21 January, 2016 1

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Response to Dr Reinhard Well's comments on the manuscript 'Application of the 15N-Gas Flux 4 method for measuring in situ N2 and N2O fluxes due to denitrification in natural and semi-natural 5 terrestrial ecosystems and comparison with the acetylene inhibition technique.' (Manuscript ID = 6 doi:10.5194/bgd-12-12653-2015) 7

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We are grateful to Dr Well for the additional comments he supplied as part of the ongoing review of 10
our manuscript. We believe that these additional comments/suggestions significantly improved the 11
clarity of our results and discussion and the overall impact of our work. We have, therefore, 12 attempted to accommodate all the suggestions where possible and amended the manuscript 13 accordingly. 14

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Response (in bold-face) to each comment (in italics) of the Reviewers follows: 17

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Major comments 19

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1) Non-linearity of fluxes (N2 + N2O and N2O) 21

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Following the reviewer's comments, we have amended the Supplementary Tables 4&5, where 23 linearity is assessed on a per plot basis by calculating the ratio of evolved gas amount between the 24 first and second hour (T2/T1) and first and last incubation interval (T3/T1). As described in the 25 Tables' captions: 'If linear evolution of N2 or N2O in a constant headspace volume is assumed then 26
T2/T1 = 2 and T3/T1 = 20'. From this analysis it becomes apparent that only in few cases 27 (highlighted in bold font) the evolution of gases approached linearity.

RW: ok, but I suggest to reword the caption:
"Evaluation of the linearity of the evolved N_2 during field incubation, per sampling plot in each field site. Only those samples that were above the MDC value are used. Linear evolution of N_2 in a constant headspace volume is proven when T2/T1 = 2 and T3/T1 = 20. T1 = 1 hour, T2 = 2 hours and T3 ~ 20 hours of incubation time. Ratios close to the ideal values are highlighted in bold font"

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please add a criterion here, e.g deviation from ideal ratio < x %.

Subsequently we have 28
amended Figure 2 in the manuscript by removing the linear regression and only showing the 29
average increase of the evolved gases with time per land use type. Additionally, we have 30
calculated the flux rate of N2 and N2O at each sampling interval and compared the means per land 31
use type with additional statistical tests and included this comparison in Figure 3 in the 32
manuscript. These additional results of N2 and N2O fluxes after 1, 2 and 20 hr incubation are 33
described in section 3.2 (Lines: 434-456).

RW: ok

Following this additional temporal analysis of N2 and N2O 34
fluxes and prompted by the reviewer's suggestion we have re-structured the discussion section 35

4.2, first to reflect the order the results are presented but also accommodating additional 36 discussion for the temporal analysis and provision of recommendations for further improvements 37 of the pitfalls in the observed methodology (Lines: 543-731)

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

Line 594-596 "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)." Information on hybrid N2 or N2O can only be obtained from the comparison of 15XN and 15N atom fraction of extracted NO3. But since you did not analyse extracts, there is no evidence for or against hybrid fluxes. So I suggest to delete this sentence

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

L 626-627: "and/or reduction of gas exchanges at the soil-atmosphere interface due to positive pressure build up in the chamber headspace (Healy et al. 1996)."
Was this addressed in Healy et al? This could not occur in vented chambers. In unvented chambers pressure fluctuations might result in both, enhanced or inhibited emissions depending on increasing or decreasing atmospheric pressure during closure. But pressure differences would hardly affect diffusion. I remember that Healy mainly focused on diffusive fluxes, showing that decreasing fluxes are due to decreasing CONCENTRATION GRADIENTS. Suggest to double check this and eventually modify accordingly. is

L 632 "enhanced N_2O reduction due to both subsoil diffusion and the increasing concentration of the N_2O in the topsoil"
N2O reduction to N2 in topsoil would not be enhanced by subsoil diffusion. Do you mean "extended enclosure time lead to lowering of N2O fluxes due to subsoil diffusion and enhanced N2O reduction to N2"?

L 666 please refer to Table S6 here
Suggest to reformulate "could potentially be explained by a delay in the *de novo* synthesis of DENITRIFICTION ENZYMES AND THE FACT THAT THE N_2O reductase enzyme is known to have a slower expression than the preceding reduction enzymes (Knowles, 1982), leading to N_2O accumulation and lower N_2 production after 2 hours of incubation." since the product ratio first increases until T2 which could not be explained by a change in N2O reductase only.

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

L 37-40
"Total denitrification 37 rates measured by the acetylene inhibition technique in the same land use types correlated (r = 38 0.58) with the denitrification rates measured under the ^{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."

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

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Overall the uncertainties are now very well addressed from my view. I really appreciate this, since these data and their interpretation will help to improve field measurement using the 15N gas flux method.

. Finally, the additional results from 38
the temporal analysis are also summarised in the abstract and the conclusion as per the reviewer's 39
request (Abstract Lines: 42-48; Conclusion Lines: 792-801). 40

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2) Non-homogeneity of labelling 43

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In the discussion (Lines: 569-606) we acknowledge the fact that our tracer distribution was sub-45 optimal when compared to the optimised protocol suggested by Wu et al. 2011, but probably a 46 necessary compromise for our large scale intensive measurements. We also clearly state that by 47 comparing the estimated total soil NO3- pool enrichment and the calculated 15XN it is shown that 48 there has been non-homogeneous mixing of the tracer with the ambient soil nitrate and this may 49 have led to the underestimation of the calculated fluxes. However, we also refer to the literature 50 to show that under field conditions, it is unlikely to achieve complete mixing of the added tracer 51 with the ambient nitrate pool and that relatively accurate measurements are still possible with a 52 less-uniformly labelled denitrifying pool. Drawing from the reviewer's suggestions we have 53 included in the discussion some hypotheses as to how the non-uniform distribution of the tracer 54 may have affected the flux rates due to soil moisture but also substrate availability effects (Lines: 55 608-617 and 647-653). 56

57

3) Moisture effect 58

We have missed to describe in the methods section that the injection of the tracer in the organic 59 soil sites (C-PB, C-UG and R-HL) was done from the surface to 15 cm depth rather than 10 cm, 60 which was the injection depth in all the other land use types. The purpose of this was to increase 61 the volume of the labelled soil in these low bulk density soils in order to increase the probability of 62
detectable denitrification activity. This information has been added in line 256. It was also our 63 oversight to report an increase of the soil moisture content equivalent or less than 2 mm 64 precipitation and this sentence has now been removed from the manuscript. Following the above 65 clarifications, the volumes of soil water in the OS plots reported in Supplementary Table 1 are 66 correct and within the expected range for soils with very low bulk density (< 0.2 g/cm3). 67

68

Moreover, we have added a clarification in the methods section (Lines: 268-270) to explain that no 69
time was allowed for the equilibration of the added tracer solution in the soil enclosure to avoid 70
significant loss of the low amount of added nitrate via plant uptake. Subsequently, in the 71
discussion we hypothesise how the moisture effect from the addition of the tracer solution 72
without equilibration with the soil water may have affected the gas flux rates (Lines: 608-617). 73

Finally, we were not able to do repeated measurements after 15N 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
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average water content in the entire soil. 105

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Please see response for major comments 2&3 above 107

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5. Table S1: Soil water numbers are questionable (up to 5 L) since the volume of labelled soil was 5 L 109
only. Please check. 110

111

Please see response for major comment 3 above 112

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6. Table S6: the fact that there were no clear time trends for the product ratio probably shows the 114
overlap of several processes (see general comments) 115

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An additional discussion has been added on the temporal variability of the N2 and N2O gas fluxes 117
as well as the denitrification product ratio to attempt an explanation for the observed inconsistent 118
patterns. Briefly, the lack of a consistent pattern of N2 flux rate change particularly with incubation 119
time among the different land use types suggested a more complex temporal variability of N2 120
fluxes that apart from the duration of incubation could have also been affected by the distribution 121
of the added nitrate tracer, with more details presented in Lines 644-676. 122

123

7. L 652-657 the conclusion with respect to hybrid N2 or N2o is incorrect (see Spott & Satnge 2007 124
and Spott et al., 2011): hybrid N2 and/or N2o would be proven by 15XN was lower than 15N atom 125
fraction of NO3 but not from the deviation between 15XN of N2 and N2O. In fact the fraction of 126
hybrid gas could be different in N2 and N2O fluxes which could lead to different values in 15XN. But 127
this could not be determined due to missing 15NO3 analysis and the large non-homogeneity in 128
labeling. 129

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The above conclusion has been removed from the discussion as incorrect. 131

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8. L 535 to 538 this statement is not well justified. Your precision for R29 and R30 is in the same 133
order 133
compared to previous studies including as early as Siegel et al., 1982 (see comparison of precision in 134

