first time in a field study.~~

Most studies using ^{15}N 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 ^{15}N enrichment applications in intact soil cores (Morse and Bernhardt 2013) and laboratory incubations (Yang et al. 2014) for a more precise and accurate detectable ^{15}N-N_2 signal. However, it should be noted that in these cases ~~where an extended incubation period was employed, the soil cores or slurries did not allow the subsoil diffusion of the evolved N_2 and N_2O back into the soil pore spaces~~the soil cores or slurries were incubated in fully enclosed systems and were thus not affected by potential bias from diffusion of evolved N_2 and N_2O to the subsoil (Clough et al. 2005). The open-bottom, un-vented static chamber design used in this study in combination with the extended incubation period up to 20 hours may have potentially allowed some loss of the evolved N_2 and N_2O through downward subsoil diffusion and/or reduction of gas exchanges at the soil-atmosphere interface due to positive pressure build

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up in the chamber headspace (Healy et al. 1996). This could partly explain the non-linear increase of the evolved N_2 and N_2O in the chamber headspace (Figures 2a & b) and also the decrease of the N_2 flux rate with increasing incubation time (Figure 3a). The N_2O flux rate increased up to 2 hours incubation followed by a decrease after 20 hours consistently across land use types (Figure 3b) and this was ~~possibly~~ an indication of potentially enhanced N_2O reduction due to both subsoil diffusion and the increasing concentration of the N_2O in the topsoil. However, due to the high spatial heterogeneity within each land use type, the mean N_2O flux rate was not significantly different between the different incubation intervals. In other words, the non-linearity of N_2O evolution had less effect on the flux rate estimation than the inherent spatial variability within each land use type, which is in agreement with the findings of Chadwick et al. (2014), who suggested that the spatial variability of N_2O fluxes far exceeds the bias due to assumed linearity of fluxes.

The lack of a consistent pattern of N_2 flux rate change with incubation time among the different land use types suggested a more complex temporal variability of N_2 fluxes that apart from the duration of incubation could have also been affected by the distribution of the added nitrate tracer. In the OS sites with the lowest average nitrate content (Table 2) and the highest water filled pore space (Mean WFPS: C-PB = 70 ± SE 3.21 %; C-UG = 66 ± SE 1.58 %; R-HL = 69 ± SE 2.00 %), non-homogeneous tracer distribution ($^{15}X_N$ = 90%) 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, ~~, while~~ 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~~-event~~ within the first 1 – 2 hours of

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incubation, until the added water started to equilibrate with the ambient soil moisture. Therefore the N_2 flux rate in C-MW may be significantly overestimated after 1 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 $N_2O/(N_2 + N_2O)$. This observation could potentially be explained by a delay in the *de novo* synthesis of the N_2O reductase enzyme, 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}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

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soil moisture and substrate availability effects due to the added tracer solution. The uncertainty in the estimated in situ N_2 fluxes can be significantly reduced if additional effort is made to increase the homogeneity of the tracer application by increasing the number of injections while reducing the volume of the applied tracer (particularly in soils where denitrification is limited by moisture ~~limited soils~~). 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 ~~interval between tracer injection and the onset of incubation and subsequent gas sampling duration to~~length of incubation, thus avoiding potential over-estimation of denitrification in nitrate and moisture limited ecosystems and potential under-estimation due to ~~back~~subsoil diffusion of evolved gases~~ during incubation times~~. 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).

