

16th November, 2015

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

We are very grateful to the two reviewers for their comprehensive comments and suggestions for the improvement of the manuscript. We have attempted to accommodate all the suggestions and amended the manuscript accordingly where possible. Due to overlap of the comments between the two reviewers, we are presenting a joint response for all received comments.

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

Major comments

1)[**Reviewer #2:** *The long enclosure (up to 20h) was used for the first time in field studies to my knowledge (previously up to 2 h, see details). Linearity check with 1, 2, 20h was not adequate due to the long interval between 2 and 20h. Previous studies (e.g. Tauchnitz et al, 2014) checked linearity by short intervals of 20 minutes. Linearity was only evaluated on the total data set, i.e. data from all sites from one system were pooled. But this check must be done for each site and sampling event. Physically linearity is extremely improbable, since concentration gradients decrease over time (e.g. Healy et al 1996). Moreover, the modelling by Healy et al. predicts that diffusion to subsoil increase with extended enclosure. This has been shown for denitrification studies (with the AIT) by Mahmood 1997. Although tests of this subsoil diffusion bias have never been published for the 15N gas flux method to my knowledge, it is evident that this bias must be very significant for enclosure periods of almost 1 day. Note that Morse et al 2013 incubated in closed vessels when accumulating > 20h. I assume subsoil diffusion is the major reason why 15N concentration did not increase significantly in many of the measurements.*

Request: - *Evaluate linearity / non-linearity of N₂ and N₂O fluxes at each site and sampling date and discuss possible bias from subsoil diffusion during extended enclosure]*

36 Response

37 In response to the reviewer's comment, we have carried out additional checks for the linearity of
38 the evolved N₂ and N₂O gases per sampling plot and sampling event, which are presented in the
39 Supplementary Information (SI) submitted with the revised manuscript (Supplementary Tables
40 4&5). This additional information is described in the results section (lines: 433-444) with reference
41 to the SI. Despite, the reviewer's expectation for significant bias of the reported fluxes due to the
42 extended enclosure period, this was not shown by the additional analysis, except for two cases,
43 which are subsequently reported in the results, and discussed in lines (559-595). We suspect that
44 subsoil diffusion may have not significantly affected our flux rates due to the relatively high water
45 filled pore space (WFPS) of our field sites (mean WFPS data per site reported in discussion: lines
46 573-576) which may have limited the downwards diffusion of gases back into the soil despite the
47 absence of a bottom barrier in our chambers. Jury et al. (1982) have shown that the wetter the soil
48 the longer it takes for steady state gas diffusion to be established and this may take several hours
49 from the start of gas production. The underestimation of flux rates due to a decreasing diffusion
50 gradient between the soil surface and the chamber headspace (as modelled by Healy et al. 1996)
51 does not constitute an issue for the N₂ gas, which is not a trace gas and is abundant in the
52 atmosphere (78%). This was the main reason why we selected an extended incubation period to
53 be able to detect a reliable ¹⁵N-N₂ signal in the N₂ rich chamber headspace. A decreasing gas
54 diffusion gradient is more likely to be observed in the case of N₂O, but only where there is
55 significant N₂O production, such as in fertilised grasslands for example (see R-IG in Supplementary
56 Table 2). However, the majority of our field sites showed a very low N₂O production rate and it is
57 unlikely that these have been affected by the gas diffusion gradient. It would have been desirable
58 to perform the linearity checks at more frequent intervals, as suggested by the reviewer, but
59 unfortunately this was not possible in the present study, where we focused more on constraining
60 the spatial variability of the denitrification fluxes, at the expense of a more detailed temporal
61 investigation (which was also the case in Tauchnitz et al. 2015). In subsequent applications of our
62 methodology we will assess the temporal variability of N₂ and N₂O gas fluxes during varying
63 incubation periods, as there seems to be a lack of conclusive results particularly for field
64 applications of the ¹⁵N Gas-Flux method.

65

66

67 *2)[Reviewer #1: The new method seems promising and the results here are certainly worthy of*
68 *publication, but there needs to be a more thorough treatment of possible fertilization and water*
69 *addition effects in the new method. The authors worked hard to minimize the amount of nitrate and*
70 *water added to the field chambers but there needs to be a more clear statement of just how much*
71 *the inorganic N pools and soil moisture content were increased by the additions. And once the extent*
72 *of the increases is clarified, there should be some comparison with the literature to see if these*
73 *increases have affected rates in previous studies.]*

74

75 *[Reviewer #2: The amount of label added: it was variable and pretty low, but this is not well justified,*
76 *since no mineral N data of sites were shown. It is thus not possible to see to which extent*
77 *denitrification was potentially enhanced by increasing nitrate. In nitrate-free soils, 1kg*

78 *NO₃-N/ha would clearly enhance denitrification.*

79 *Request: -Show mineral N and 15N label amendment for each site (in an appendix) and discuss based*
80 *on that the possible dilution and consumption of the label]*

81

82 Response

83 **In response to the above comments by both reviewers we have added ambient soil nitrate data as**
84 **well as the estimated soil nitrate pool enrichment for each land use type in Table 2 at the end of**
85 **the manuscript. Moreover, in the Supplementary Information we have added Supplementary**
86 **Table 2 that details the ¹⁵N label amendment per field site for the present study and compares**
87 **with the annual average soil nitrate pool enrichment for the period April 2013 to October 2014.**
88 **Based on these data, the range of soil nitrate enrichment was quite variable (range: 2- 40 %) and**
89 **above our annual average and this was attributed to discrepancies between the soil nitrate**
90 **content on the day of the measurement and the data used for calculating the required tracer**
91 **concentration (data from previous campaign). Our aim was to enrich the total soil nitrate pool by**
92 **no more than 10% with ¹⁵N-NO₃⁻, but clearly this was not always possible unless we were able to**
93 **measure the ambient soil nitrate pool on the day of the ¹⁵N amendment, which was logistically**
94 **impossible. To our knowledge only Kulkarni et al. (2014) have applied the ¹⁵N Gas-Flux method in**
95 **the field with soil nitrate enrichment levels lower than in our study, but in their case this resulted**
96 **in poorly detected ¹⁵N-N₂ fluxes. Even at slightly higher soil nitrate enrichment levels that we**
97 **originally aimed for, our tracer application rates corresponded to daily N atmospheric deposition**
98 **rates in the case of the organic soils and daily fertilizer application rates for the improved**
99 **grasslands. Therefore, we believe that our field denitrification rates using the ¹⁵N Gas-Flux method**
100 **reflect as close as possible ‘true’ in situ rates. (Manuscript changes: Lines: 411-416 and 597-621)**

101

102 **The range of the augmented water content was between 3 and 5 %. Detailed data from each**
103 **sampling plot are presented in Supplementary Table 1. The manuscript has been amended in lines**
104 **265-269.**

105

106 *3)[Reviewer #2: The 15N distribution was not well explained since the grid distance of injection was*
107 *not given. It is thus not possible to judge potential non-homogeneity of labeling. For this, the volume*
108 *of each injection and the distance must be reported. You might compare your pattern to Wu et al*
109 *2011 who optimized injection volume to achieve homogeneity.]*

110

111 Response

112 The information on number of injections, volume per injection and the distances of the grid have
113 been added to the methods section (Lines: 254-257). Wu et al. (2011) have optimised the number
114 of injections and the volume of tracer needed to achieve homogeneous labelling of a soil core
115 (diameter 15 cm; height 20 cm) and reported that 38 injections of 4 mL volume each were
116 necessary. We have used only 10 injections of 5- 20 mL volume (depending on the soil water
117 content of each land use type) to minimise the disturbance of the soil pore water:air matrix,
118 particularly in highly porous media such as peatland soils, and this may have affected the
119 homogeneous distribution of the tracer. This comparison has been added to the Discussion (Lines:
120 633-639).

121

122 4)[**Reviewer #2:** Another artefact from long enclosures is the decrease in $N_2O/(N_2+N_2O)$ ratio due to
123 increasing N_2O reduction as N_2O concentration increases during accumulation. This is
124 straightforward and has been repeatedly shown (unfortunately I have no reference at hand). This
125 effect is not addressed at all in this paper and might in part explain why ratios were mostly very
126 small.

127 Request: -Evaluate the change in product ratio during 1, 2, 20h sampling for each site and discuss the
128 bias of the 20h values]

129

130 Response

131 The change of the denitrification product ratio with incubation time was evaluated in each
132 sampling plot where both N_2 and N_2O fluxes were available (data shown in Supplementary Table
133 6). Generally, the product ratio increased with increasing incubation time with the exception of
134 the grassland soils, where the maximum product ratio was observed after 2 hours of incubation.
135 This was indeed an indication of some further reduction of the denitrification derived N_2O to N_2
136 during the extended closure period of up to 20 hours, even though the N_2O increased linearly
137 during 20 hours incubation (apart from the R-IG), as discussed in the response for the major
138 comment 1. This observation has been included in the Results section (Lines: 460-466). We refer to
139 this observation in the discussion as well where we make the recommendation that in soils
140 displaying high denitrification activity (e.g. improved grasslands) the incubation period should not
141 exceed 2 hours for a more accurate estimation of the $N_2O/ N_2 + N_2O$ ratio. A longer incubation is
142 warranted under conditions of low flux seasons (winter) or low flux sites such as the organic soils
143 (Lines: 681-691).