Well ea 1998). So please formulate more cautious or give exact numbers in identical units (eg. 135
Standard dev for R29 and R30) to show to which extent your analysis was better. 136

137

The above statement has been changed to: ‘Therefore, the analytical precision achieved for both 138
15N-N2 and 15N-N2O analyses, using smaller gas sample volumes than previously reported, allowed
139
us to quantify in situ N2 and N2O fluxes with low tracer addition under field conditions.’ Moreover,
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our achieved precision for R29 and R30 is presented in Table 1 and in the discussion (Lines: 512-141
517) it is stated that it was comparable to the recent studies by Lewicka-Szczebak et al. (2013) and 142
Yang et al. (2014). 143

144

9. L 563 to 567 it is not well clarified what this means. Suggest: “the soil cores or slurries were 145
incubated in fully enclosed systems and were thus not affected by potential bias from diffusion of 146
evolved N2 and N2O to the subsoil (Clough et al. 2005). But please check if the reference still fits to 147
this modification. 148

149

The sentence has been adapted following the reviewer’s suggestion (Lines: 624-627) 150

151

10. L 570 -572 this is indeed by no means the case (see first general comment). So you have to keep 152
the possibility that increasing subsoil diffusion during extended chamber closure was a potential 153
source of bias. 154

155

The above sentence has been removed and replaced by additional discussion to explain the 156
temporal patterns of gas fluxes during the incubation period (Lines: 627-642). 157

158

11. L 681-684 this would not only result from subsoil diffusion of N2O but also from enhanced 159
reduction in the topsoil due to increasing N2O concentration during extended cover periods. 160

161

The above suggestion has been added in the discussion (Lines: 633-637) 162

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12. L 734 please cite also Bollman & Conrad 1996, who were the first to show the artefacts by 164
catalytic NO decomposition and to clarify that this artefact is known since long. 165

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The above citation was added in Lines 107 & 771. 167

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13. In the entire manuscript: use consistently the correct spelling of the product ratio: 169
N2O/(N2+N2O), one or both brackets were often missing 170

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Spelling consistency checked and corrected throughout the manuscript 172

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Minor comments to the response file with marked changes of the text: 175

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1. L 682: suggest: " to maintain natural drainage and root growth during the measurements" since 177
natural drainage is also needed if the ground water table is far below 178

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Sentence changed to the reviewer's suggestion (Lines: 240-241) 180

181

2. L 699 delete "equal" since 4*6 is not equally spaced. A pattern with triangles of equal side length 182
would be optimal. So the distance between your injections varied between 4, 6 and about 7.5 cm, 183
isn't it? 184

185

The word equal has been deleted 186

187

3. L 881-895 these statements are not justified, see general comments 188

The results section the above comment refers to has been completely re-written (Lines: 434-456) 189
to reflect the additional temporal analysis of gas fluxes. 190

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4. L 913-914 but this statement only applies for landuse average, whereas individual sites could have 192
any pattern. Please be more detailed here and explain that there was no consistent pattern for all 193
sites. 194

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The sentence has been amended in response to the above comment (Lines: 470-472). 196

197

5. L 1070 to 1073 sentence not clear to me. Do you want to highlight that you could detect fluxes in 198
view of low enrichment? But in fact your active pool was close to the enrichment of the tracer 199
solution since 15XN was around 90 at%. So you can't state that your method worked at low 200

enrichment. 201

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The overall aim of this study and the larger scale one presented in Sgouridis & Ullah (2015) was to measure in situ N2 and N2O 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 N2 and N2O fluxes and to also make recommendations for future method improvements.

Application of the 15N-Gas Flux method for measuring in situ N2 and N2O fluxes due to 229 denitrification in natural and semi-natural terrestrial ecosystems and comparison with 230 the acetylene inhibition technique. 231

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Keywords: Organic soils, forest, grassland, 15N tracer, acetylene inhibition technique, nitrous 241 oxide. 242

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Abstract 249

Soil denitrification is considered the most un-constrained process in the global N cycle due to 250 uncertain in situ N2 flux measurements, particularly in natural and semi-natural terrestrial 251 ecosystems. 15N tracer approaches can provide in situ measurements of both N2 and N2O 252 simultaneously, but their use has been limited to fertilised agro-ecosystems due to the need 253 for large 15N additions in order to detect 15N2 production against the high atmospheric N2. For 254 15N-N2 analyses, we have used an 'in house' laboratory designed and manufactured N2 255 preparation instrument which can be interfaced to any commercial continuous flow isotope 256 ratio mass spectrometer (CF-IRMS). The N2 prep unit has gas purification steps, a copper 257 based reduction furnace, and allows the analysis of small gas injection volumes (4 µL) for 258 15N-N2 analysis. For the analysis of N2O, an automated Tracegas Pre-concentrator (Isoprime 259 Ltd) coupled to an IRMS was used to measure the 15N-N2O (4 mL gas injection volume). 260 Consequently, the coefficient of variation for the determination of isotope ratios for N2 in air 261 and in standard N2O (0.5 ppm) was better than 0.5 ‰. The 15N Gas-Flux method was adapted 262 for application in natural and semi-natural land use types (peatlands, forests and grasslands) 263 by lowering the 15N tracer application rate to 0.04 – 0.5 kg 15N ha-1. For our chamber design 264 (volume/ surface = 8:1 cm3:cm2) and up to 20 h incubation period, theThe minimum 265 detectable flux rates were 4 µg N m-2 h-1 and 0.2 ng N m-2 h-1 for the N2 and N2O fluxes, 266 respectively. Total denitrification rates measured by the acetylene inhibition technique in the 267 same land use types correlated (r = 0.58) with the denitrification rates measured under the 15N 268 Gas-Flux method but were underestimated by a factor of 4 and this was partially attributed to 269 the incomplete inhibition of N2O reduction to N2 under a relatively high soil moisture 270 content. Even though relatively robust for in situ denitrification measurements so far, 271 methodological Uuncertainties still exists in the estimation of N2 and N2O fluxes with the 272 15N Gas-Flux method were associated withdue to issues related to non-homogenous 273
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distribution of the added tracerthe inhomogeneity of the tracer distribution, and subsoil gas 274 diffusion using open-bottom chambers, and decreasing gas diffusion gradients due to 275 extended incubation period (up to 20 hours) particularly , during longer incubation 276 duration.The N2 flux ranged between 2.4 and 416.6 µg N m-2 h-1, and the grassland soils 277 showed on average 3 and 14 times higher denitrification rates than the woodland and organic 278 soils respectively. The N2O flux was on average 20 to 200 times lower than the N2 flux, 279 while the denitrification product ratio (N2O/ N2 + N2O) was low, ranging between 0.03 and 280 13 %. Total denitrification rates measured by the acetylene inhibition technique in the same 281 land use types correlated (r = 0.58) with the denitrification rates measured under the 15N Gas 282 Flux method but were underestimated by a factor of 4 and this was partially attributed to the 283 incomplete inhibition of N2O reduction to N2 under relatively high soil moisture 284 contentDespite these uncertainties, the 15N Gas Flux method constitutes a more reliable field 285 technique . The results show that the 15N Gas-Flux method can be used for large scale 286 quantifying quantification of N2 and N2O production ratesfluxes in natural terrestrial 287 ecosystems, thus significantly improving our ability to constrain ecosystem N budgets. 288

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1. Introduction 305

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

capacity of denitrification for its removal with important consequences of chronic N 318
enrichment of natural terrestrial ecosystems (Galloway et al. 2008, Limpens et al. 2003). 319
Moreover, nitrous oxide (N2O), an obligate intermediate of denitrification, is a potent 320
greenhouse gas involved in the breakdown of stratospheric ozone (Ravishankara et al. 2009). 321
Therefore, a reliable estimation of the relative magnitude of the major denitrification end 322
products (N2 + N2O) in soils is crucial in evaluating the role of denitrification as an Nr sink 323
(Kulkarni et al. 2008). 324