~~However, we have demonstrated that the N_2 flux and more importantly the N_2O flux increased linearly with time through the 20 hour incubation period, probably as a result of a slow N_2O diffusion rate due to the high water filled pore space (WFPS) (Jury et al. 1982) in our field sites (Mean WFPS: C-PB = 70 ± SE 3.21 %; C-UG = 66 ± SE 1.58 %; R-HL = 69 ± SE 2.00 %; C-MW = 42 ± SE 0.76 %; R-DW = 65 ± SE 1.79 %; R-UG = 64 ± SE 1.41 %; C-IG = 60 ± SE 1.45 %; R-IG = 61± SE 2.46 %). In the case of the C-MW, the N_2 flux may have been underestimated due to a faster decrease in the gas concentration gradient between the soil surface and the chamber headspace as a result of higher air-filled porosity (Healy et al. 1996) and the subsequent diffusion of N_2 back into~~

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subsoil. In the case of the R-IG, where N_2O flux was not found linear up to 20 hours incubation, some of the N_2O may have been diffused into the subsoil and further reduced to N_2 (Clough et al. 2005), thus leading to an underestimated N_2O flux rate. In this study, we have chosen to report flux rates based on linear regression up to 20 hours incubation period (where available), for comparison purposes between land use types exhibiting marked differences in potential denitrifer activity (Sgouridis and Ullah 2014). It has been shown that a linear flux model is less sensitive to noisy datasets hovering close to the limit of detection (particularly the OS land use type in our case), in spite of the possibility of underestimation of true fluxes (Levy et al. 2011). However, when our objective was to estimate annual in situ flux rates of N_2 and N_2O due to denitrification from natural and semi-natural land use types between April 2013 and October 2014 (Sgouridis and Ullah 2015), the flux rate estimation was based on the maximum evolved N_2 and N_2O rate at any valid (above the MDC) time step, thus reporting maximum flux rates per land use type to possibly avoid the risk of underestimation. Therefore, we suggest using varying incubation times under field conditions to capture a more reliable ^{15}N signal, particularly for N_2 gas, from sites exhibiting significant seasonal variability of flux rates.

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 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 N-rich C-MW site in the woodland soils, tracer application rates were higher than the daily atmospheric N deposition rates, thus 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, and potentially enhance the denitrification activity, in excess of the amount of nitrogen normally deposited via natural processes and common management practices.

The major assumptions of the ^{15}N Gas Flux method and the associated 'non-equilibrium equations' are that the denitrifying soil NO_3^- pool is uniformly labelled with ^{15}N and that the N_2 and N_2O originate from the same denitrifying pool (Stevens and Laughlin 1998). The ^{15}N fraction in the denitrifying pool ($^{15}X_N$), calculated non-destructively from the measured isotope ratios, ranged between 65 and 93 % and was well above the 10 % threshold for the correct application of the 'non-equilibrium equations' (Lewicka-Szczebak et al. 2013). However, the calculated $^{15}X_N$ was higher than the estimated total

soil NO_3^- pool enrichment (range: 2 – 40 %) suggesting only partial mixing of the added tracer (98 ^{15}N at %) with the ambient soil nitrate at natural abundance despite the elaborate effort for uniform tracer application with multiple injections across 10 cm 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 highly porous media such as peatland soils, and this may have affected the homogeneous distribution of the tracer. 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, it is unlikely under field conditions to achieve complete mixing of the added tracer with the ambient nitrate; and experimental studies (Mulvaney 1988, Mulvaney and Van den Heuvel 1988) have shown that the 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). Moreover, the similar $^{15}X_N$ values obtained from both the N_2 and the N_2O isotope ratio data for the woodland and grassland soils (Supplementary Table 3), was an additional indication that the effect of hybrid N_2

~~fluxes was negligible and thus it was appropriate to use the $^{15}X_N$, calculated from the N_2O isotope ratios, for calculating N_2 flux rates using the more reliable R30 measurements (Stevens and Laughlin 2001). 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 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). ~~We have managed to further lower the limit of detection for N_2 and N_2O fluxes due to the high precision of our preparative devices coupled to the IRMS systems, but also by lowering the volume to surface area ratio of our chambers from 16:1 to 8:1 (cm^3/cm^2) and by~~

~~extending the incubation time to approximately 20 hours, for the first time in a field study.~~