144

145

146 5)[**Reviewer #2:** AIT was used as a reference but the major bias from this method was not discussed
147 i.e. catalytic NO decomposition (Bollman & Conrad 1997, Nadeem et al 2013). Hence this method is
148 today considered to be inadequate for field quantification (e.g. Felber et

149 *al, 2013). Moreover, the C₂H₂ treated cores were sealed from the bottom thus avoiding subsoil*
150 *diffusion. If the ¹⁵N labeled cores had been sealed from the bottom, discrepancies between the*
151 *methods would certainly have been even larger than reported.*

152 *-Discuss all factors of bias of the AIT and take into account the absence of subsoil*
153 *diffusion.]*

154

155 Response

156 **Our intention was to use the AIT as an alternative field method to compare against and in essence**
157 **‘fool-proof’ our measurements with the adapted ¹⁵N Gas-Flux method that was applied for the**
158 **first time in the field. The good agreement we got between the two methods gives an additional**
159 **indication that our adapted method gives reasonable estimates of in situ denitrification. However**
160 **we agree with the comment here and are aware of the several drawbacks of the AIT as a field**
161 **quantification method and the fact that subsoil diffusion was not possible with the AIT, which also**
162 **did not receive any nitrate amendment, preclude the direct comparison of the two methods. In**
163 **response to the reviewer’s comments we have adapted the respective section of the discussion**
164 **4.3 (Lines: 697-737) to reflect several possible sources of uncertainty that may be responsible for**
165 **the discrepancies observed between the two field methods.**

166

167

168 **Minor comments**

169

170 **Reviewer #2:**

171

172 *[P 12654 (54), L 15 : check reported precision, do you mean 0.5% of 0.367 at%? This*
173 *would be d¹⁵N of 5 per mil, i.e. one to two orders worth than previous methods. L 18*
174 *give units of volume/surface ratio L18 20h accumulation time far too long, see above L*
175 *24 C₂H₂ bias not fully addressed (see above)]*

176

177 Response

178 **The coefficient of variation (CV) of 0.5 % refers to the R29 and R30 precision reported in Table 1.**
179 **The units for the chamber volume/surface are cm³:cm².**

180

181 *[P55 L9 but not only with respect to EXCESS nitrogen]*

182

183 Response

184 **The word 'excess' is deleted**

185

186 *[P56 L 18-20 AIT not adequately discussed (see above, check Bollman & Conrad, 1997*

187 *and Nadeem et al. 2013)]*

188

189 Response

190 **The effect of acetylene on the catalytic decomposition of NO has been added as a significant**
191 **drawback of the AIT for quantifying in situ denitrification rates with reference to Nadeem et al.**
192 **2013 (Lines: 105-109).**

193

194 *[P57 L1 This statement is incorrect since the ¹⁵N gas flux method is inadequate for*

195 *saturated soils (see Tauchnitz et al 2014 and references therein) where only the push-pull method is*
196 *suitable for quantification. L7 refer also to Tauchnitz et al 2014]*

197

198 Response

199 **The statement on the suitability of the ¹⁵N Gas-Flux method for saturated soils has been deleted.**
200 **The reference to the study by Tauchnitz et al (2015) in restored peatland soils has been added to**
201 **the literature review in Lines 119-122.**

202

203 *[P59 L18-21; P60 L11: Not clear what per mil means here L12 not clear what 3 mL **

204 *100 mL means]*

205

206 Response

207 **The per mil units refer to the standard deviation of $\delta^{15}\text{N}$. The clarification has been added to the**
208 **section 2.1. In L12 the mistake is a typo. It reads now three 100 mL flasks.**

209

210 *[P61 L2 Small insertion depth of 10 cm further enhances subsoil diffusion (see Healy et al, 1996) L10*
211 *the purpose of a vent in incorrectly addressed her. It is needed to allow pressure pumping, and this is*
212 *independent of cover volume. Exclusion of pressure pumping affects fluxes, please discuss. L12 did*
213 *you check temperature during 20h closure? If so, please report data L15 report number of injections*
214 *and grid dimensions*

215 *L25 since water content is among the main drivers: more detail is needed here: what*
216 *was the range of augmented water content and discuss potential effects. An increase*
217 *of 5% (g/g) is quite a lot.]*

218

219 Response

220 **According to Healy et al. (1996), inserting the chamber walls into the soil up to the depth of gas**
221 **production could minimise the error due to the distortion of the gas concentration gradient by**
222 **increasing vertical (upward) diffusion and minimising any radial diffusion. The collars were**
223 **inserted at approximately 10 cm depth, which was also the depth of the tracer injection.**
224 **Therefore, the top 10 cm of soil was considered our gas production depth and this was surrounded**
225 **by the collar walls, thus minimising radial diffusion. Deeper insertion of the collars would not have**
226 **affected subsoil diffusion downward, as the reviewer suggests, but it would rather minimised any**
227 **further radial diffusion (see Healy et al, 1996).**

228

229 **We did not use a vent tube (as suggested by Hutchinson and Mosier, 1981) in our chamber design,**
230 **which could have diluted the chamber headspace with atmospheric N₂, as part of our effort to**
231 **increase the probability of a detectable ¹⁵N-N₂ signal in the chamber headspace. The build-up of**
232 **positive pressure within the chamber's headspace, particularly during the extended 20 hours**
233 **incubation, may have potentially led to underestimations of the N₂ and N₂O fluxes and thus we**
234 **amended the manuscript to recognize this underestimation. (See manuscript amendments: Lines**
235 **248-252 and 567-570).**

236

237 **The soil temperature was not recorded inside the soil enclosure during the incubation, since we**
238 **wanted to minimise any further disturbance of the soil matrix but measured within the m² plots**
239 **assuming similar temperature inside and outside of the chamber. To avoid any over-heating of the**
240 **enclosed soils, we covered our chambers with reflective foil**

241

242 **The number of injections and grid dimensions are reported in line 255.**

243

244 **The range of the augmented water content was between 3 and 5 %. Detailed data from each**
245 **sampling plot are presented in Supplementary Table 1. The manuscript has been amended in lines**
246 **265-269.**

247

248 *[P62 L 10 capping the bottom precludes comparison with 15N gas fluxes since the soil was not*
249 *capped at the bottom in the 15N treated microplots (see comments on subsoil diffusion)]*

250

251 Response

252 **The intact soil cores used in the AIT technique were capped at both ends to make sure that cored**
253 **soil (up to 10 cm depth) is retained during incubation within the tube without falling out to avoid**
254 **any overdose of soil with C₂H₂ and to maintain similar soil pore and headspace C₂H₂ across the**
255 **sites A significant effect of subsoil diffusion was not demonstrated for the ¹⁵N Gas-Flux method in**
256 **the majority of the sampling plots (as shown in Supplementary Tables 4 &5) and this mismatch of**
257 **the two methods in terms of sealing is discussed in the major comments above. Therefore, we**
258 **believe that a comparison of denitrification rates between the two field methods cannot be**
259 **precluded on the basis of the subsoil diffusion effect**

260

261 *[P63 suggest to give also an equation for evolved N₂O]*

262

263 Response

264 **The equation for calculating the evolved N₂O is exactly the same with equation (5), where N₂**
265 **concentration is replaced by the total N₂O concentration. This is described in the manuscript in**
266 **lines 346-350, and therefore we believe that repeating the same equation for a second time would**
267 **be redundant.**

268

269 *[In section 2.3: please explain how you calculate N₂O flux from other sources.]*

270

271 Response

272 **We did not partition the sources of N₂O in this study, but rather measured total N₂O flux (from all**
273 **possible sources) to be used in equation (5) for estimating the evolved N₂O due to denitrification.**
274 **This is explained in the methods section in lines 346-350.**

275

276 *[P64 L1-5 linearity is not expected for 20h closure. Please address time course data and linearity for*
277 *each site and sampling (see above)]*

278 *P67 L 1 this analysis is not adequate. Each site and date must be checked individually (see above,*
279 *data might be shown in appendix). Please check also which values were significantly different from*
280 *background air. Data not significantly different must be excluded from linearity checks.]*

281

282 Response

283 **Please see response for major comment 1 above.**

284

285 *[P64 L22 15XN of N₂ and N₂O can be very different due to inhomogeneity of labeling and formation*
286 *of hybrid N₂ or N₂O (Spott et al 2007). Please discuss uncertainty from assuming equal 15XN of N₂*
287 *and N₂O. Did you get useful 15XN of N₂ in high flux plots? If so how 15XN of N₂ and N₂O agreed in*
288 *those cases. (data of individual sites should be given in an appendix)*

289

290 *[P71 L 6-24 in this discussion please also address that you did not measure 15XN of*

291 *N₂]*

292

293 *[P71 L27 the arguing for hybrid N fluxes should better explained. You can only check this*

294 *precisely if you have good estimates for the enrichment of NO₃ (15a_NO3). If 15XN*

295 *< 15a_NO3 then you obtain positive values for hybrid N according to Spott et al 2007.*

296 *But this might be also due to non-homogeneity. You did not measure 15a-NO3 but have*

297 *initial estimates which are lower than 15XN. So this indicates strong non-homogeneity.*

298 *This is an important observation. Would be good to show the data (15XN and calculated*

299 *15a_NO3, should be shown in appendix) and discuss more in detail.]*

300

301 Response

302 **We were able to calculate ¹⁵X_N from the N₂ isotope ratio data mostly from the woodland and**
303 **grassland plots. Data from all plots where the ¹⁵X_N could be calculated from both the N₂ and the**
304 **N₂O isotope ratio data are shown in Supplementary Table 3. When comparing the mean ¹⁵X_N from**
305 **the two data sources for each land use type, these were not significantly different, thus indicating**
306 **negligible effect from hybrid N₂ and N₂O fluxes. This comparison has been added in the results,**
307 **lines: 427-431 and the discussion for further clarification, lines: 652-657.**

308

309 *[P69 l 1-3: the lower NO_x formation is probably due to the different geometry of the ion source of the*
310 *IRMS and not due to injection volume. L10 note that true values are needed when using the*
311 *equations by Spott et al 2007 to calculate hybrid N₂ and/or N₂O L 16 but note that your precision*
312 *was not better than older data, eg Well et al 1998.]*

313

314 Response

315 **Clarifications were added in the discussion section 4.1 (Lines: 509-538) to address the above**
316 **comments by the reviewer.**

317

318 *[P70 L14 note that Morse and Bernhard incubated in closed systems which did not allow subsoil*
319 *diffusion. 20 h closure has never before been employed for 15N gas flux studies in the field, to my*
320 *knowledge. L18 this is not adequately proven because it was only tested using averages of all sites of*
321 *one system, but it needs to be shown on individual sites /dates (see above) L20 please show WFPS*
322 *data]*