325

N2 comprises ~78 % of the atmosphere and thus it is extremely difficult to measure small N2 326
fluxes from soil against this high background, particularly in natural terrestrial ecosystems 327
(Groffman et al. 2006). Available methods for measuring both N2 and N2O are limited and 328
can be categorised into the direct flux and 15N isotope tracer methods (Kulkarni et al. 2014), 329
whilst micrometeorological approaches (Eddy covariance) are impossible in the N2 rich 330
atmosphere (Felber et al. 2012). The gas-flow soil core method (Burgin and Groffman 2012, 331
Butterbach-Bahl et al. 2002, Scholefield et al. 1997, Wang et al. 2011) allows the direct 332
measurement of N2 flux (without the addition of any substrate such as nitrate) from intact soil 333
cores where the soil atmosphere is replaced by a mixture of He/O2. However, despite the high 334
precision of the technique, cores still need to be extracted from the field and conditioned over 335
lengthy periods of time for the complete removal of N2 from the soil atmosphere. This 336
method is therefore time and resource intensive which limits its application to intensive 337
temporal and large spatial scales (Kulkarni et al. 2014). Moreover, the gas-flow soil core 338
method cannot discriminate between sources of N2O thus overestimating the denitrification 339
product ratio N2O/ (N2 + N2O) (Butterbach-Bahl et al. 2013, Morse et al. 2015). The 340
acetylene inhibition technique (AIT) is also a direct flux method that exploits the ability of 341
acetylene (C2H2) at high concentrations (10 % v/v) to inhibit the reduction of N2O to N2 342

(Tiedje et al. 1989), thus total denitrification (N2 + N2O) is measured in C2H2 amended soil cores in situ, whilst N2 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 C2H2 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 15N Gas-Flux method (Mosier and Klemedtsson 1994) has the advantage of providing in situ measurements of both N2 and N2O simultaneously, thus allowing its application over large temporal and spatial scales. It requires the addition of a 15N-labelled tracer in a soil enclosure in the field which is subsequently covered by a chamber while the chamber headspace is progressively enriched with 15N-N2 and 15N-N2O produced by denitrification (Stevens and Laughlin 1998). Assuming that both N2 and N2O 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 15N 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 15N 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 15N-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 15N Gas-Flux technique method can be applied to a range of natural and semi-natural terrestrial ecosystems allowing

~~the quantification of the relative magnitude of N2 and N2O fluxes due to denitrification from these under-represented ecosystems.~~

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

~~The major challenge in measuring 15N-N2 at near natural abundance levels is the possibility of interference at m/z 30 (30N2) due to the reaction of oxygen in the ion source with N and the formation of NO+ ions that also have m/z 30 (Stevens et al. 1993). Commonly, this issue is addressed in continuous flow isotope ratio mass spectrometers (CF-IRMS) with the inclusion of a copper (Cu) oven for reducing O2 in the gas sample (Russow et al. 1996). Recently, it has been suggested that the interference at m/z 30 can be further reduced by including a molecular sieve column in gas chromatograph IRMS (GC-IRMS) systems to not only separate N2 and O2 in the gas sample, but also to quantitatively remove O2 and other trace gases such as carbon monoxide (Lewicka-Szczebak et al. 2013, Yang et al. 2014). We hypothesise that the precision for m/z 30 determination can be greatly improved by using a custom-built preparative unit for the removal of H2O, CO2, N2O, NO+ and CO; a device which also permits the micro-scale injection of volumes of < 5 µL. These injection volumes are much smaller than have previously been reported in the literature.~~

~~392~~

~~Studies that have compared the 15N Gas-Flux method with the AIT in the field are rare and 393 have exclusively focused on highly fertilised agro-ecosystems with moderate to low soil 394 moisture contents (Aulakh et al. 1991, Mosier et al. 1986, Rolston et al. 1982). These studies 395 have measured comparable denitrification rates by both field techniques, although the 396 relatively low soil moisture contents have probably allowed greater diffusion of C2H2 to the 397 anaerobic microsites where denitrification occurs (Malone et al. 1998), whilst the high nitrate 398 application rates have probably favoured nitrate reduction over N2O reduction (Dendooven 399 and Anderson 1995) resulting in high denitrification rates from the AIT. Conversely, 400 laboratory studies have shown that the AIT significantly underestimates total denitrification 401 compared to the 15N tracer approach (Yu et al. 2010) and the direct N2 flux approach (Qin et 402 al. 2012) due to the incomplete inhibition of N2O reduction to N2 by C2H2 in wet soils (Yu et 403 al. 2010) or in soils with low nitrate content, where N2O reduction is more energetically 404 favourable (Qin et al. 2013, Qin et al. 2014). A comparison of the 15N Gas-Flux method with 405 the AIT under in situ conditions across a range of natural and semi-natural terrestrial 406 ecosystems has not been attempted before. It can provide valuable insights in terms of the 407 validity and applicability of the two field techniques for measuring denitrification rates across 408 broad spatial and temporal scales. 409~~

~~410~~

~~The objectives of the present study were: (1) to determine the precision and suitability of our 411 preparative-IRMS instrumentation for measuring 15N-N2 and 15N-N2O at low enrichment 412 levels, (2) to adapt the 15N Gas-Flux method for application across natural and semi-natural 413 terrestrial ecosystems and (3) to compare the validity and applicability of the 15N Gas-Flux 414 method with the AIT for measuring in situ denitrification rates. 415~~

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2. Materials and methods 428
2.1. IRMS system 429

For N2 gas isotopic analysis we used an Isoprime isotope ratio mass spectrometer 430 (Isoprime Ltd, UK, Wythenshawe) coupled to an in house built N2 preparative interface 431 (Figure 1). Headspace gas (4 µL) was manually injected with a gas tight syringe (SGE 432 Analytical science) into the preparative interface via an open split. Prior to its 433 introduction into the IRMS, the sample was treated as follows: a) dried by passing 434 through $Mg(ClO_4)_2$ (Elemental Microanalysis Ltd, Devon, UK), b) CO_2 removed with 0.7 435 - 1.2 mm Carbosorb (Elemental Microanalysis Ltd, Devon, UK), c) N_2O cryogenically 436 trapped under liquid nitrogen, and d) O_2 removed over a copper packed reduction furnace 437

heated at 600°C. The N2 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 (28N2, 29N2 and 30N2) 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 N2 (BOC special gases) until a standard deviation of d15N 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 d15N 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 TraceGasTM Preconcentrator coupled to an IsoprimeTM IRMS (GV instruments Ltd, UK) whereupon the sample was directed through a series of chemical traps designed to remove H2O and CO2. The N2O was cryogenically trapped under liquid nitrogen. The waste was flushed out of the instrument. The N2O was further cryofocused in a second liquid nitrogen trap prior to being introduced onto a 25 m x 0.32 mm Poraplot Q gas chromatography column (Chrompack column, Varian, Surrey, U.K). The column separated N2O from any residual CO2, and both entered the IRMS via an open split. The retention time between the first eluting CO2 (< 2E-10 amplitude) and second eluting N2O peak typically fell in the range between 60 - 70 seconds to avoid isobaric interference of the CO2 with the calculated 15N. The N2O was directed towards the triple collectors of the isotope ratio mass spectrometer where m/z 44, m/z 45 and m/z 46 mass ions were measured and recorded. Instrument stability checks were performed prior to each analysis by running a series of 10 reference pulses of N2O (BOC special gases) until a standard deviation of d15N better than 0.05 ‰

was achieved. Prior to each sample batch analysis, trace gas N2O measurements were made on three 100 mL flasks containing atmospheric air collected from outside the stable isotope laboratory. d15N precisions using the Trace gas Preconcentrator and Isoprime IRMS were better than 0.3 ‰ respectively at 600 µAmp trap current.

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

In situ measurements of N2 and N2O were made using static chambers according to the 15N Gas Flux method (Mosier and Klemedtsson 1994). Five plots were randomly established in June 2013 in each of four study sites in the Ribble - Wyre River catchments (area 1145 km2; NW England, 53o59'99" N, 2o41'79" W). The study sites were a heathland (R-HL), a deciduous woodland (R-DW), an unimproved grassland (R-UG) and an improved grassland (R-IG). In August 2013, four more study sites were tested in the Conwy River catchment (area 345 km2; N. Wales, 52o59'82" N, 3o46'06" W) following a similar sampling design. These sites were an acid grassland (C-UG), an ombrotrophic peat bog (C-PB), a mixed deciduous and coniferous woodland (C-MW) and an improved grassland (C-IG). Further details on the location, land management status and major soil properties for all study sites can be found in Sgouridis & Ullah (2014).