~~We were able to measure appreciable *in situ* fluxes of both N_2 and N_2O due to denitrification in all three land use types.~~ Our N_2 fluxes from woodland soils compare well with the rates reported in the literature for restored forested wetlands in North America (Morse and Bernhardt 2013) and with the rates from northern hardwood forests in 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 N_2 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 N_2O fluxes were up to 200 times lower than the N_2 fluxes leading to low denitrification product ratios in all land use types, a result which is in line with the N_2O yields reported from ^{15}N tracer studies in forest (Kulkarni et al. 2014, Morse and Bernhardt 2013) and grassland soils (Baily et al. 2012, Bergsma et al. 2001). ~~It is likely that the denitrification product ratio in the grassland soils has been underestimated due to the extended incubation period (up to 20 hours), during which some of the denitrification derived N_2O may have diffused back into the soil and was further reduced to N_2. Therefore, we would recommend that in soils displaying high denitrification activity (e.g. improved grasslands) the incubation period should not exceed 2 hours for a more accurate estimation of the $N_2O/ N_2 + N_2O$ ratio.~~ In the present study we have compared the in situ denitrification rates between three major land use types

using an extended field incubation period to increase the probability of detecting a reliable ^{15}N-N_2 signal, particularly under conditions of low denitrifier activity due to seasonality of denitrification and/or inherent capacity of soils (for example organic and deciduous forest soils). However, these rates should be considered conservative since confounding issues such as subsoil diffusion and non-homogeneous labelling of the soil nitrate pool may in some cases have led to underestimations of the in situ denitrification rates.

4.3. Comparison with the AIT

The total denitrification rates measured with the C_2H_2 amended intact soil cores followed the same trend as the total denitrification (N_2 and N_2O fluxes combined) from the ^{15}N Gas-Flux measurements, while they were on average 168 times lower than the denitrification potential measured in the same land use types in anaerobic soil slurries amended with acetylene and nitrate in a previous study (Sgouridis and Ullah 2014), thus reflecting lower in situ rates. The AIT denitrification rates were between 3 and 5 times lower than the ^{15}N Gas-Flux rates despite the fact that the AIT intact soil cores were capped at the bottom, thus not allowing any subsoil diffusion of the evolved gases due to denitrification. Therefore, the AIT rates should have been higher than the ^{15}N Gas-Flux rates if serious underestimation was occurring due to subsoil diffusion in the open-bottom static chambers, which was not the case. Adding nitrate to the C_2H_2 amended cores would have been desirable for directly evaluating the priming effect of the added substrate on denitrification rates. The ^{15}N tracer addition to the static chambers corresponded to the amounts of N naturally deposited in these land use types either via management practices and/or atmospheric deposition, thus avoiding excessive N fertilisation of the sampling

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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_2O reduction to N_2 by C_2H_2 in wet soils (Yu et al. 2010) or in soils with low nitrate content (Qin et al. 2013, 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 $^{15}N\text{-}NO_3^-$ tracer application rate was low (< 1 kg N ha^{-1}). Moreover, the disturbance of the soil structure during the extraction of the soil cores and the effect of the acetylene addition to microbial activity were not significant as it was suggested by the similar CO_2 production rates (Aulakh et al. 1991), representing soil respiration (Felber et al. 2012), in the static chambers and the C_2H_2 amended and un-amended intact soil cores. Therefore, we could argue that it is possible that the AIT underestimated total denitrification rates compared to the ^{15}N Gas-Flux method due to the likely incomplete inhibition of N_2O reduction to N_2 under relatively high soil moisture contents, although the shorter incubation time (2h for the intact cores) may have limited the ability of C_2H_2 to fully equilibrate within soil pore spaces. Other confounding factors such as the catalytic decomposition of NO in the presence of C_2H_2 (Bollmann and Conrad 1996, Nadeem et al. 2013) may have also contributed to the lower denitrification rates

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measured by the AIT~~.~~. This study has confirmed some of the drawbacks of the AIT as a quantification method of in situ denitrification rates compared to the ^{15}N Gas ~~-~~Flux~~.~~.