323

324 Response

325 **The difference between our approach and the one described in Morse and Bernhard (2013) has**
326 **been made explicit in the Discussion (Lines: 564-567). The rest of this comment is addressed in our**
327 **response above (comment 1). The mean WFPS data per field site are presented in the Discussion**
328 **(Lines: 573-576).**

329

330 *[P72 L4 not clear to me. I agree that nitrification might dilute the 15N in NO₃ causing a decrease in*
331 *15XN. But N₂O from nitrification is another issue. You can calculate that based on the Bergsma*
332 *(2001) equations and it would be a valuable extension of your data.]*

333

334 Response

335 **A clarification has been added to the Discussion (Lines: 658-660) to address the above comment. It**
336 **now reads ‘...the slope of ¹⁵X_N with time was negative suggesting dilution of the ¹⁵N-labelled soil**
337 **NO₃⁻ pool by the oxidation of the ambient ammonium (nitrification).’ The source partitioning of**
338 **the N₂O is the subject of a separate publication and we do not think that adding this information**
339 **here is within the scope of this methodological study.**

340

341 *[P72 L 9 to 20. This discussion is too simple as it only compares ranges of values without addressing*
342 *denitrification controls. So if you want to keep this, compare soil types, mineral N level, organic C,*
343 *moisture and so on, and discuss in which cases agreement or disagreement of data was expected.]*

344

345 Response

346 **In this part of the Discussion (Lines: 665-677, revised manuscript) we are comparing our measured**
347 **in situ denitrification rates with the published literature, where similar methodological**
348 **approaches were used. There is a general agreement of our rates with the rates reported for low**
349 **¹⁵N field applications, whereas our rates are significantly lower compared to fertiliser level**
350 **applications of ¹⁵N. We believe that this part of the discussion is important as it shows that the**
351 **denitrification rates measured with our adapted method generally agree with the literature and**
352 **are not unreasonable. We do not expand our discussion to discuss the observed differences in**
353 **denitrification rates between land use types and the effect of soil variables in controlling process**
354 **rates, as this discussion would be beyond the scope of this methodological study, but instead we**
355 **make reference to the separate publication that focuses on ‘The relative magnitude and controls**
356 **of *in situ* N₂ and N₂O fluxes due to denitrification in natural and semi-natural terrestrial**
357 **ecosystems using ¹⁵N tracers’ (Sgouridis and Ullah, accepted). In response to the reviewer’s**
358 **comment we have removed the part of the discussion between P12672 L25 and P12673 L15 (Pages**
359 **and lines refer to the pdf of the manuscript published in the Biogeosciences Discussion forum).**

360

361 *[P73 L 5 This does not apply to all organic soils, i.e. to bogs, but not to fens L9 this needs clarification.*
362 *Not adequate to leave BD values out, but include them as zero fluxes or 50 % of detection limit.*
363 *Which option is advisable depends on the number of BD values. If you have only few, then 50% of*
364 *detection limit would be adequate from my view.]*

365

366 Response

367 **The comment for P73 L5 does no longer apply as this part of the discussion has been removed (see**
368 **previous comment). As for the comment for P73 L9, although this statement has also been**
369 **removed from the discussion we would like to provide a clarification. By ‘N₂ fluxes below the**
370 **detection limit’ we meant those samples that did not pass our minimum detectable concentration**
371 **filter (MDC, described in the manuscript) and therefore they were not regarded as valid samples.**
372 **As to the reason why these samples were not valid we cannot be certain as it may had to do with**
373 **the sampling procedure, or simply that the ¹⁵N-N₂ signal even over 20 hour incubation may have**
374 **been too low to be detected by our IRMS. Therefore, we chose to not use these samples as invalid,**
375 **rather than assuming a potentially false 0 flux, which would have seriously underestimated the**
376 **mean flux rate calculation.**

377

378 *[P73 L22-26 since the AIT is not quantitative this arguing is not suitable (see above)]*

379

380 Response

381 **This argument has been removed from the discussion. See also response to major comment 5.**

382

383 *[P74 L12-15 this is a weak argument since N₂O flux is by no means equal to denitrification.*

384

385 **And also reviewer #1:** *The authors correctly point out that “adding nitrate to the*

386 *C₂H₂ amended cores would have been desirable for evaluating directly the priming effect of the*
387 *added substrate on denitrification rates”, yet they did not do this. As a result, they cannot really*
388 *conclude that the AIT rates were lower due to incomplete blockage of N₂O reduction from the data*
389 *you have. The idea that “if the ¹⁵N tracer addition in the static chambers, even at such low rate (< 1*
390 *kg N/ha), were to stimulate the denitrification activity, this might have been reflected through high*
391 *bulk N₂O flux from the chamber compared to the intact cores” is not really valid, as the vast majority*
392 *of the denitrification flux went to N₂. So it would be hard to see a fertilization effect in the bulk N₂O*
393 *flux.]*

394

395 Response

396 **We agree with the comments of both reviewers and in response we amended the respective**
397 **section of the discussion as such: ‘Adding nitrate to the C₂H₂ amended cores would have been**
398 **desirable for evaluating directly the priming effect of the added substrate on denitrification rates.**
399 **Even though the ¹⁵N tracer addition to the static chambers corresponded to the amounts of N**
400 **naturally deposited in these land use types either via management practices and/or atmospheric**
401 **deposition, thus avoiding excessive N fertilisation of the sampling plots, it cannot be conclusively**
402 **argued that the same amount of applied nitrate would not have led to similar denitrification rates**
403 **between the AIT and the ¹⁵N Gas-Flux methods.’ Discussion Lines: 702-714.**

404

405 *[Fig.1: the meaning of N₂ and N₂O in the Fig. is not clear. NO is not removed in the furnace but*
406 *reduced to N₂]*

407

408 Response

409 **Figure 1 was adapted to clarify the above comment. N₂O is removed in the liquid nitrogen trap. NO**
410 **is not removed but reduced to N₂ in the furnace and finally N₂ is directed to the IRMS.**

411

412 *[Fig 2: Units: _g N/m²/h?]*

413

414 Response

415 The units in Figure 2 are $\mu\text{g N}$ as the evolved N_2 and N_2O refer to amounts of gas accumulated in
416 the chamber headspace at the different incubation times.

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437 **Application of the ^{15}N -Gas Flux method for measuring *in situ* N_2 and N_2O fluxes due to**
438 **denitrification in natural and semi-natural terrestrial ecosystems and comparison with**
439 **the acetylene inhibition technique.**

440

441 **F. Sgouridis^{1*}, ~~S. Ullah¹~~ and A. Stott² and S. Ullah¹**

442

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450

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

453

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458

459 **Abstract**

460 Soil denitrification is considered the most un-constrained process in the global N cycle due to
461 uncertain *in situ* N₂ flux measurements, particularly in natural and semi-natural terrestrial
462 ecosystems. ¹⁵N tracer approaches can provide *in situ* measurements of both N₂ and N₂O
463 simultaneously, but their use has been limited to fertilised agro-ecosystems due to the need
464 for large ¹⁵N additions in order to detect ¹⁵N₂ production against the high atmospheric N₂. For
465 ¹⁵N-N₂ analyses, we have used an 'in house' laboratory designed and manufactured N₂
466 preparation instrument which can be interfaced to any commercial continuous flow isotope
467 ratio mass spectrometer (CF-IRMS). The N₂ prep unit has gas purification steps, a copper
468 based reduction furnace, and allows the analysis of small gas injection volumes (4 µL) for
469 ¹⁵N-N₂ analysis. For the analysis of N₂O, an automated Tracegas Pre-concentrator (Isoprime
470 Ltd) coupled to an IRMS was used to measure the ¹⁵N-N₂O (4 mL gas injection volume).
471 Consequently, the coefficient of variation for the determination of isotope ratios for N₂ in air
472 and in standard N₂O (0.5 ppm) was better than 0.5 %. The ¹⁵N Gas-Flux method was adapted
473 for application in natural and semi-natural land use types (peatlands, forests and grasslands)
474 by lowering the ¹⁵N tracer application rate to 0.04 - 0.5 kg ¹⁵N ha⁻¹. For our chamber design
475 (volume/ surface = 8:1 cm³:cm²) and a-up to 20 h incubation period, the minimum detectable
476 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.
477 The N₂ flux ranged between 2.4 and 416.6 µg N m⁻² h⁻¹, and the grassland soils showed on
478 average 3 and 14 times higher denitrification rates than the woodland and organic soils
479 respectively. The N₂O flux was on average 20 to 200 times lower than the N₂ flux, while the
480 denitrification product ratio (N₂O/ N₂ + N₂O) was low, ranging between 0.03 and 13 %. Total
481 denitrification rates measured by the acetylene inhibition technique under the same field
482 conditions in the same land use types correlated ($r = 0.58$) with the denitrification rates

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483 measured under the ^{15}N Gas-Flux method but were underestimated by a factor of 4 and this
484 was partially attributed to the incomplete inhibition of N_2O reduction to N_2 under relatively
485 high soil moisture content. The results show that the ^{15}N Gas-Flux method can be used for
486 quantifying N_2 and N_2O production rates in natural terrestrial ecosystems, thus significantly
487 improving our ability to constrain ecosystem N budgets.