In each plot a round PVC collar (basal area 0.05 m2; chamber volume 4 L) was inserted into the soil at c. 10 cm depth (15 cm for the R-HL and C-PB plots) 2 - 4 weeks before the measurement date. Thedate. The collars were open at the bottom to permit natural water table levelsmaintain 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 486 acrylic cylindrical cover (chamber) providing a gas-tight seal when filled with water 487 (Ullah and Moore 2011). The gas leak rate from the chamber was determined in the 488 laboratory by placing the sealed collar and chamber over a tray of water, injecting CH4 489 (10 ppm), and determining the change in CH4 concentration within the chamber 490 headspace over time (Yang et al. 2011). The CH4 concentration change within 24 hours 491 was negligible with the relative standard deviation (RSD) being < 5 %. We did not use a 492 vent tube for pressure equilibration, as suggested by Hutchinson and Mosier (1981), in 493 our chamber design, which could have diluted the chamber headspace with atmospheric 494 N2, as part of our effort to increase the probability of a detectable 15N-N2 signal in the 495 chamber headspace. Instead chambers were covered with reflective foil for minimising 496 temperature increase within the chamber headspace during the incubation period (Ullah 497 and Moore 2011). Labelled K15NO3- (98 at. % 15N, Sigma-Aldrich) was applied in each 498 plot via ten injections of equal volume through an equally spaced grid (4 x 6 cm) using 499 custom-made 10 cm long lumber needles (15 cm for the R-HL and C-PB plots) attached 500 to a plastic syringe (Ruetting et al. 2011). The 15N tracer was delivered as the needle was 501 pushed into the soil from the surface up to 10 or 15 cm depth aiming to achieve as 502 uniform as possible labelling of the soil volume enclosed by the collar, as required by the 503 15N gas flux method (Mosier and Klemedtsson 1994). The volume and concentration of 504 the labelled K15NO3- tracer solution was determined from measurements of soil nitrate 505 and moisture content, as well as bulk density adjacent to each plot made during the 506 installation of the collars (Morse and Bernhardt 2013). Lower application rates (< 0.1 kg 507 N ha-1) were administered to natural study sites (e.g. peat bog, heathland) and higher rates 508 (< 1 kg N ha-1) administered to semi-natural (e.g. unimproved and improved grasslands). 509 The tracer solution (50 - 200 mL) was adjusted between 3 and 5 % of the ambient 510

volumetric water content (see Supplementary Table 1 for detailed data from each 511 sampling plot). It should be noted that no time was allowed for the equilibration of the 512 added tracer solution in the soil enclosure to avoid significant loss of the low amount of 513 added nitrate via plant uptake. Since the volume of the added solution corresponded to a 514 precipitation amount of = 2 mm, the increase of the volumetric water content was 515 considered minor (Tauchnitz et al. 2015). 516

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Following the 15N tracer application the collars were covered with the acrylic chamber 518 fitted with a rubber septum for gas sampling. Two sets of gas samples (20 mL each) were 519 collected with a gas tight syringe (SGE Analytical science) through the septum of the 520 chamber cover at T = 1h, T = 2h and T ~ 20h after the tracer injection, while a T = 0h 521 sample was collected immediately after tracer injection above the plot surface before 522 fitting the chamber cover. The gas samples were transferred into pre-evacuated (<100 Pa) 523 12 mL borosilicate glass vials with butyl rubber septa (Exetainer vial; Labco Ltd., High 524 Wycombe, United Kingdom) for storage under positive pressure and were analysed 525 within 8 weeks from collection without any significant change of the gas concentration 526 (Laughlin and Stevens 2003). 527

528

Adjacent to each PVC collar in each plot, two intact soil cores (50 mm I.D., 15 cm long) 529 were extracted from 10 cm depth leaving the top 5 cm void as a headspace volume. The 530 cores were capped on both ends with the top cap fitted with a rubber septum for gas 531 sampling. One set of cores was amended with pure C2H2 with 5 mL injected through the 532 septum directly in the middle of the soil core before 10 % of the headspace being also 533 replaced with pure C2H2. The second set of cores was not amended with C2H2 and both 534

cores were placed back in the ground where they came from. Gas samples (5 mL) were 535 collected with a gas tight syringe (SGE Analytical science) through the septa of the cores 536 at T = 1h and T = 2h after amendment with acetylene. The gas samples were transferred 537 into pre-evacuated (<100 Pa) 3 mL borosilicate glass vials with butyl rubber septa 538 (Exetainer vial; Labco Ltd., High Wycombe, United Kingdom) for storage under positive 539 pressure. 540

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2.3. Flux calculations 542

The 15N content of the N2 in each 12 mL vial was determined using the IRMS system 543 described above and the ratios R29 (29N2/28N2) and R30 (30N2/28N2) were measured in 544 both enriched (T=1, 2 and 20 hours) and reference samples (T=0 hours). The inclusion of 545 air reference standards between every 10 samples indicated an upward drift for R30 over 546 time, potentially due to the formation of NO+ in the ion source despite the inclusion of the 547 Cu reduction step (Lewicka-Szczebak et al. 2013). Subsequently, every sample batch was 548 drift corrected by fitting a linear regression through the air reference standards and 549 calculating an offset correction for both R29 and R30 (Yang et al. 2014). The minimum 550 detectable change (MDC) in R29 and R30 was defined with repeated manual analyses of 551 air reference standards (n=10) and was calculated using the following equation (Matson et 552 al. 2009): 553

.....=..... .....+(2..... .....) (1) 554

where μ is the mean difference of all possible unique pairs of air reference standards 555 (n=45) and s is the standard deviation between sample pairs. The MDC for R29 was 7.7 x 556 10-7 and for R30 was 6.1 x 10-7 and these values were used to determine if each time step 557

sample was significantly different from ambient reference samples (T=0 hours), and if not 558 they were excluded from the flux calculations. 559

560

For calculating the total N2 flux from a uniformly labelled soil nitrate pool when both 561 R29 and R30 are measured, the 'non-equilibrium' equations were applied as described by 562 Mulvaney (1984) for estimating first the 15N fraction in the soil NO3- denitrifying pool 563 (15XN) as: 564

..15
..=2(....30/....29)/(1+2(....30/....29)) (2) 565

where .R29 and .R30 is the difference between R29 and R30 respectively between 566 enriched (T=1, 2 and 20 hours) and reference samples (T=0 hours). Subsequently, the 567 15XN allows the quantification of the fraction of the N2 evolved from the 15N-labelled pool 568 (d) using either the .R30 or the .R29: 569

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....30(..15
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2 (3) 570

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2 (4) 571

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Using d and the concentration of [N2] (µg N) in the chamber headspace, the evolved N2 573 from the soil pool was calculated: 574

....2=..[..2]/(1-..) (5) 575

The N2 flux was then calculated using linear regression between the maximum evolved 576 N2 and the respective incubation time per plot surface area and was expressed in µg N m-2 577

h-1 representing the total N2 flux from the mixture of the 15N-labelled tracer and the soil N 578 at natural abundance (Stevens and Laughlin 1998). 579

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The 15N content of the N2O in the same 12 mL vials as well as the ratios R45 (45N2O 581 /44N2O) and R46 (46N2O /44N2O) were measured in both enriched (T=1, 2 and 20 hours) 582 and reference samples (T=0 hours). The application of the 'non-equilibrium' equations to 583 N2O is analogous to N2 after correcting for the naturally occurring oxygen isotopes 584 (Bergsma et al. 2001). Therefore, the ratios R45 and R46 were converted to ratios of R29 585 and R30 respectively by applying the following equations: 586

..29=..45-..17 (6) 587

..30=(..46-(..29..17))-..18 (7) 588

where for R17 (17O/16O) the value 0.000373 was used and for R18 (18O/16O) the value 589 0.0020052 was used (Bergsma et al. 2001). There was no significant instrumental drift for 590 the ratios R45 and R46 over time. The MDC was defined, for the converted R29 and R30, 591 with repeated automatic analyses of 0.5 ppm N2O standards (n=15) as 3.4 x 10-5 and 2.9 x 592 10-5 respectively. The second set of gas samples collected at the same time in the field 593 were analysed for total N2O on a GC-μECD (7890A GC Agilent Technologies Ltd., 594 Cheshire, UK) and the concentration of [N2O] (μg N) was used in Eq. (5) to calculate the 595 N2O flux due to denitrification of the mixture of the 15N-labelled tracer and the soil N and 596 expressed in μg N-N2O m-2 h-1. Assuming that the N2O originates from the same 597 uniformly labelled pool as N2, the 15XN from N2O was used to estimate d for N2 using 598 either R30 (Eq. 3) or R29 (Eq. 4), thus lowering the limit of detection for N2 (Stevens and 599

Laughlin 2001) and allowing measurement of N2 gas flux from natural terrestrial 600 ecosystems at low 15N-tracer application rates. 601

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Gas samples collected from the intact soil cores with or without acetylene amendment 603 were analysed for N2O on a GC μECD (7890A GC Agilent Technologies Ltd., Cheshire, 604 UK) and for CO2 on a GC-FID (7890A GC Agilent Technologies Ltd., Cheshire, UK) 605 and flux rates were determined by linear regression between 0 and 2 hours. The 606 instrument precision was determined from repeated analyses of 6 ppm N2O and 200 ppm 607 CO2 standards respectively (n = 8) and the RSD was <1%. 608