The estimation of the denitrification product ratio using the AIT method, from the un-amended cores (N_2O only) and the C_2H_2 amended cores (N_2 + N_2O), is usually overestimated since the source of N_2O cannot be discriminated with the AIT, whilst the N_2 flux is underestimated due to the incomplete inhibition of N_2O reduction (Butterbach-Bahl et al. 2013). This was confirmed in the present study for all the land use types and even the maximum denitrification product ratio after 2 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_2O 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_2O analyses ~~was greatly improved by using smaller sample volumes than previously reported, thus~~allowed us ~~allowing us~~ to quantify in situ N_2 and N_2O fluxes with low ^{15}N ~~enrichment~~ tracer addition under field conditions in natural and semi-natural land use types~~, which was previously not possible~~ 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

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moisture and substrate priming effects due to the added nitrate tracer solution. The spatial variability of N_2O fluxes superseded any bias associated with non-linear fluxes due to the extended incubation period. The uncertainty in the estimated N_2 and N_2O fluxes can be significantly reduced by increasing the homogeneity of the tracer application and by closely monitoring the linear evolution of the produced gases with more frequent gas sampling at shorter equal incubation intervals to avoid under or over estimation of denitrification. ~~The ^{15}N Gas-Flux method was applied for the first time across a range of natural and semi-natural land use types at ^{15}N tracer application rates mimicking current estimates of atmospheric N deposition (natural systems) or grassland fertiliser application rates and yielded analytically valid flux rates for both N_2 and N_2O in all the land use types. A possible limitation of the adapted ^{15}N Gas-Flux method when applied at low ^{15}N enrichment levels is the uncertainty associated with the estimation of the soil NO_3^- pool enrichment and the possibility for subsoil diffusion of the evolved gases in cases of extended incubation (> 2 hr) that may result in the underestimation of denitrification rates.~~ 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 ~~overestimates~~ overestimated the denitrification product ratio compared to the ^{15}N Gas-Flux method. The ^{15}N Gas-Flux method holds much promise as a more reliable field technique for measuring in situ denitrification rates and its wider application across a range of terrestrial ecosystems can lead to its refinement and improvement and in the long term can significantly contribute to our understanding of the role of denitrification as a reactive nitrogen sink.

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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

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1443 **Table 2:** The ambient soil nitrate pool, the ^{15}N tracer application rate, the estimated
1444 enrichment of the total soil nitrate pool, the calculated $^{15}X_N$ value from N_2O and the slope of
1445 the $^{15}X_N$ change with incubation time in the three land use types. Data are means with
1446 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)

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1448 **Table 3**: Comparison of mean flux rates and ratios between land use types for the two field
1449 methods using One-Way ANOVA. All variables are log-transformed. *F*; *F* statistic, *P*;
1450 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