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503 **1. Introduction**

504 There has been a renewed interest recently in developing new or enhancing existing
505 measurement approaches for improving our ability to constrain dinitrogen (N₂) fluxes due to
506 denitrification in terrestrial ecosystems (Kulkarni et al. 2014, Lewicka-Szczebak et al. 2013,
507 Wang et al. 2011, Yang et al. 2014). Denitrification, the reduction within soils of nitrogen
508 oxides (NO₃⁻ and NO₂⁻) to NO, N₂O and ultimately N₂ gas, constitutes the most important
509 mechanism for the removal of ~~excess~~ reactive nitrogen (Nr) in terrestrial ecosystems
510 (Galloway et al. 2008, Groffman 2012). Despite its importance, denitrification is considered
511 the most un-constrained process in the global N cycle (Groffman 2012, Kulkarni et al. 2008)
512 due to uncertainties in N₂ flux estimations that are likely leading to underestimations of
513 denitrification rates at multiple scales (Butterbach-Bahl et al. 2013). Considering
514 contemporary atmospheric N deposition rates globally including UK (Dore et al. 2012,
515 Galloway et al. 2008, Payne 2014), the available Nr pool in soils may be greater than the
516 capacity of denitrification for its removal with important consequences of chronic N
517 enrichment of natural terrestrial ecosystems (Galloway et al. 2008, Limpens et al. 2003).
518 Moreover, nitrous oxide (N₂O), an obligate intermediate of denitrification, is a potent
519 greenhouse gas involved in the breakdown of stratospheric ozone (Ravishankara et al. 2009).
520 Therefore, a reliable estimation of the relative magnitude of the major denitrification end
521 products (N₂ + N₂O) in soils is crucial in evaluating the role of denitrification as an Nr sink
522 (Kulkarni et al. 2008).

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524 N₂ comprises ~78 % of the atmosphere and thus it is extremely difficult to measure small N₂
525 fluxes from soil against this high background, particularly in natural terrestrial ecosystems
526 (Groffman et al. 2006). Available methods for measuring both N₂ and N₂O are limited and

527 can be categorised into the direct flux and ¹⁵N isotope tracer methods (Kulkarni et al. 2014),
528 whilst micrometeorological approaches (Eddy covariance) are impossible in the N₂ rich
529 atmosphere (Felber et al. 2012). The gas-flow soil core method (Burgin and Groffman 2012,
530 Butterbach-Bahl et al. 2002, Scholefield et al. 1997, Wang et al. 2011) allows the direct
531 measurement of N₂ flux (without the addition of any substrate such as nitrate) from intact soil
532 cores where the soil atmosphere is replaced by a mixture of He/O₂. However, despite the high
533 precision of the technique, cores still need to be extracted from the field and conditioned over
534 lengthy periods of time for the complete removal of N₂ from the soil atmosphere. This
535 method is therefore time and resource intensive which limits its application to intensive
536 temporal and large spatial scales (Kulkarni et al. 2014). Moreover, the gas-flow soil core
537 method cannot discriminate between sources of N₂O thus overestimating the denitrification
538 product ratio (N₂O/ N₂ + N₂O) (Butterbach-Bahl et al. 2013, Morse et al. 2015). The
539 acetylene inhibition technique (AIT) is also a direct flux method that exploits the ability of
540 acetylene (C₂H₂) at high concentrations (10 % v/v) to inhibit the reduction of N₂O to N₂
541 (Tiedje et al. 1989), thus total denitrification (N₂ + N₂O) is measured in C₂H₂ amended soil
542 cores *in situ*, whilst N₂ flux is estimated indirectly by difference from un-amended soil cores.
543 Despite its simplicity and cost-effectiveness, the AIT is becoming increasingly unpopular due
544 its several limitations (~~reviewed in~~ Groffman et al. (2006), ~~of which the catalytic~~
545 ~~decomposition of NO in~~ ~~the presence of C₂H₂ under oxic or suboxic conditions in the field~~
546 ~~(Nadeem et al. 2013) that in particular,~~ precludes its use for reliable estimates of *in situ*
547 denitrification rates (Felber et al. 2012).

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549 The ¹⁵N Gas-Flux method (Mosier and Klemmedtsson 1994) has the advantage of providing *in*
550 *situ* measurements of both N₂ and N₂O simultaneously, thus allowing its application over
551 large temporal and spatial scales. It requires the addition of a ¹⁵N-labelled tracer in a soil

552 enclosure in the field which is subsequently covered by a chamber while the chamber
553 headspace is progressively enriched with $^{15}\text{N-N}_2$ and $^{15}\text{N-N}_2\text{O}$ produced by denitrification
554 (Stevens and Laughlin 1998). Assuming that both N_2 and N_2O originate from the same
555 uniformly labelled soil NO_3^- pool (Stevens and Laughlin 2001), the true denitrification
556 product ratio can be more accurately estimated as opposed to the direct flux approaches
557 (Bergsma et al. 2001). ~~The ^{15}N Gas-Flux method is suitable for both well and poorly drained~~
558 ~~soil applications and allows for broader areal coverage compared to ^{15}N tracer ‘push-pull’~~
559 ~~techniques constrained to ‘point’ measurements in fully saturated soils and sediments (Harms~~
560 ~~and Jones 2012, Sanders and Trimmer 2006, Whitmire and Hamilton 2005).~~ Field
561 applications of the ^{15}N Gas-Flux method so far have been limited to fertilised agro-
562 ecosystems (Baily et al. 2012, Cuhel et al. 2010, Graham et al. 2013) and more recently
563 restored peatland soils (Tauchnitz et al. 2015) with high ^{15}N tracer application rates (between
564 10 - 200 kg N ha^{-1}), with the exception of Kulkarni et al. (2014) who have measured
565 denitrification rates in Northern hardwood forests of the US by adding tracer amounts of ^{15}N -
566 labelled nitrate and Morse and Bernhardt (2013) who applied the same technique in intact soil
567 cores collected from mature and restored forested wetlands in North Carolina, USA. These
568 recent studies hold much promise that the ^{15}N Gas-Flux technique can be applied to a range
569 of natural and semi-natural terrestrial ecosystems allowing the quantification of the relative
570 magnitude of N_2 and N_2O fluxes due to denitrification from these under-represented
571 ecosystems.

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573 Natural and semi-natural terrestrial ecosystems in the UK (i.e. peatlands, heathlands, acid
574 grasslands, deciduous and coniferous forests), where there is no fertiliser use and the impact
575 from grazing and commercial forestry is minimal (Mills et al. 2013), along with improved
576 and unimproved grasslands (grazed and/or fertilised) constitute approximately 49 % and 85

577 % of rural land use cover in England and Wales, respectively (Morton et al. 2011). Unlike
578 arable agriculture, these land use types have been poorly investigated for their role in Nr loss
579 through denitrification.

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581 The major challenge in measuring $^{15}\text{N-N}_2$ at near natural abundance levels is the possibility
582 of interference at m/z 30 ($^{30}\text{N}_2$) due to the reaction of oxygen in the ion source with N and the
583 formation of NO^+ ions that also have m/z 30 (Stevens et al. 1993). Commonly, this issue is
584 addressed in continuous flow isotope ratio mass spectrometers (CF-IRMS) with the inclusion
585 of a copper (Cu) oven for reducing O_2 in the gas sample (Russow et al. 1996). Recently, it
586 has been suggested that the interference at m/z 30 can be further reduced by including a
587 molecular sieve column in gas chromatograph IRMS (GC-IRMS) systems to not only
588 separate N_2 and O_2 in the gas sample, but also to quantitatively remove O_2 and other trace
589 gases such as carbon monoxide (Lewicka-Szczebak et al. 2013, Yang et al. 2014). We
590 hypothesise that the precision for m/z 30 determination can be greatly improved by using a
591 custom-built preparative unit for the removal of H_2O , CO_2 , N_2O , NO^+ and CO ; a device
592 which also permits the micro scale injection of volumes of $< 5 \mu\text{L}$. These injection volumes
593 are much smaller than have previously been reported in the literature.

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595 | Studies that have ~~directly~~ compared the ^{15}N Gas-Flux method with the AIT in the field are
596 rare and have exclusively focused on highly fertilised agro-ecosystems with moderate to low
597 soil moisture contents (Aulakh et al. 1991, Mosier et al. 1986, Rolston et al. 1982). These
598 studies have measured comparable denitrification rates by both field techniques, although the
599 relatively low soil moisture contents have probably allowed greater diffusion of C_2H_2 to the
600 anaerobic microsites where denitrification occurs (Malone et al. 1998), whilst the high nitrate

601 application rates have probably favoured nitrate reduction over N₂O reduction (Dendooven
602 and Anderson 1995) resulting in high denitrification rates from the AIT. Conversely,
603 laboratory studies have shown that the AIT significantly underestimates total denitrification
604 compared to the ¹⁵N tracer approach (Yu et al. 2010) and the direct N₂ flux approach (Qin et
605 al. 2012) due to the incomplete inhibition of N₂O reduction to N₂ by C₂H₂ in wet soils (Yu et
606 al. 2010) or in soils with low nitrate content, where N₂O reduction is more energetically
607 favourable (Qin et al. 2013, Qin et al. 2014). A ~~direct~~-comparison of the ¹⁵N Gas-Flux
608 method with the AIT under *in situ* conditions across a range of natural and semi-natural
609 terrestrial ecosystems has not been attempted before. It can provide valuable insights in terms
610 of the validity and applicability of the two field techniques for measuring denitrification rates
611 across broad spatial and temporal scales.

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

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627 2. Materials and methods

628 2.1. IRMS system

629 For N₂ gas isotopic analysis we used an Isoprime isotope ratio mass spectrometer
630 (Isoprime Ltd, UK, Wythenshawe) coupled to an in house built N₂ preparative interface
631 (Figure 1). Headspace gas (4 µL) was manually injected with a gas tight syringe (SGE
632 Analytical science) into the preparative interface via an open split. Prior to its
633 introduction into the IRMS, the sample was treated as follows: a) dried by passing
634 through Mg(ClO₄)₂ (Elemental Microanalysis Ltd, Devon, UK), b) CO₂ removed with 0.7
635 - 1.2 mm Carbosorb (Elemental Microanalysis Ltd, Devon, UK), c) N₂O cryogenically
636 trapped under liquid nitrogen, and d) O₂ removed over a copper-packed reduction furnace
637 heated at 600°C. The N₂ was then directed towards the triple collectors of the isotope
638 ratio mass spectrometer where *m/z* 28, *m/z* 29 and *m/z* 30 mass ions were measured.
639 Mass/charge ratios for the *m/z* 28, *m/z* 29 and *m/z* 30 nitrogen (²⁸N₂, ²⁹N₂ and ³⁰N₂) were
640 recorded for each sample at a trap current of 300 µAmps. Instrument stability checks were
641 performed prior to each analysis by running a series of 10 reference pulses of N₂ (BOC
642 special gases) until a standard deviation of $\delta^{15}\text{N}$ better than 0.05 ‰ was achieved.
643 Additionally, 10 consecutive injections (4 µL) of atmospheric air were analysed prior to
644 the analysis of actual samples. Precision of the instrument was better than $\delta^{15}\text{N}$ 0.08 ‰ in
645 all quality control tests.