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2.4. Statistical analysis 610

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

~~Comparisons between the 15N Gas-Flux and AIT techniques were made with independent 623 samples t-test. All statistical analyses were performed using SPSS® 21.0 for Windows 624 (IBM Corp., 2012, Armonk, NY). 625~~

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3. Results 644

3.1. IRMS system evaluation 645

The precision of the IRMS systems was evaluated using repeated analyses of ambient air 646 samples for N2 (n=10) injected manually in one batch and repeated analyses of N2O gas 647 standard at natural abundance and 0.5 ppm concentration (n=15) using automated 648 injections. The mean measured ratios of R29 and R30 for N2 and of R45 and R46 for N2O 649 are shown in Table 1. Measurement precision was defined as the coefficient of variation 650 (%) and it was lower for R29 compared to R30 and lower for R45 compared to R46, but 651 still less than 0.5 % for all four measured ratios. We estimated the 15N atom% abundance 652 for both gases as per Yang et al. (2014) and the precision was less than 0.01 % for N2 in 653 air and 0.26 % for standard N2O at natural abundance. The mean measured R30 (5.16 x 654 10-5) was higher than the theoretical value of 1.35 x 10-5 for N2 in ambient air suggesting 655 some interference at m/z 30 potentially due to the formation of NO+ ions in the ion source 656 of the mass spectrometer despite the inclusion of the Cu reduction oven. The contribution 657 of NO+ ions (R30 measured - R30 theoretical) was 3.81 x 10-5, whilst the ratio of R30 658 theoretical/ R30 measured was 0.26. Correcting the R30 ratio for the contribution of NO+ 659 ions results in a lower 'true' precision for the R30 (CV = 1.67 %). 660

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3.2. Field application of the 15N Gas-Flux method 662

The 15N tracer application rate was variable between land use types and ranged between 663 0.03 and 1 kg 15N ha-1 while it was lower in the case of the organic soils and higher for 664

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

The 15N fraction in the denitrifying pool (15XN), as calculated from the measured isotopic ratios of the N2O after 1 hour of incubation using Eq. (2), ranged between 65 and 93 15N at%. The average change of the 15XN with incubation time, indicated by the slope shown in Table 2, was not different from 0 in case of the organic (t-test; t = 0.520, df = 18, p > 0.05) and grassland soils (t-test; t = 0.047, df = 28, p > 0.05), whilst it was significantly below 0 for the woodland soils (t-test; t = 2.917, df = 18, p < 0.05). Separating the woodland soils to C-MW and R-DW sites, only the former displayed a significant negative slope of 15XN with incubation time (t-test; t = 3.306, df = 8, p < 0.05), suggesting N2O production from a second nitrate pool, possibly nitrate produced from the oxidation of NH_4^+ via nitrification, in the C-MW. In cases where the 15XN could be calculated from the N2 isotope ratio data (woodland and grassland soils; data shown in Supplementary Table 3), this was not significantly different from their respective 15XN calculated from the N2O isotope ratio data (t-test; t-WL = 0.929, df = 12, p > 0.05; t-GL = 1.511, df = 20, p > 0.05).

The mean evolved amount of N2 and N2O gases due to denitrification in each land use type increased with increasing incubation time (Figure 2). The increase in the evolved N2 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

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hours incubation in Figure 2a, it was not significant for the OS and WL soils. The amount of N2O accumulated after 20 hours (Figure 2b) was significantly higher than in the previous time points for all land use types (ANOVA; FOS = 4.6, FWL = 5.1, FGL = 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 N2 accumulations were observed after the first or second hour of incubation, whilst in most cases the increase in N2 and N2O 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 antagonisticcontrolling factorsenvironmental controls of denitrification effects and the length of the incubation period (details below). Consequently, the N2 flux rate decreased with increasing incubation time (Figure 3a) and this decrease was significant between each time interval in the OS (Kruskal-Wallis; .2=11.35, p=0.003), between 1 and 20 hours in the WL (Kruskal-Wallis; .2=10.78, p=0.005) and between 1 and 2 hours in the GL (Kruskal-Wallis; .2=10.10, p=0.006). Conversely, the N2O 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; .2OS = 3.58, .2WL = 3.47, .2GL = 3.01, p > 0.05).

The linearity of the evolved N2 and N2O 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 N2 flux, significant deviation from linearity was observed only in C-MW (mean r2 = 0.59, n = 5), whilst in C-PB, C-UG, R-HL and R-IG the per site analysis was Formatted: Not HighlightFormatted: Not HighlightFormatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: Not HighlightFormatted: SubscriptFormatted: Font: ItalicFormatted: Font: Italic, SuperscriptFormatted: Font: ItalicFormatted: English (United Kingdom) Formatted: English (United Kingdom)
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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 N2 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 N2O flux, this was found to increase linearly with time in all the field sites (Supplementary Table 5), with the exception of the R-IG (mean r2 = 0.49, n = 4). When data were pooled per land use type (Figure 2b), the amount of N2O accumulated after 20 hours was significantly higher than in the previous time points for all land use types (ANOVA; FOS = 4.6, FWL = 5.1, FGL = 14.7, p < 0.05). Therefore, N2 and N2O flux rates were estimated using linear regression (when r2 > 0.95) between 1 and 20 hours incubation using only those time points that were above the MDC values estimated for each gas.

The N2 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 tThe grassland soils showeding on average 3 and 14 times higher denitrification rates than the woodland and organic soils respectively (Figure 3a4a). A similar pattern was observed for the N2O 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 N2O than the woodland and organic soils respectively (Figure 3b4b), whilst the N2O flux was on average 20 to 200 times lower than the N2 flux among land use types. Consequently, the denitrification product ratio (N2O/ (N2 + N2O) was low, ranging between 0.03 and 13 % and was highest in the GL and similar between the WL and OS (Figure 3c4c). The change of the denitrification product ratio with incubation time was evaluated in each sampling plot where both N2 and N2O fluxes were available (data shown in

~~Supplementary Table 6). Generally, the product ratio increased with increasing incubation 739 timethere was no consistent pattern between individual sampling plots with the exception 740 of the grassland soils, where the maximum product ratio was observed after 2 hours of 741 incubation (ANOVA; $F = 6.11$, $p < 0.05$). This was an indication of some reduction of the 742 denitrification derived N_2O to N_2 during the extended closure period (up to 20 hours) in 743 the grassland soils. 744~~

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~~3.3. Comparison with the AIT 746~~

~~The total denitrification rate measured from the C_2H_2 amended intact soil cores in the 747 same land use types ranged between 0.5 and 325.2 µg N m-2 h-1 and correlated positively 748 with the total denitrification rate (N_2 and N_2O fluxes combined) measured with the 15N 749 Gas-Flux method (Pearson; $r = 0.581$, $n = 25$, $p < 0.01$) following a similar trend among 750 land use types, albeit only the OS being significantly lower than the grassland and 751 woodland soils (Table 3). The AIT denitrification rates were between 3 and 5 times lower 752 than the total denitrification from the 15N Gas-Flux (Figure 4a5a) with the difference 753 being significant in woodland (t-test; $t = 3.914$, $df = 18$, $p < 0.01$) and grassland soils (t-754 test; $t = 3.521$, $df = 25$, $p < 0.01$). 755~~

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~~The total N_2O flux measured from the un-amended intact soil cores ranged between 0.15 757 and 86.6 µg N m-2 h-1 and was between 1 and 3 times lower than the total denitrification 758 rate from the C_2H_2 amended cores. There were no significant differences between bulk 759 N_2O fluxes measured with the static chambers and the un-amended intact soil cores 760 (Figure 4b5b), which indicated that total N_2O emissions were comparable between the 761 two field techniques. Consequently, estimating the denitrification product ratio from the 762~~

un-amended and C2H2 amended intact soil cores resulted in significantly higher ratios 763 compared to the 15N Gas-Flux approach (Figure 4c5c), which were on average between 764 50 and 60 % and not significantly different among land use types (Table 3). 765

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The mean CO2 production rate was similar irrespective of whether it was measured in 767 static chambers, in C2H2 amended or un-amended intact soil cores (Figure 56), indicating 768 that soil respiration (including both microbial and plant respiration) was not affected by 769 the measurement technique. 770

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4. Discussion 785
4.1. IRMS system evaluation 786