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1459 **Figures**

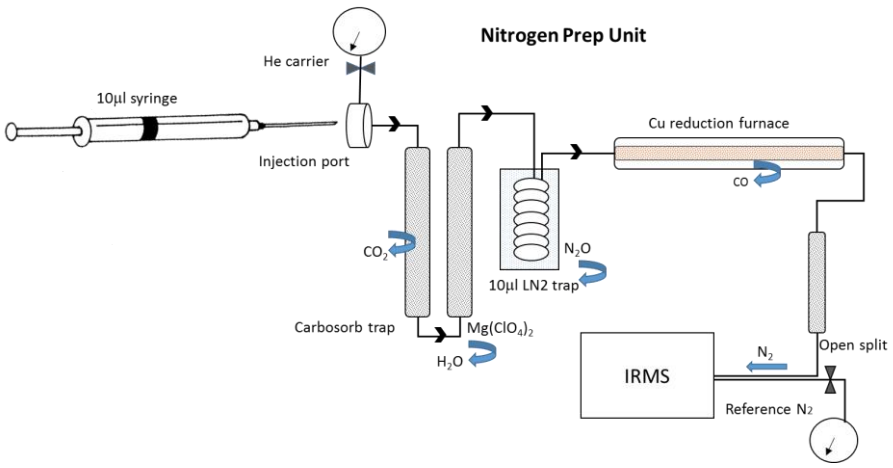

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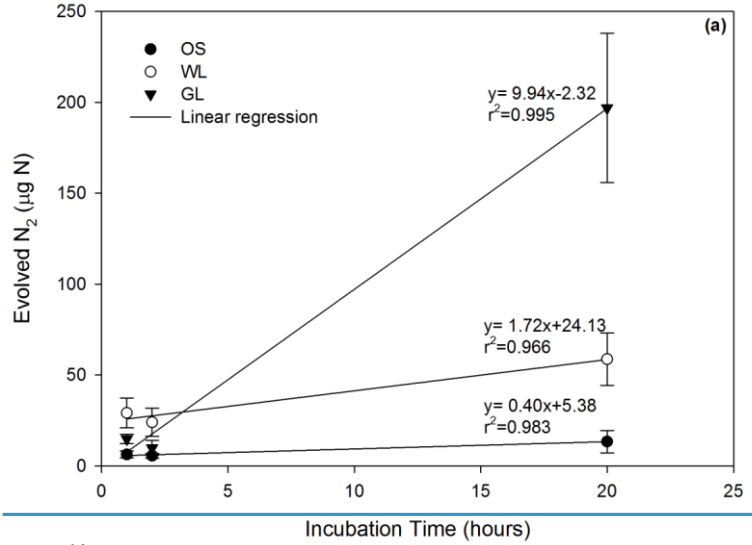
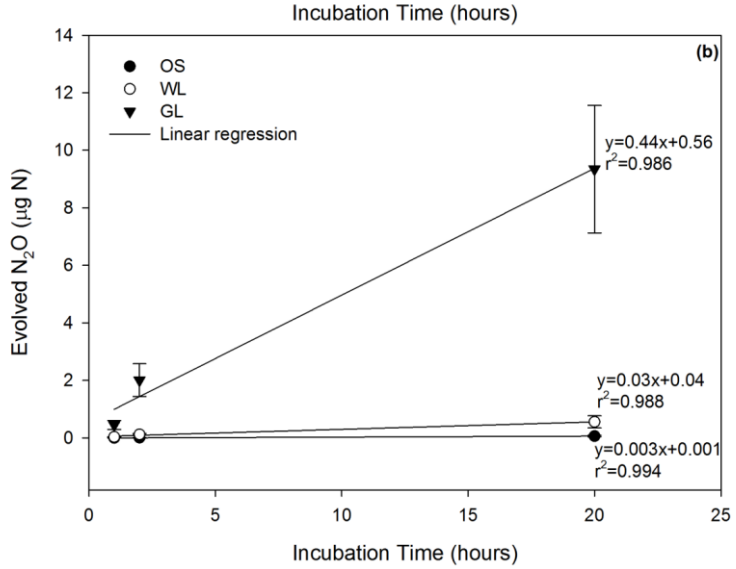
Figure 1: Schematic of the ^{15}N-N_2 analysis system


(a)
OS
WL
GL
Linear regression
y= 9.94x-2.32
r²=0.995
y= 1.72x+24.13
r²=0.966
y= 0.40x+5.38
r²=0.983
Evolved N2 (μg N)
Incubation Time (hours)


(b)
OS
WL
GL
Linear regression
y=0.44x+0.56
r²=0.986
y=0.03x+0.04
r²=0.988
y=0.003x+0.001
r²=0.994
Evolved N2O (μg N)
Incubation Time (hours)