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647 Nitrous oxide was analysed using modified headspace methods described for the analysis
648 of nitrogen gas above. Headspace gas (*ca.* 4 mL) was injected into a TraceGas™
649 Preconcentrator coupled to an Isoprime™ IRMS (GV instruments Ltd, UK) whereupon
650 the sample was directed through a series of chemical traps designed to remove H₂O and
651 CO₂. The N₂O was cryogenically trapped under liquid nitrogen. The waste was flushed
652 out of the instrument. The N₂O was further cryofocused in a second liquid nitrogen trap
653 prior to being introduced onto a 25 m x 0.32 mm Poraplot Q gas chromatography column
654 (Chrompack column, Varian, Surrey, U.K). The column separated N₂O from any residual
655 CO₂, and both entered the IRMS via an open split. The retention time between the first
656 eluting CO₂ (< 2^{E-10} amplitude) and second eluting N₂O peak typically fell in the range
657 between 60 - 70 seconds to avoid isobaric interference of the CO₂ with the calculated ¹⁵N.
658 The N₂O was directed towards the triple collectors of the isotope ratio mass spectrometer
659 where *m/z* 44, *m/z* 45 and *m/z* 46 mass ions were measured and recorded. Instrument
660 stability checks were performed prior to each analysis by running a series of 10 reference
661 pulses of N₂O (BOC special gases) until a standard deviation of $\delta^{15}\text{N}$ better than 0.05
662 ‰ was achieved. Prior to each sample batch analysis, trace gas N₂O measurements were
663 made on ~~3~~ three 100 mL flasks containing atmospheric air collected from outside the
664 stable isotope laboratory. $\delta^{15}\text{N}$ precisions using the Trace gas Preconcentrator and
665 Isoprime IRMS were better than 0.3 ‰ respectively at 600 μAmp trap current.

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667 2.2. Field application of the ¹⁵N Gas-Flux and AIT techniques

668 *In situ* measurements of N₂ and N₂O were made using static chambers according to the
669 ¹⁵N Gas-Flux method (Mosier and Klemmedtsson 1994). Five plots were randomly
670 established in June 2013 in each of four study sites in the Ribble - Wyre River catchments

671 (area 1145 km²; NW England, 53°59'99" N, 2°41'79" W). The study sites were a
672 heathland (R-HL), a deciduous woodland (R-DW), an unimproved grassland (R-UG) and
673 an improved grassland (R-IG). In August 2013, four more study sites were tested in the
674 Conwy River catchment (area 345 km²; N. Wales, 52°59'82" N, 3°46'06" W) following a
675 similar sampling design. These sites were an acid grassland (C-UG), an ombrotrophic
676 peat bog (C-PB), a mixed deciduous and coniferous woodland (C-MW) and an improved
677 grassland (C-IG). Further details on the location, land management status and major soil
678 properties for all study sites can be found in Sgouridis & Ullah (2014).

679

680 In each plot a round PVC collar (basal area 0.05 m²; chamber volume 4 L) was inserted
681 into the soil at c. 10 cm depth 2 - 4 weeks before the measurement date. The collars were
682 open at the bottom to permit natural water table levels during the measurements. The
683 natural vegetation cover at the soil surface of each installed collar remained unchanged.

684 The PVC collars were fitted with a circular groove of 25 mm depth to fit in an acrylic
685 cylindrical cover (chamber) providing a gas-tight seal when filled with water (Ullah and
686 Moore 2011). The gas leak rate from the chamber was determined in the laboratory by
687 placing the sealed collar and chamber over a tray of water, injecting CH₄ (10 ppm), and
688 determining the change in CH₄ concentration within the chamber headspace over time
689 (Yang et al. 2011). The CH₄ concentration change within 24 hours was negligible with

690 the relative standard deviation (RSD) being < 5 % . We did not use a vent tube for
691 pressure equilibration, as suggested by Hutchinson and Mosier (1981), in our chamber
692 design, which could have diluted the chamber headspace with atmospheric N₂, as part of
693 our effort to increase the probability of a detectable ¹⁵N-N₂ signal in the chamber
694 headspace~~Due to the relatively small volume of the chamber's headspace there was no~~
695 ~~need for air circulation within the chamber or a vent for pressure equilibration (Mulvaney~~

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696 | ~~and Kurtz 1984).~~ Instead chambers were covered with reflective foil for minimising
697 | temperature increase within the chamber headspace during the incubation period (Ullah
698 | and Moore 2011). Labelled $\text{K}^{15}\text{NO}_3^-$ (98 at. % ^{15}N , Sigma-Aldrich) was applied in each
699 | plot via ~~multiple-ten~~ injections of equal volume through an equally-spaced grid (4 x 6 cm)
700 | using custom-made 10 cm long lumber needles attached to a plastic syringe (Ruetting et
701 | al. 2011). The ^{15}N tracer was delivered as the needle was pushed into the soil from the
702 | surface up to 10 cm depth aiming to achieve as uniform as possible labelling of the soil
703 | volume enclosed by the collar, as required by the ^{15}N gas flux method (Mosier and
704 | Klemmedtsson 1994). The volume and concentration of the labelled $\text{K}^{15}\text{NO}_3^-$ tracer solution
705 | was determined from measurements of soil nitrate and moisture content, as well as bulk
706 | density adjacent to each plot made during the installation of the collars (Morse and
707 | Bernhardt 2013). Lower application rates ($< 0.1 \text{ kg N ha}^{-1}$) were administered to natural
708 | study sites (e.g. peat bog, heathland) and higher rates ($< 1 \text{ kg N ha}^{-1}$) administered to
709 | semi-natural (e.g. unimproved and improved grasslands). The tracer solution (50 - ~~2050~~
710 | mL) was adjusted ~~between~~ within 3 and 5 % of the ambient volumetric water content (see
711 | Supplementary Table 1 for detailed data from each sampling plot). Since the volume of
712 | the added solution corresponded to a precipitation amount of $\leq 2 \text{ mm}$, the increase of the
713 | volumetric water content was considered minor (Tauchnitz et al. 2015).

714

715 | Following the ^{15}N tracer application the collars were covered with the acrylic chamber
716 | fitted with a rubber septum for gas sampling. Two sets of gas samples (20 mL each) were
717 | collected with a gas tight syringe (SGE Analytical science) through the septum of the
718 | chamber cover at $T = 1\text{h}$, $T = 2\text{h}$ and $T \approx 20\text{h}$ after the tracer injection, while a $T = 0\text{h}$
719 | sample was collected immediately after tracer injection above the plot surface before
720 | fitting the chamber cover. The gas samples were transferred into pre-evacuated ($<100 \text{ Pa}$)

721 12 mL borosilicate glass vials with butyl rubber septa (Exetainer vial; Labco Ltd., High
722 Wycombe, United Kingdom) for storage under positive pressure and were analysed
723 within 8 weeks from collection without any significant change of the gas concentration
724 (Laughlin and Stevens 2003).

725

726 Adjacent to each PVC collar in each plot, two intact soil cores (50 mm I.D., 15 cm long)
727 were extracted from 10 cm depth leaving the top 5 cm void as a headspace volume. The
728 cores were capped on both ends with the top cap fitted with a rubber septum for gas
729 sampling. One set of cores was amended with pure C₂H₂ with 5 mL injected through the
730 septum directly in the middle of the soil core before 10 % of the headspace being also
731 replaced with pure C₂H₂. The second set of cores was not amended with C₂H₂ and both
732 cores were placed back in the ground where they came from. Gas samples (5 mL) were
733 collected with a gas tight syringe (SGE Analytical science) through the septa of the cores
734 at T = 1h and T = 2h after amendment with acetylene. The gas samples were transferred
735 into pre-evacuated (<100 Pa) 3 mL borosilicate glass vials with butyl rubber septa
736 (Exetainer vial; Labco Ltd., High Wycombe, United Kingdom) for storage under positive
737 pressure.

738

739 2.3. Flux calculations

740 The ¹⁵N content of the N₂ in each 12 mL vial was determined using the IRMS system
741 described above and the ratios R29 (²⁹N₂/²⁸N₂) and R30 (³⁰N₂/²⁸N₂) were measured in
742 both enriched (T=1, 2 and 20 hours) and reference samples (T=0 hours). The inclusion of
743 air reference standards between every 10 samples indicated an upward drift for R30 over

744 time, potentially due to the formation of NO^+ in the ion source despite the inclusion of the
745 Cu reduction step (Lewicka-Szczebak et al. 2013). Subsequently, every sample batch was
746 drift corrected by fitting a linear regression through the air reference standards and
747 calculating an offset correction for both R29 and R30 (Yang et al. 2014). The minimum
748 detectable change (MDC) in R29 and R30 was defined with repeated manual analyses of
749 air reference standards (n=10) and was calculated using the following equation (Matson et
750 al. 2009):

$$751 \quad MDC = \mu_{pair\ diff} + (2\sigma_{pair\ diff}) \quad (1)$$

752 where μ is the mean difference of all possible unique pairs of air reference standards
753 (n=45) and σ is the standard deviation between sample pairs. The MDC for R29 was $7.7 \times$
754 10^{-7} and for R30 was 6.1×10^{-7} and these values were used to determine if each time step
755 sample was significantly different from ambient reference samples (T=0 hours), and if not
756 they were excluded from the flux calculations.