The precision of our trace gas isotope ratio mass spectrometer (TG-IRMS) for manual 787 analysis of 15N-N2 in gas samples was comparable for both R29 and R30 ratios to the 788 recently developed gas chromatograph-IRMS (GC-IRMS) systems that included a 789 combination of a copper reduction oven and a molecular sieve (Lewicka-Szczebak et al. 790 2013) or only a molecular sieve (Yang et al. 2014) for the removal of O2 from the 791 samples. This was achieved while injecting a trace amount of headspace gas sample (4 792 µL), which is less than half of what is used by Lewicka-Szczebak et al. (2013) and ten 793 times less than the required sample volume by Yang et al. (2014). Furthermore, the 794 interference at m/z 30 by NO+ ions was reduced by an order of magnitude (3.81 x 10-5) 795 compared to the value (1.6 x 10-4) reported by Lewicka-Szczebak et al. (2013). 796 Consequently, correcting the R30 ratio for the NO+ ions interference led to a CV value of 797 < 2%, which was significantly lower than the precision reported for natural abundance 798 samples in previous studies (Lewicka-Szczebak et al. 2013, Russow et al. 1996, Stevens 799 et al. 1993), thus constituting a significant improvement in m/z 30 determination in N2 gas 800 samples with low 15N enrichment. However, the correction of the R30 ratio is only useful 801 for estimating the 'true' instrument precision for m/z 30 and is not necessary for 802 calculating N2 fluxes as shown by Lewicka-Szczebak et al. (2013), unless using the 803 mathematical formulations of Spott and Stange (2007). 804

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~~The TraceGasTM Preconcentrator IRMS system used for 15N-N2O analysis displayed 806 similar precision for the determination of R45 and R46 in standard N2O gas at circa 807 ambient concentration to a similar system used by Bergsma et al.(2001), while injecting 808 only 4 mL of gas sample as opposed to 0.5 L used by Bergsma et al. (2001). When 809 expressed in delta values (d15N), the precision of our system was better than 0.05 ‰, 810 which is significantly better than the respective precisions reported in Lewicka-Szczebak 811 et al. (2013) and Yang et al. (2014), but comparable to Well et al. (1998). Therefore, the 812 improved analytical precision achieved for both 15N-N2 and 15N-N2O analyses, using 813 smaller gas sample volumes than previously reported, allowed us to quantify in situ N2 814 and N2O fluxes with low 15N enrichmenttracer addition under field conditions, which 815 was previously not possible. 816~~

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~~4.2. Field application of the 15N Gas-Flux method 818~~

~~The average 15N tracer application rate (0.04 - 0.5 kg 15N ha-1 or 0.4 - 1.2 mg 15N kg-1 dry 819 soil) across land use types was one to two orders of magnitude lower than previous 820 applications of the 15N Gas-Flux method in highly fertilised agricultural systems (Baily et 821 al. 2012, Bergsma et al. 2001, Cuhel et al. 2010, Graham et al. 2013) and in restored 822 peatland soils (Tauchnitz et al. 2015). The estimated enrichment of the total soil NO3- 823 pool was variable (2 – 40 %, Supplementary Table 2) and this wide range was due to the 824 fact that the tracer concentration was calculated based on the previous campaign's soil 825 nitrate data, which in some cases did not reflect the soil nitrate content on the day of the 826 tracer application a month later. It should be noted that the soil nitrate enrichment levels 827 reported in this study correspond to the high end of the average soil NO3- pool enrichment 828 (10 – 15 %, Supplementary Table 2) for the period April 2013 to October 2014, which is 829~~
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presented in a separate publication (Sgouridis and Ullah 2015). To our knowledge, only Kulkarni et al. (2014) have applied the 15N 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 15N-N2 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 NO3- -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 15N tracer at these low rates should not be expected to enrich the soil nitrate pool significantly, and potentially enhance the denitrification activity, in excess of the amount of nitrogen normally deposited via natural processes and common management practices.

The major assumptions of the 15N Gas-Flux method and the associated 'non-equilibrium equations' are that the denitrifying soil NO3- pool is uniformly labelled with 15N and that the N2 and N2O originate from the same denitrifying pool (Stevens and Laughlin 1998). The 15N fraction in the denitrifying pool (15XN), 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 15XN was higher than the estimated total soil NO3- pool enrichment (range: 2 - 40 %) suggesting non-homogeneous mixing of the
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added tracer (98 15N at %) with the ambient soil nitrate at natural abundance despite our effort for uniform tracer application with multiple injections across the investigated soil depth (Ruetting et al. 2011). Wu et al. (2011) have optimised the number of injections and the volume of tracer needed to achieve homogeneous labelling of a soil core (diameter 15 cm; height 20 cm) and reported that 38 injections of 4 mL volume each were necessary. We have used only 10 injections of 5- 20 mL volume (depending on the soil water content of each land use type) to minimise the disturbance of the soil matrix, particularly in the highly porous media such as peatland soils, and this was clearly sub-optimal for the homogenous labelling of the soil enclosure but probably a necessary compromise for large scale intensive measurements. We were not able to sample the soil within the chamber collars for directly estimating the $^{15}NO_3^-$ content of the soil pool due to time and budget constraints. However, in cases where destructive soil sampling was used to measure the soil nitrate pool enrichment (Kulkarni et al. 2014), the results were significantly different from the estimated enrichment due to sampling bias of the volume of soil affected by the tracer application. Non-uniform mixing of the 15N label may lead to overestimation of the 15XN and underestimation of the denitrification flux rates (Boast et al. 1988). However, under field conditions, it is unlikely under field conditionsto achieve complete mixing of the added tracer with the ambient nitrate pool; and experimental studies (Mulvaney 1988, Mulvaney and Van den Heuvel 1988) have shown that the associated error is well-constrained and that accurate measurements can be made even with a less-uniformly labelled denitrifying pool. The non-significant change of 15XN with incubation time suggested only one denitrifying pool for both N_2 and N_2O, assuming negligible N_2 production from anammox and co-denitrification (Spott and Stange 2007). Only in the case of the C-MW well-drained forest site, shown to exhibit the highest nitrification potential (Sgouridis and Ullah 2014), the slope of 15XN with time was

negative suggesting dilution of the 15N-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 15XN, but Bergsma et al. (1999) showed that temporal changes of the soil NO_3^- pool enrichment are negligible at 15N 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 15XN 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 N2-N m-2 h-1 and 0.04 - 0.21 µg N2O-N m-2 h-1) reported by Bergsma et al. 903 (2001), Kulkarni et al (2014) and Tauchnitz et al (2015), using similar field 15N tracer 904 approaches, and comparable to the minimum rates measured by a high precision 15N gas 905 flux approach in a laboratory soil incubation (Yang et al. 2014) and the gas flow soil core 906 method (8 µg N2-N m-2 h-1 and < 1 µg N2O-N m-2 h-1) by Wang et al. (2011). We have 907 managed to further lower the limit of detection for N2 and N2O fluxes due to the high 908 precision of our preparative devices coupled to the IRMS systems, but also by lowering 909 the volume to surface area ratio of our chambers from 16:1 to 8:1 (cm3/cm2) and by 910 extending the incubation time to approximately 20 hours, for the first time in a field 911 study. 912~~

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~~Most studies using 15N tracers and static chambers in highly fertilised systems typically 914 deploy their chambers between 1 and 2 hours (Baily et al. 2012, Cuhel et al. 2010, 915 Tauchnitz et al. 2015), but it has been shown that longer incubation periods (up to 24 or 916 48 hours) may be needed in case of low 15N enrichment applications in intact soil cores 917 (Morse and Bernhardt 2013) and laboratory incubations (Yang et al. 2014) for a more 918 precise and accurate detectable 15N-N2 signal. However, it should be noted that in these 919 cases where an extended incubation period was employed, the soil cores or slurries did 920 not allow the subsoil diffusion of the evolved N2 and N2O back into the soil pore 921 spacesthe soil cores or slurries were incubated in fully enclosed systems and were thus 922 not affected by potential bias from diffusion of evolved N2 and N2O to the subsoil 923 (Clough et al. 2005). The open-bottom, un-vented static chamber design used in this study 924 in combination with the extended incubation period up to 20 hours may have potentially 925 allowed some loss of the evolved N2 and N2O through downward subsoil diffusion and/or 926 reduction of gas exchanges at the soil-atmosphere interface due to positive pressure build 927~~
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~~up in the chamber headspace (Healy et al. 1996). This could partly explain the non-linear increase of the evolved N2 and N2O in the chamber headspace (Figures 2a & b) and also the decrease of the N2 flux rate with increasing incubation time (Figure 3a). The N2O 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 N2O reduction due to both subsoil diffusion and the increasing concentration of the N2O in the topsoil. However, due to the high spatial heterogeneity within each land use type, the mean N2O flux rate was not significantly different between the different incubation intervals. In other words, the non-linearity of N2O 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 N2O fluxes far exceeds the bias due to assumed linearity of fluxes.~~