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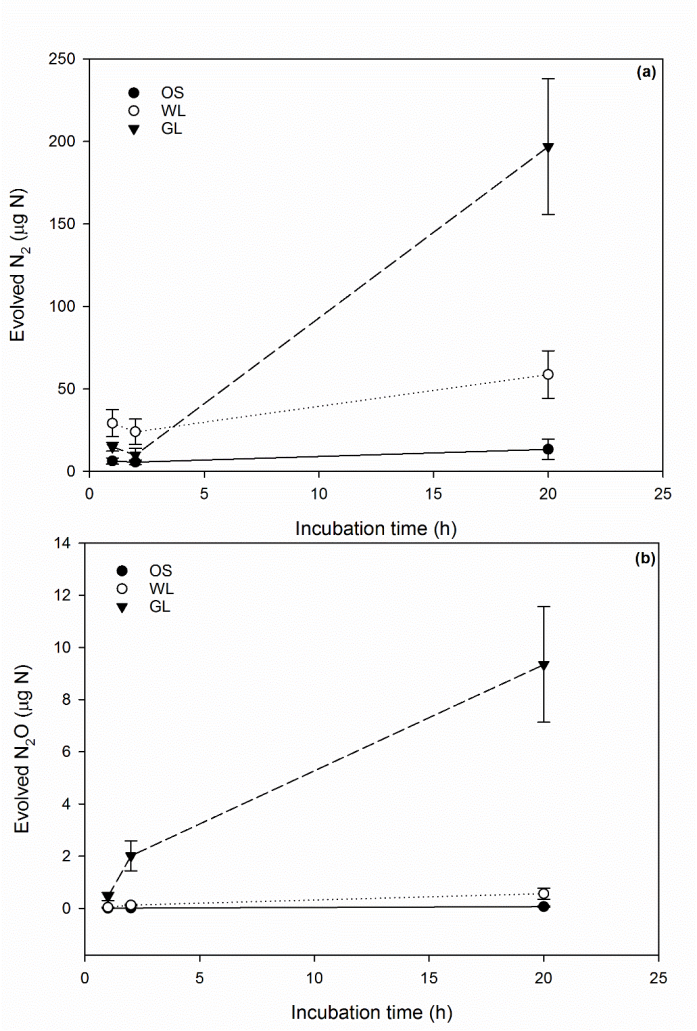

Figure 2: Evolved (a) N_2 and (b) N_2O gas measured between 1, 2 and 20 hours incubation time ~~points~~ intervals using the ^{15}N Gas-Flux method in the organic soil (OS), woodland (WL) and grassland (GL) land use types. Data points are means and the error bars represent standard errors.