757

758 For calculating the total N_2 flux from a uniformly labelled soil nitrate pool when both
759 R29 and R30 are measured, the 'non-equilibrium' equations were applied as described by
760 Mulvaney (1984) for estimating first the ^{15}N fraction in the soil NO_3^- denitrifying pool
761 ($^{15}X_N$) as:

$$762 \quad ^{15}X_N = 2(\Delta R30/\Delta R29)/(1 + 2(\Delta R30/\Delta R29)) \quad (2)$$

763 where $\Delta R29$ and $\Delta R30$ is the difference between R29 and R30 respectively between
764 enriched (T=1, 2 and 20 hours) and reference samples (T=0 hours). Subsequently, the
765 $^{15}X_N$ allows the quantification of the fraction of the N_2 evolved from the ^{15}N -labelled pool

766 (d) using either the $\Delta R30$ or the $\Delta R29$:

$$767 \quad d = \frac{\Delta R30}{({}^{15}X_N)^2} \quad (3)$$

$$768 \quad d = \frac{\Delta R29}{2({}^{15}X_N)(1-{}^{15}X_N)^2} \quad (4)$$

769

770 Using d and the concentration of $[N_2]$ ($\mu\text{g N}$) in the chamber headspace, the evolved N_2
771 from the soil pool was calculated:

$$772 \quad \text{Evolved } N_2 = d[N_2]/(1 - d) \quad (5)$$

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

777

778 The ^{15}N content of the $N_2\text{O}$ in the same 12 mL vials as well as the ratios $R45$ ($^{45}\text{N}_2\text{O}$
779 $/^{44}\text{N}_2\text{O}$) and $R46$ ($^{46}\text{N}_2\text{O} /^{44}\text{N}_2\text{O}$) were measured in both enriched (T=1, 2 and 20 hours)
780 and reference samples (T=0 hours). The application of the 'non-equilibrium' equations to
781 $N_2\text{O}$ is analogous to N_2 after correcting for the naturally occurring oxygen isotopes
782 (Bergsma et al. 2001). Therefore, the ratios $R45$ and $R46$ were converted to ratios of $R29$
783 and $R30$ respectively by applying the following equations:

$$784 \quad R29 = R45 - R17 \quad (6)$$

$$785 \quad R30 = (R46 - (R29R17)) - R18 \quad (7)$$

786 where for $R17$ ($^{17}\text{O}/^{16}\text{O}$) the value 0.000373 was used and for $R18$ ($^{18}\text{O}/^{16}\text{O}$) the value
787 0.0020052 was used (Bergsma et al. 2001). There was no significant instrumental drift for
788 the ratios $R45$ and $R46$ over time. The MDC was defined, for the converted $R29$ and $R30$,
789 with repeated automatic analyses of 0.5 ppm N_2O standards ($n=15$) as 3.4×10^{-5} and $2.9 \times$
790 10^{-5} respectively. The second set of gas samples collected at the same time in the field
791 were analysed for ~~bulk-total~~ N_2O on a GC- μECD (7890A GC Agilent Technologies Ltd.,
792 Cheshire, UK) and the concentration of $[\text{N}_2\text{O}]$ ($\mu\text{g N}$) was used in Eq. (5) to calculate the
793 N_2O flux due to denitrification of the mixture of the ^{15}N -labelled tracer and the soil N and
794 expressed in $\mu\text{g N-N}_2\text{O m}^{-2} \text{ h}^{-1}$. Assuming that the N_2O originates from the same
795 uniformly labelled pool as N_2 , the $^{15}\text{X}_\text{N}$ from N_2O was used to estimate d for N_2 using
796 either $R30$ (Eq. 3) or $R29$ (Eq. 4), thus lowering the limit of detection for N_2 (Stevens and
797 Laughlin 2001) and allowing measurement of N_2 gas flux from natural terrestrial
798 ecosystems at low ^{15}N -tracer application rates.

799

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

806

807 2.4. Statistical analysis

808 Using factor analysis on selected soil physico-chemical properties, the samples from the 8
809 field sites were ordinated in three broad land use types: organic soils (C-PB, C-UG, R-
810 HL); forest soils (C-MW, R-DW) and grassland soils (C-IG, R-UG, R-IG) according to
811 Sgouridis and Ullah (2014). All subsequent statistical analyses were performed on the
812 broad land use types rather than individual field sites. The data were analysed for
813 normality and homogeneity of variance with the Kolmogorov-Smirnov test and the
814 Levene statistic respectively and logarithmic transformations were applied as necessary.
815 One-Way ANOVA combined with the Hochberg's GT2 *post hoc* test for unequal sample
816 sizes or the Games-Howell *post hoc* test for unequal variances was performed for
817 comparing the variance of the means between land use types for all gas fluxes. Pearson
818 correlation was used between log-transformed flux rates. Comparisons between the ¹⁵N
819 Gas-Flux and AIT techniques were made with independent samples *t*-test. All statistical
820 analyses were performed using SPSS® 21.0 for Windows (IBM Corp., 2012, Armonk,
821 NY).

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839 3. Results

840 3.1. IRMS system evaluation

841 The precision of the IRMS systems was evaluated using repeated analyses of ambient air
842 samples for N₂ (n=10) injected manually in one batch and repeated analyses of N₂O gas
843 standard at natural abundance and 0.5 ppm concentration (n=15) using automated
844 injections. The mean measured ratios of R29 and R30 for N₂ and of R45 and R46 for N₂O
845 are shown in Table 1. Measurement precision was defined as the coefficient of variation
846 (%) and it was lower for R29 compared to R30 and lower for R45 compared to R46, but
847 still less than 0.5 % for all four measured ratios. We estimated the ¹⁵N atom% abundance
848 for both gases as per Yang et al. (2014) and the precision was less than 0.01% for N₂ in
849 air and 0.26 % for standard N₂O at natural abundance. The mean measured R30 (5.16 x
850 10⁻⁵) was higher than the theoretical value of 1.35 x 10⁻⁵ for N₂ in ambient air suggesting
851 some interference at m/z 30 potentially due to the formation of NO⁺ ions in the ion source

852 of the mass spectrometer despite the inclusion of the Cu reduction oven. The contribution
853 of NO^+ ions (R30 measured - R30 theoretical) was 3.81×10^{-5} , whilst the ratio of R30
854 theoretical/ R30 measured was 0.26. Correcting the R30 ratio for the contribution of NO^+
855 ions results in a lower 'true' precision for the R30 (CV = 1.67 %).

856

857 3.2. Field application of the ^{15}N Gas-Flux method

858 The ^{15}N tracer application rate was variable between land use types and ranged between
859 0.03 and $1 \text{ kg } ^{15}\text{N ha}^{-1}$ or between 0.1 and 2.2 $\text{mg } ^{15}\text{N kg}^{-1}$ dry soil and while it was lower
860 in the case of the organic soils and higher for the woodland and grassland soils (Table 2).

861 Based on the soil nitrate content on the day of the tracer amendments (Table 2), the
862 estimated enrichment of the total soil nitrate pool was on average between 13 and 25 %
863 (detailed data on the ^{15}N tracer application per field site are shown in Supplementary
864 Table 2).

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866 The ^{15}N fraction in the denitrifying pool ($^{15}\text{X}_\text{N}$), as calculated from the measured isotopic
867 ratios of the N_2O after 1 hour of incubation using Eq. (2), ranged between 65 and 93 ^{15}N
868 at%. The average change of the $^{15}\text{X}_\text{N}$ with incubation time, indicated by the slope shown
869 in Table 2, was not different from 0 in case of the organic (t-test; $t = 0.520$, $df = 18$, $p >$
870 0.05) and grassland soils (t-test; $t = 0.047$, $df = 28$, $p > 0.05$), whilst it was significantly
871 below 0 for the woodland soils (t-test; $t = 2.917$, $df = 18$, $p < 0.05$). Separating the
872 woodland soils to C-~~WL-MW~~ and R-~~DWWL~~ sites, only the former displayed a significant
873 negative slope of $^{15}\text{X}_\text{N}$ with incubation time (t-test; $t = 3.306$, $df = 8$, $p < 0.05$), suggesting
874 ~~that~~ N_2O may be deriving production from a second nitrate pool, possibly nitrate produced

875 from the oxidation of NH_4^+ via nitrification, in the C-WLMW. ~~In the cases where the~~
876 ~~$^{15}\text{X}_\text{N}$ could be calculated from the N_2 isotope ratio data (woodland and grassland soils;~~
877 ~~data shown in Supplementary Table 3), this was not significantly different from their~~
878 ~~respective $^{15}\text{X}_\text{N}$ calculated from the N_2O isotope ratio data (t-test; $t_{\text{WL}} = 0.929$, $df = 12$, p~~
879 ~~> 0.05 ; $t_{\text{GL}} = 1.511$, $df = 20$, $p > 0.05$).~~

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881 ~~The linearity of the evolved N_2 and N_2O fluxes in the chamber headspace between 1 and~~
882 ~~20 hours of incubation time was evaluated in each sampling plot when all three time steps~~
883 ~~were above the MDC values (data presented in Supplementary Tables 4 & 5). With~~
884 ~~respect to the N_2 flux, significant deviation from linearity was observed only in C-MW~~
885 ~~(mean $r^2 = 0.59$, $n = 5$), whilst in C-PB, C-UG, R-HL and R-IG the per site analysis was~~
886 ~~not possible due to missing flux data between time steps. When the data were pooled per~~
887 ~~land use type (Figure 2a), the linear increase in the evolved N_2 . ~~The evolved N_2 in the~~~~
888 ~~chamber headspace increased linearly from 1 to 20 hours of incubation in all three land~~
889 ~~use types (Figure 2a). ~~The increase~~ was statistically significant after 20 hours incubation~~
890 ~~in GL (ANOVA; $F = 19.8$, $p < 0.01$), whilst due to the high variability among plots,~~
891 ~~shown by the large error bars at 20 hours incubation in Figure 2a, it was not significant~~
892 ~~for the OS and WL soils. ~~Regarding the N_2O flux, this was found to increase linearly with~~~~
893 ~~time in all the field sites (Supplementary Table 5), with the exception of the R-IG (mean~~
894 ~~$r^2 = 0.49$, $n = 4$). When data were pooled per land use type (Figure 2b), ~~Similarly, the~~~~
895 ~~evolved N_2O also increased linearly between incubation time points (Figure 2b), and the~~
896 ~~amount of N_2O accumulated after 20 hours was significantly higher than in the previous~~
897 ~~time points for all land use types (ANOVA; $F_{\text{OS}} = 4.6$, $F_{\text{WL}} = 5.1$, $F_{\text{GL}} = 14.7$, $p < 0.05$).~~
898 Therefore, N_2 and N_2O flux rates were estimated using linear regression (when $r^2 > 0.95$)

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899 between 1 and 20 hours incubation using only those time points that were above the MDC
900 values estimated for each gas.