~~The lack of a consistent pattern of N2 flux rate change with incubation time among the different land use types suggested a more complex temporal variability of N2 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 (15XN = 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~~ Formatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: Not HighlightFormatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: SuperscriptFormatted: Subscript

incubation, until the added water started to equilibrate with the ambient soil moisture. 953 Therefore the N2 flux rate in C-MW may be significantly overestimated after 1 hour of 954 incubation. In the grassland sites and the R-DW forest site with intermediate soil 955 moistures (Mean WFPS: R-DW = 65 ± SE 1.79 %; R-UG = 64 ± SE 1.41 %; C-IG = 60 ± 956 SE 1.45 %; R-IG = 61± SE 2.46 %) and nitrate content, the tracer injection is unlikely to 957 have significantly affected the denitrification rate when all the conditions (i.e. soil 958 moisture and substrate availability) were favourable, and therefore flux rates estimated 959 after one hour of incubation should be more reliable as long as the bias from analytical 960 uncertainty was low. In these sites denitrification rates estimated after one or 20 hours of 961 incubation were not significantly different (Figure 3a), suggesting a quasi-linear N2 962 evolution throughout the incubation period (at least in 37.5% of the sampling plots, see 963 Supplementary Table 4). However, the N2 flux rates were significantly lower after 2 964 hours of incubation, whereas the N2O flux rates were maximum at 2 hours of incubation 965 consequently leading to an increased product ratio N2O/ (N2 + N2O). This observation 966 could potentially be explained by a delay in the de novo synthesis of the N2O reductase 967 enzyme, known to have a slower expression than the preceding reduction enzymes 968 (Knowles, 1982), leading to N2O accumulation and lower N2 production after 2 hours of 969 incubation. After 20 hours incubation, the decrease in the product ratio could be 970 explained by a higher reduction rate of N2O to N2 due to probably higher N2O reductase 971 activity but also slower soil-atmosphere exchange of N2O due to the decreasing 972 concentration gradient (Healy et al. 1996). 973

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It has been shown that the N2 flux estimation with the 15N Gas Flux method is sensitive to 975 the incubation time interval and the homogeneity of the tracer distribution due to the 976 combination of several antagonistic effects such as decreasing gas diffusion gradients and 977 Formatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: Font: ItalicFormatted: SubscriptFormatted: Not HighlightFormatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: SubscriptFormatted: Superscript

~~soil moisture and substrate availability effects due to the added tracer solution. The 978 uncertainty in the estimated in situ N2 fluxes can be significantly reduced if additional 979 effort is made to increase the homogeneity of the tracer application by increasing the 980 number of injections while reducing the volume of the applied tracer (particularly in soils 981 where denitrification is limited by moisture limited soils). Moreover, allowing the 982 equilibration of the added tracer solution with the ambient soil water before gas sampling 983 commences and by closely monitoring the linear evolution of the produced gases with 984 more frequent gas sampling at shorter equal incubation intervals could help in identifying 985 the appropriate interval between tracer injection and the onset of incubation and 986 subsequent gas sampling duration tolength of incubation, thus avoiding potential over-987 estimation of denitrification in nitrate and moisture limited ecosystems and potential 988 under-estimation due to backsubsoil diffusion of evolved gases during incubation times. 989 The detailed uncertainty analysis of the N2 and N2O flux estimation presented in this 990 study complements the large scale application of the 15N Gas Flux method in the same 991 land use types between April 2013 and October 2014 for estimating annual rates of 992 denitrification and N2O emission, which is presented in Sgouridis and Ullah (2015). 993~~

~~However, we have demonstrated that the N2 flux and more importantly the N2O flux 994 increased linearly with time through the 20 hour incubation period, probably as a result of 995 a slow N2O diffusion rate due to the high water filled pore space (WFPS) (Jury et al. 996 1982) in our field sites (Mean WFPS: C-PB = 70 ± SE 3.21 %; C-UG = 66 ± SE 1.58 %; 997 R-HL = 69 ± SE 2.00 %; C-MW = 42 ± SE 0.76 %; R-DW = 65 ± SE 1.79 %; R-UG = 64 998 ± SE 1.41 %; C-IG = 60 ± SE 1.45 %; R-IG = 61± SE 2.46 %). In the case of the C-MW, 999 the N2 flux may have been underestimated due to a faster decrease in the gas 1000 concentration gradient between the soil surface and the chamber headspace as a result of 1001 higher air-filled porosity (Healy et al. 1996) and the subsequent diffusion of N2 back into 1002~~
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subsoil. In the case of the R-IG, where N2O flux was not found linear up to 20 hours incubation, some of the N2O may have been diffused into the subsoil and further reduced to N2 (Clough et al. 2005), thus leading to an underestimated N2O 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 N2 and N2O 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 N2 and N2O 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 15N signal, particularly for N2 gas, from sites exhibiting significant seasonal variability of flux rates.

The average 15N tracer application rate (0.04 - 0.5 kg 15N ha-1 or 0.4 - 1.2 mg 15N kg-1 dry soil) across land use types was one to two orders of magnitude lower than previous applications of the 15N 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 NO3- 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 15N 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 15N-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 15N 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 15N Gas-Flux method and the associated 'non-equilibrium equations' are that the denitrifying soil NO_3^- pool is uniformly labelled with 15N and that the N_2 and N_2O originate from the same denitrifying pool (Stevens and Laughlin 1998). The 15N fraction in the denitrifying pool (15XN), 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 15XN was higher than the estimated total

soil NO_3^- pool enrichment (range: 2 – 40 %) suggesting only partial mixing of the added tracer (98 15N 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 $15NO_3^-$ 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 15N label may lead to overestimation of the 15XN 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 15XN 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 15XN 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 15XN, calculated from the N2O isotope ratios, for calculating N2 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 15XN with time was negative suggesting dilution of the 15N-labelled soil NO3- pool by the oxidation of the ambient ammonium (nitrification). It is therefore possible that N2 flux rates may be overestimated in C-MW, due to the underestimation of the 15XN, but Bergsma et al. (1999) showed that temporal changes of the soil NO3- pool enrichment are negligible at 15N enrichment levels similar to ours.

The minimum detectable N2 and N2O fluxes depend on the precision of the IRMS systems, the soil NO3- 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 N2 and N2O) for calculating a 15XN 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 N2 and N2O fluxes respectively. These were significantly better than the minimum rates (175 - 900 µg N2-N m-2 h-1 and 0.04 - 0.21 µg N2O-N m-2 h-1) reported by Bergsma et al. (2001), Kulkarni et al (2014) and Tauchnitz et al (2015), using similar field 15N tracer approaches, and comparable to the minimum rates measured by a high precision 15N gas flux approach in a laboratory soil incubation (Yang et al. 2014) and the gas-flow soil core method (8 µg N2-N m-2 h-1 and < 1 µg N2O-N m-2 h-1) by Wang et al. (2011). We have managed to further lower the limit of detection for N2 and N2O 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 (cm3/cm2) 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. 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 1128
reliable 15N-N2 signal, particularly under conditions of low denitrifier activity due to 1129
seasonality of denitrification and/or inherent capacity of soils (for example organic and 1130
deciduous forest soils). However, these rates should be considered conservative since 1131
confounding issues such as subsoil diffusion and non-homogeneous labelling of the soil 1132
nitrate pool may in some cases have led to underestimations of the in situ denitrification 1133
rates. 1134

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4.3. Comparison with the AIT 1136

The total denitrification rates measured with the C2H2 amended intact soil cores followed 1137
the same trend as the total denitrification (N2 and N2O fluxes combined) from the 15N 1138
Gas-Flux measurements, while they were on average 168 times lower than the 1139
denitrification potential measured in the same land use types in anaerobic soil slurries 1140
amended with acetylene and nitrate in a previous study (Sgouridis and Ullah 2014), thus 1141
reflecting lower in situ rates. The AIT denitrification rates were between 3 and 5 times 1142
lower than the 15N Gas-Flux rates despite the fact that the AIT intact soil cores were 1143
capped at the bottom, thus not allowing any subsoil diffusion of the evolved gases due to 1144
denitrification. Therefore, the AIT rates should have been higher than the 15N Gas-Flux 1145
rates if serious underestimation was occurring due to subsoil diffusion in the open-bottom 1146
static chambers, which was not the case. Adding nitrate to the C2H2 amended cores would 1147
have been desirable for directly evaluating the priming effect of the added substrate on 1148
denitrification rates. The 15N tracer addition to the static chambers corresponded to the 1149
amounts of N naturally deposited in these land use types either via management practices 1150
and/or atmospheric deposition, thus avoiding excessive N fertilisation of the sampling 1151
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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 15N Gas-Flux methods. Previous comparisons between the AIT and the 15N 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 15N tracer approach (Yu et al. 2010) and the direct N2 flux approach (Qin et al. 2012) due to the incomplete inhibition of N2O reduction to N2 by C2H2 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 15N-NO3- 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 CO2 production rates (Aulakh et al. 1991), representing soil respiration (Felber et al. 2012), in the static chambers and the C2H2 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 15N Gas-Flux method due to the likely incomplete inhibition of N2O reduction to N2 under relatively high soil moisture contents, although the shorter incubation time (2h for the intact cores) may have limited the ability of C2H2 to fully equilibrate within soil pore spaces. Other confounding factors such as the catalytic decomposition of NO in the presence of C2H2 (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 1176 quantification method of in situ denitrification rates compared to the 15N Gas--Flux . 1177