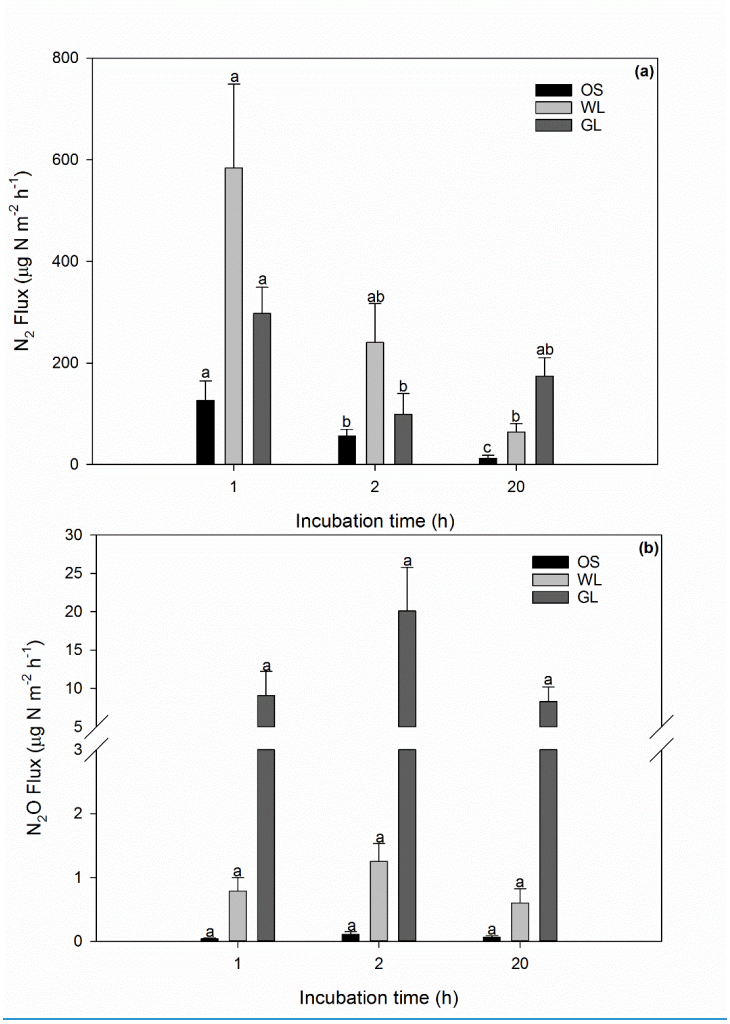

Figure 3: Mean rates of: (a) N_2 flux and (b) N_2O flux due to denitrification at the three incubation time intervals in the three land use types (OS; organic soils, WL; woodland and GL; grassland). Same lower case letters indicate no significant differences ($p > 0.05$) between incubation time intervals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

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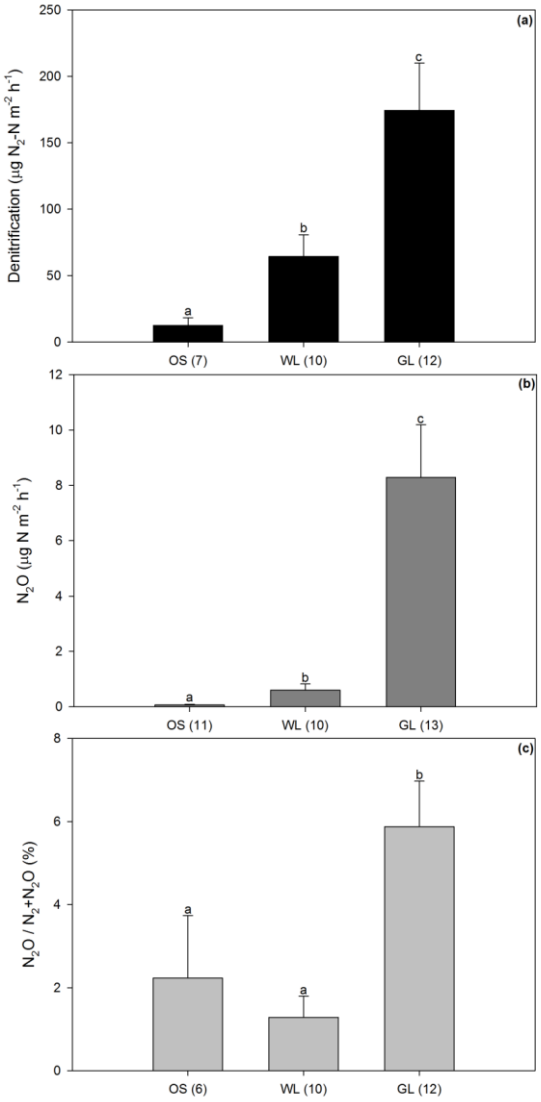
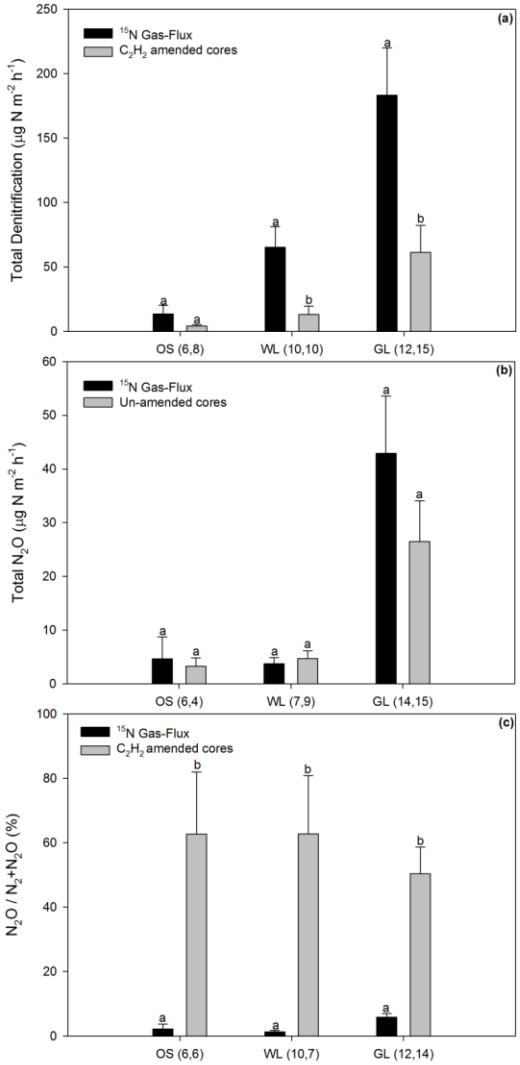