901

902 The N₂ flux ranged between 2.4 and 416.6 µg N m⁻² h⁻¹ and was significantly different
903 among land use types (Table 3) with the grassland soils showing on average 3 and 14
904 times higher denitrification rates than the woodland and organic soils respectively (Figure
905 3a). A similar pattern was observed for the N₂O flux due to denitrification (range: 0.003 -
906 20.8 µg N m⁻² h⁻¹) with the grassland soils emitting on average 14 and 120 times more
907 N₂O than the woodland and organic soils respectively (Figure 3b), whilst the N₂O flux
908 was on average 20 to 200 times lower than the N₂ flux among land use types.
909 Consequently, the denitrification product ratio (N₂O/ N₂ + N₂O) was low, ranging
910 between 0.03 and 13 % and was highest in the GL and similar between the WL and OS
911 (Figure 3c). The change of the denitrification product ratio with incubation time was
912 evaluated in each sampling plot where both N₂ and N₂O fluxes were available (data
913 shown in Supplementary Table 6). Generally, the product ratio increased with increasing
914 incubation time with the exception of the grassland soils, where the maximum product
915 ratio was observed after 2 hours of incubation (ANOVA; $F = 6.11, p < 0.05$). This was an
916 indication of some ~~further~~ reduction of the denitrification derived N₂O to N₂ during the
917 extended closure period (up to 20 hours).

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3.3. Comparison with the AIT

921 The total denitrification rate measured from the C₂H₂ amended intact soil cores in the
922 same land use types ranged between 0.5 and 325.2 µg N m⁻² h⁻¹ and correlated positively
923 with the total denitrification rate (N₂ and N₂O fluxes combined) measured with the ¹⁵N
924 Gas-Flux method (Pearson; $r = 0.581$, $n = 25$, $p < 0.01$) following a similar trend among
925 land use types, albeit only the OS being significantly lower than the grassland and
926 woodland soils (Table 3). The AIT denitrification rates were between 3 and 5 times lower
927 than the total denitrification from the ¹⁵N Gas-Flux (Figure 4a) with the difference being
928 significant in woodland (t-test; $t = 3.914$, $df = 18$, $p < 0.01$) and grassland soils (t-test; $t =$
929 3.521 , $df = 25$, $p < 0.01$).

930

931 The ~~bulk-total~~ N₂O flux measured from the un-amended intact soil cores ranged between
932 0.15 and 86.6 µg N m⁻² h⁻¹ and was between 1 and 3 times lower than the total
933 denitrification rate from the C₂H₂ amended cores. There were no significant differences
934 between bulk N₂O fluxes measured with the static chambers and the un-amended intact
935 soil cores (Figure 4b), which indicated that total N₂O emissions were comparable
936 between the two field techniques. Consequently, estimating the denitrification product
937 ratio from the un-amended and C₂H₂ amended intact soil cores resulted in significantly
938 higher ratios compared to the ¹⁵N Gas-Flux approach (Figure 4c), which were on average
939 between 50 and 60 % and not significantly different among land use types (Table 3).

940

941 The mean CO₂ production rate was similar irrespective of whether it was measured in
942 static chambers, in C₂H₂ amended or un-amended intact soil cores (Figure 5), indicating
943 that soil respiration (including both microbial and plant respiration) was not affected by
944 the measurement technique.

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963 **4. Discussion**

964 4.1. IRMS system evaluation

965 The precision of our trace gas isotope ratio mass spectrometer (TG-IRMS) for manual
966 analysis of $^{15}\text{N-N}_2$ in gas samples was comparable for both R29 and R30 ratios to the
967 recently developed gas chromatograph-IRMS (GC-IRMS) systems that included a
968 combination of a copper reduction oven and a molecular sieve (Lewicka-Szczebak et al.
969 2013) or only a molecular sieve (Yang et al. 2014) for the removal of O_2 from the
970 samples. ~~In fact, injecting only~~This was achieved while injecting a trace amount of
971 headspace gas sample (4 μL), which is less than half of what is used by Lewicka-
972 Szczebak et al. (2013) and ten times less than the required sample volume by Yang et al.
973 (2014). ~~we have reduced~~Furthermore, the interference at m/z 30 by NO^+ ions was
974 reduced by an order of magnitude (3.81×10^{-5}) compared to the value (1.6×10^{-4})
975 reported by Lewicka-Szczebak et al. (2013). Consequently, correcting the R30 ratio for
976 the NO^+ ions interference led to a CV value of $< 2\%$, which was significantly lower than
977 the precision reported for natural abundance samples in previous studies (Lewicka-
978 Szczebak et al. 2013, Russow et al. 1996, Stevens et al. 1993), thus constituting a
979 significant improvement in m/z 30 determination in N_2 gas samples with low ^{15}N
980 enrichment. However, the correction of the R30 ratio is only useful for estimating the
981 ‘true’ instrument precision for m/z 30 and is not necessary for calculating N_2 fluxes as
982 shown by Lewicka-Szczebak et al. (2013). unless using the mathematical formulations of
983 Spott and Stange (2007).

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985 The TraceGasTM Preconcentrator IRMS system used for $^{15}\text{N-N}_2\text{O}$ analysis displayed
986 similar precision for the determination of R45 and R46 in standard N_2O gas at circa
987 ambient concentration to a similar system used by Bergsma et al.(2001), while injecting

988 only 4 mL of gas sample as opposed to 0.5 L used by Bergsma et al. (2001). When
989 expressed in delta values ($\delta^{15}\text{N}$), the precision of our system was better than 0.05 ‰,
990 which is significantly better than the respective precisions reported in Lewicka-Szczebak
991 et al. (2013) and Yang et al. (2014), but comparable to Well et al. (1998). Therefore, the
992 improved analytical precision achieved for both $^{15}\text{N-N}_2$ and $^{15}\text{N-N}_2\text{O}$ analyses using
993 smaller sample volumes than previously reported, allowed us to quantify *in situ* N_2 and
994 N_2O fluxes with low ^{15}N enrichment under field conditions, which was previously not
995 possible.

996

997 4.2. Field application of the ^{15}N Gas-Flux method

998 The minimum detectable N_2 and N_2O fluxes depend on the precision of the IRMS
999 systems, the soil NO_3^- pool enrichment and the incubation parameters, such as the
1000 dimensions of the static chamber and the incubation time (Bergsma et al. 2001, Stevens
1001 and Laughlin 2001). For our chamber design, an incubation time of up to 20 hours, and
1002 using the estimated MDC values (for both N_2 and N_2O) for calculating a $^{15}\text{X}_\text{N}$ value of
1003 0.6, the minimum detectable flux rates were $4 \mu\text{g N m}^{-2} \text{h}^{-1}$ and $0.2 \text{ ng N m}^{-2} \text{h}^{-1}$ for the N_2
1004 and N_2O fluxes respectively. These were significantly better than the minimum rates (175
1005 $- 900 \mu\text{g N}_2\text{-N m}^{-2} \text{h}^{-1}$ and $0.04 - 0.21 \mu\text{g N}_2\text{O-N m}^{-2} \text{h}^{-1}$) reported by Bergsma et al.
1006 (2001), ~~and~~ Kulkarni et al (2014) and Tauchnitz et al (2015), using similar field ^{15}N
1007 tracer approaches, and comparable to the minimum rates measured by a high precision
1008 ^{15}N gas flux approach in a laboratory soil incubation (Yang et al. 2014) and the gas-flow
1009 soil core method ($8 \mu\text{g N}_2\text{-N m}^{-2} \text{h}^{-1}$ and $< 1 \mu\text{g N}_2\text{O-N m}^{-2} \text{h}^{-1}$) by Wang et al. (2011). We
1010 have managed to further lower the limit of detection for N_2 and N_2O fluxes due to the
1011 high precision of our preparative devices coupled to the IRMS systems, but also by

1012 lowering the volume to surface area ratio of our chambers from 16:1 to 8:1 (cm^3/cm^2) and
1013 by extending the incubation time to approximately 20 hours, for the first time in a field
1014 study.