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The estimation of the denitrification product ratio using the AIT method, from the un-1179 amended cores (N_2O only) and the C_2H_2 amended cores (N_2 + N_2O), is usually 1180 overestimated since the source of N_2O cannot be discriminated with the AIT, whilst the 1181 N_2 flux is underestimated due to the incomplete inhibition of N_2O reduction (Butterbach-1182 Bahl et al. 2013). This was confirmed in the present study for all the land use types and 1183 even the maximum denitrification product ratio after 2 hours incubation in the case of the 1184 grassland soils (23 %), was still significantly lower than the respective ratio from the AIT 1185 (50 %). Therefore, the much lower denitrification product ratio estimated from the 15N 1186 Gas-Flux measurements is significantly more reliable and the wider application of this 1187 field technique across a range of land use types can have important implications for 1188 evaluating the role of denitrification as a reactive nitrogen sink and as a source of N_2O 1189 emissions (Butterbach-Bahl et al. 2013, Kulkarni et al. 2008). 1190

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5. Conclusion 1192

The improved analytical precision for both ^{15}N-N_2 and ^{15}N-N_2O analyses was greatly 1193 improved by using smaller sample volumes than previously reported, thusallowed us 1194 allowing us to quantify in situ N_2 and N_2O fluxes with low ^{15}N enrichment tracer addition 1195 under field conditions in natural and semi-natural land use types, which was previously 1196 not possible for the first time. The estimation of N_2 fluxes was sensitive to the incubation 1197 time interval and the homogeneity of the tracer distribution due to the combination of 1198 several antagonistic effects such as decreasing gas diffusion gradients over time and soil 1199
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moisture and substrate priming effects due to the added nitrate tracer solution. The spatial 1200 variability of N2O fluxes superseded any bias associated with non-linear fluxes due to the 1201 extended incubation period. The uncertainty in the estimated N2 and N2O fluxes can be 1202 significantly reduced by increasing the homogeneity of the tracer application and by 1203 closely monitoring the linear evolution of the produced gases with more frequent gas 1204 sampling at shorter equal incubation intervals to avoid under or over estimation of 1205 denitrification. The 15N Gas Flux method was applied for the first time across a range of 1206 natural and semi-natural land use types at 15N tracer application rates mimicking current 1207 estimates of atmospheric N deposition (natural systems) or grassland fertiliser application 1208 rates and yielded analytically valid flux rates for both N2 and N2O in all the land use types. 1209 A possible limitation of the adapted 15N Gas Flux method when applied at low 15N 1210 enrichment levels is the uncertainty associated with the estimation of the soil NO3- pool 1211 enrichment and the possibility for subsoil diffusion of the evolved gases in cases of 1212 extended incubation (> 2 hr) that may result in the underestimation of denitrification rates. 1213 Comparing the 15N Gas Flux method with the AIT confirmed the drawbacks of the AIT as 1214 a reliable quantification method of in situ denitrification rates. Moreover, the AIT method 1215 overestimates overestimated the denitrification product ratio compared to the 15N Gas 1216 Flux method. The 15N Gas Flux method holds much promise as a more reliable field 1217 technique for measuring in situ denitrification rates and its wider application across a 1218 range of terrestrial ecosystems can lead to its refinement and improvement and in the long 1219 term can significantly contribute to our understanding of the role of denitrification as a 1220 reactive nitrogen sink. 1221

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Tables 1434

Table 1: Measured ratios of R29 and R30 for N2 in ambient air (n=10), ratios of R45 and 1435
R46 in standard N2O gas (0.5 ppm concentration, n=15) and 15N at% abundance calculated 1436
from the respective ratios for both gases. SD; standard deviation, CV; coefficient of variation. 1437

	R29 (N2)	R30 (N2)	R45 (N2O)	R46 (N2O)	15N at% (N2)	15N at% (N2O)
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~~

~~1438~~

~~1439~~

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Table 2: The ambient soil nitrate pool, the 15N tracer application rate, the estimated 1443
enrichment of the total soil nitrate pool, the calculated 15XN value from N2O and the slope of 1444
the 15XN change with incubation time in the three land use types. Data are means with 1445
standard errors in parentheses. 1446

Land Use Type

Ambient

NO_3^-

(kg N ha-1)

Tracer
application rate
(kg 15N ha-1)

Enrichment of
total soil NO_3^-
pool (%)

15XN (%)

15XN 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)~~

~~1447~~

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

~~15N Gas-Flux~~

~~F~~

~~P~~

~~Denitrification~~

~~19.4~~

~~< 0.001~~

~~N2O emission~~

~~31.1~~

~~< 0.001~~

~~N2O/ (N2 + N2O)~~

~~7.4~~

~~< 0.01~~

~~Total bulk N2O~~

~~19.4~~

~~< 0.001~~

~~CO2 production~~

~~19.8~~

~~< 0.001~~

~~AIT~~

~~Denitrification~~

~~12.7~~

~~< 0.001~~

~~Total bulk N2O~~

~~9.4~~

~~< 0.01~~

~~N2O/ (N2 + N2O)~~

~~0.3~~

~~> 0.05~~

~~CO2 production (un-amended cores)~~

~~11.2~~

~~< 0.001~~

~~CO2 production (C2H2 amended cores)~~

~~11.7~~

~~< 0.001~~

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~~Figures 1459~~

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~~Figure 1: Schematic of the 15N-N2 analysis system 1461~~

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Figure 2: Evolved (a) N2 and (b) N2O gas measured between 1, 2 and 20 hours incubation 1477 time points intervals using the 15N Gas Flux method in the organic soil (OS), woodland (WL) 1478 and grassland (GL) land use types. Data points are means and the error bars represent 1479 standard errors. 1480

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~~Figure 3: Mean rates of: (a) N2 flux and (b) N2O flux due to denitrification at the three 1484 incubation time intervals in the three land use types (OS; organic soils, WL; woodland and 1485 GL; grassland). Same lower case letters indicate no significant differences (p > 0.05) between 1486 incubation time intervals according to the non-parametric Kruskal-Wallis test. Error bars 1487 represent standard errors. 1488~~

~~1489~~

1490

Figure 34: Mean rates of: (a) N2 flux, (b) N2O emission due to denitrification and (c) the 1491 denitrification product ratio N2O/ (N2 + N2O) in the three land use types (OS; organic soils, 1492 WL; woodland and GL; grassland). Same lower case letters indicate no significant 1493 differences ($p > 0.05$) between land use types according to One-way ANOVA and the 1494 Games-Howell post hoc test. The sample size (n) is given in parenthesis for each land use 1495 type on the x-axis. Error bars represent standard errors. 1496

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Figure 45: (a) Mean total denitrification measured with the 15N Gas-Flux method and the 1498 AIT, (b) Mean bulk N2O emission measured in the static chambers of the 15N Gas-Flux 1499 method and in un-amended intact soil cores and (c) the denitrification product ratio N2O/ (N2 1500 + N2O) with the 15N Gas-Flux method and the AIT in the three land use types (OS; organic 1501 soils, WL; woodland and GL; grassland). Same lower case letters indicate no significant 1502 differences ($p > 0.05$) between measurement methods according to independent samples t-1503 test. The sample size (n) is given in parenthesis for each land use type and each method on 1504 the x-axis. Error bars represent standard errors. 1505

1506

Figure 56: Mean CO_2 production measured in the static chambers of the 15N Gas Flux 1507 method, in un-amended and C_2H_2 amended intact soil cores in the three land use types (OS; 1508 organic soils, WL; woodland and GL; grassland). Same lower case letters indicate no 1509 significant differences ($p > 0.05$) between measurement methods according to independent 1510 samples t test. The sample size (n) is given in parenthesis for each land use type on the x 1511 axis. Error bars represent standard errors. 1512

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