Figure 34: Mean rates of: (a) N_2 flux, (b) N_2O emission due to denitrification and (c) the denitrification product ratio N_2O/ (N_2 + N_2O) in the three land use types (OS; organic soils, WL; woodland and GL; grassland). Same lower case letters indicate no significant differences (p > 0.05) between 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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1498 **Figure 45**: (a) Mean total denitrification measured with the ^{15}N Gas-Flux method and the
1499 AIT, (b) Mean bulk N_2O emission measured in the static chambers of the ^{15}N Gas-Flux
1500 method and in un-amended intact soil cores and (c) the denitrification product ratio N_2O/ (N_2
1501 + N_2O) with the ^{15}N Gas-Flux method and the AIT in the three land use types (OS; organic
1502 soils, WL; woodland and GL; grassland). Same lower case letters indicate no significant
1503 differences ($p > 0.05$) between measurement methods according to independent samples t-
1504 test. The sample size (n) is given in parenthesis for each land use type and each method on
1505 the x-axis. Error bars represent standard errors.

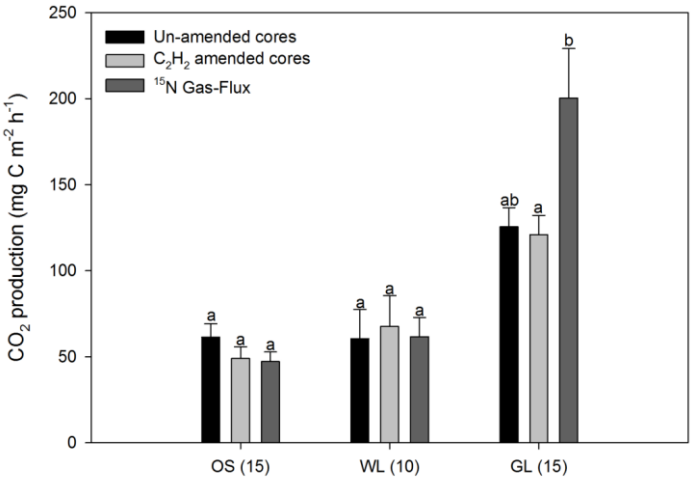

Figure 56: Mean CO_2 production measured in the static chambers of the ^{15}N Gas-Flux method, in un-amended and C_2H_2 amended intact soil cores in the three land use types (OS; organic soils, WL; woodland and GL; grassland). Same lower case letters indicate no significant differences ($p > 0.05$) between measurement methods according to 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.