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1016 Most studies using ^{15}N tracers and static chambers in highly fertilised systems typically
1017 deploy their chambers between 1 and 2 hours (Baily et al. 2012, Cuhel et al. 2010,
1018 Tauchnitz et al. 2015), but it has been shown that longer incubation periods (up to 24 or
1019 48 hours) may be needed in case of low ^{15}N enrichment applications in intact soil cores
1020 (Morse and Bernhardt 2013) and laboratory incubations (Yang et al. 2014) for a more
1021 precise and accurate detectable ^{15}N - N_2 signal. However, it should be noted that in these
1022 cases where an extended incubation period was employed, the soil cores or slurries did
1023 not allow the subsoil diffusion -of the evolved N_2 and N_2O back into the soil pore spaces
1024 (Clough et al. 2005). The open-bottom, un-vented static chamber design used in this study
1025 may have allowed some loss of the evolved and/or produced N_2 and N_2O through
1026 downward subsoil diffusion and/or reduction of gas mass-exchanges at the soil-
1027 atmosphere interfaceflow due to positive pressure build up in the chamber headspaces.
1028 However, wWe have demonstrated that the N_2 flux and more importantly the N_2O flux
1029 increased linearly with time through the 20 hour incubation period, probably as a result of
1030 a slow N_2O diffusion rate due to the high water filled pore space (WFPS) (Jury et al.
1031 1982) in our ~~land use types~~ field sites (Mean WFPS: C-PB = $70 \pm \text{SE } 3.21 \%$; C-UG = 66
1032 $\pm \text{SE } 1.58 \%$; R-HL = $69 \pm \text{SE } 2.00 \%$; C-MW = $42 \pm \text{SE } 0.76 \%$; R-DW = $65 \pm \text{SE } 1.79$
1033 $\%$; R-UG = $64 \pm \text{SE } 1.41 \%$; C-IG = $60 \pm \text{SE } 1.45 \%$; R-IG = $61 \pm \text{SE } 2.46 \%$ ~~range: 60-~~
1034 ~~70 %~~, apart from the C-WL site with an average WFPS of 42 %). In the case of the C-
1035 MW, the N_2 flux may have been underestimated due to a faster decrease in the gas
1036 concentration gradient between the soil surface and the chamber headspace as a result of

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1037 higher air-filled porosity (Healy et al. 1996) and the subsequent diffusion of N₂ ~~in the back~~
1038 ~~into~~ subsoil. In the case of the R-IG, where N₂O flux was not found linear up to 20 hours
1039 incubation, some of the N₂O may have been diffused into the subsoil and further reduced
1040 to N₂ (Clough et al. 2005), thus leading to an underestimated N₂O flux rate. In this study,
1041 we have chosen to report flux rates based on linear regression up to 20 hours incubation
1042 period (where available), for comparison purposes between land use types exhibiting
1043 marked differences in potential denitrifier activity (Sgouridis and Ullah, 2014). It has been
1044 shown that a linear flux model is less sensitive to noisy datasets hovering close to the
1045 limit of detection (particularly the OS land use type in our case), in spite of the even if
1046 the possibility of underestimation of true fluxes may be somehow underestimated (Levy
1047 et al. 2011). However, when our objective was to estimate annual in situ flux rates of N₂
1048 and N₂O due to denitrification from natural and semi-natural land use types between
1049 April 2013 and October 2014 (Sgouridis and Ullah ~~in review~~ 2015), the flux rate
1050 estimation was based on the maximum evolved N₂ and N₂O rate at any valid (above the
1051 MDC) time step, thus reporting maximum flux rates per land use type to possibly avoid
1052 the risk of underestimation. Therefore, ~~based on results so far,~~ we suggest using varying
1053 incubation times under field conditions to capture a more reliable ¹⁵N signals,
1054 ~~particularly~~ particularly of for N₂ gas, from sites exhibiting low and/or greater significant
1055 seasonal variability of flux rates variability, (e.g. OS and DW in our sites), and were
1056 therefore confident in estimating N₂ and N₂O flux rates by linear regression across the
1057 incubation period.
1058 Moreover, an extended incubation period with closed chambers aggregates any diurnal
1059 fluctuations of denitrification activity (Aulakh et al. 1991) providing a more accurate
1060 daily flux rate.

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1062 The average ^{15}N tracer application rate ($0.04 - 0.5 \text{ kg } ^{15}\text{N ha}^{-1}$ or $0.4 - 1.2 \text{ mg } ^{15}\text{N kg}^{-1}$ dry
1063 soil) across land use types was one to two orders of magnitude lower than previous
1064 applications of the ^{15}N Gas-Flux method in highly fertilised agricultural systems (Baily et
1065 al. 2012, Bergsma et al. 2001, Cuhel et al. 2010, Graham et al. 2013) and in restored
1066 peatland soils (Tauchnitz et al. 2015). The estimated enrichment of the total soil NO_3^-
1067 pool was variable (2 – 40 %, Supplementary Table 2) and this wide range was due to the
1068 fact that the tracer concentration was calculated based on the previous campaign's soil
1069 nitrate data, which in some cases did not reflect the soil nitrate content on the day of the
1070 tracer application a month later. -It should be noted that the soil nitrate enrichment levels
1071 reported in this study correspond to the high end of the average soil NO_3^- pool enrichment
1072 (10 – 15 %, Supplementary Table 2) for the period April 2013 to October 2014, which is
1073 presented in a separate publication (Sgouridis and Ullah 2015)2015. To our knowledge,
1074 only Kulkarni et al. (2014) have applied the ^{15}N Gas-Flux method in the field with soil
1075 nitrate enrichment levels (5 %) lower than in our study, but this had as a consequence,
1076 poorly detected $^{15}\text{N-N}_2$ fluxes. Nevertheless, fFor the organic soils, the average tracer
1077 application rate ~~reflected~~ corresponded to the current estimates of daily atmospheric N
1078 deposition ($0.05 \text{ kg N ha}^{-1} \text{ d}^{-1}$) in the UK ($\sim 15 - 20 \text{ kg N ha}^{-1} \text{ y}^{-1}$) (Dore et al. 2012, Payne
1079 2014), whilst for the grassland soils the tracer application mimicked a daily fertiliser
1080 application rate of $0.5 \text{ kg N ha}^{-1} \text{ d}^{-1}$. Due to the inclusion of the N-rich C-~~WL-MW~~ site in
1081 the woodland soils, tracer application rates were higher than the daily atmospheric N
1082 deposition rates, thus also ~~to~~ reflecting internal N cycling processes (e.g. nitrification) as
1083 an additional source of nitrate in these well-drained forest soils. Therefore, the application
1084 of the ^{15}N tracer at these low rates should not be expected to enrich the soil nitrate pool,
1085 and potentially enhance the denitrification activity, in excess of the amount of nitrogen
1086 normally deposited via natural processes and common management practices.

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1088 The major assumptions of the ^{15}N Gas-Flux method and the associated “non-equilibrium
1089 equations” are that the denitrifying soil NO_3^- pool is uniformly labelled with ^{15}N and that
1090 the N_2 and N_2O originate from the same denitrifying pool (Stevens and Laughlin 1998).
1091 The ^{15}N fraction in the denitrifying pool ($^{15}\text{X}_\text{N}$), calculated non-destructively from the
1092 measured isotope ratios, ranged between 65 and 93 % and was well above the 10 %
1093 threshold for the correct application of the “non-equilibrium equations” (Lewicka-
1094 Szczebak et al. 2013). However, the calculated $^{15}\text{X}_\text{N}$ was higher than the estimated total
1095 soil NO_3^- pool enrichment (range: 13-2 - 40 %) suggesting only partial mixing of the
1096 added tracer (98 ^{15}N at %) with the ambient soil nitrate at natural abundance despite the
1097 elaborate effort for uniform tracer application with multiple injections across 10 cm soil
1098 depth (Ruetting et al. 2011). Wu et al. (2011) have optimised the number of injections and
1099 the volume of tracer needed to achieve homogeneous labelling of a soil core (diameter 15
1100 cm; height 20 cm) and reported that 38 injections of 4 mL volume each were necessary.
1101 We have used only 10 injections of 5- 20 mL volume (depending on the soil water
1102 content of each land use type) to minimise the disturbance of the soil matrix, particularly
1103 in highly porous media such as peatland soils, and this may have affected the
1104 homogeneous distribution of the tracer. We ~~did not~~ were not able to sample the soil within
1105 the chamber collars for directly estimating the $^{15}\text{NO}_3^-$ content of the soil pool due to time
1106 and budget constraints. However, in cases where destructive soil sampling was used to
1107 measure the soil nitrate pool enrichment (Kulkarni et al. 2014), the results were
1108 significantly different from the estimated enrichment due to sampling bias of the volume
1109 of soil affected by the tracer application. Non-uniform mixing of the ^{15}N label may lead to
1110 overestimation of the $^{15}\text{X}_\text{N}$ and underestimation of the denitrification flux rates (Boast et
1111 al. 1988). However, it is unlikely under field conditions to achieve complete mixing of

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1112 the added tracer with the ambient nitrate; and experimental studies (Mulvaney 1988,
1113 Mulvaney and Van den Heuvel 1988) have shown that the error is well-constrained and
1114 that accurate measurements can be made even with a less-uniformly labelled denitrifying
1115 pool. The non-significant change of $^{15}\text{X}_\text{N}$ with incubation time suggested only one
1116 denitrifying pool for both N_2 and N_2O , assuming negligible N_2 production from anammox
1117 and co-denitrification (Spott and Stange 2007). ~~Moreover, the similar $^{15}\text{X}_\text{N}$ values~~
1118 ~~obtained from both the N_2 and the N_2O isotope ratio data for the woodland and grassland~~
1119 ~~soils (Supplementary Table 3), was an additional indication that the effect of hybrid N_2~~
1120 ~~fluxes was negligible and,~~ thus ~~permitting the use of the~~ it was appropriate to use the $^{15}\text{X}_\text{N}$,
1121 calculated from the N_2O isotope ratios, for calculating N_2 flux rates using the more
1122 reliable R30 measurements (Stevens and Laughlin 2001). Only in the case of the C-~~WL~~
1123 ~~MW~~ well-drained forest site, shown to exhibit the highest nitrification potential
1124 (Sgouridis and Ullah 2014), the slope of $^{15}\text{X}_\text{N}$ with time was negative suggesting ~~a second~~
1125 ~~co-occurring nitrifying pool for N_2O production~~ dilution of the ^{15}N -labelled soil NO_3^- pool
1126 by the oxidation of the ambient ammonium (nitrification). It is therefore possible that N_2
1127 flux rates may be overestimated in C-~~WLMW~~, due to the underestimation of the $^{15}\text{X}_\text{N}$
1128 ~~from the N_2O data~~, but Bergsma et al. (1999) showed that temporal changes of the soil
1129 NO_3^- pool enrichment are negligible at ^{15}N enrichment levels similar to ours.

1130

1131 We were able to measure appreciable *in situ* fluxes of both N_2 and N_2O due to
1132 denitrification in all three land use types. Our N_2 fluxes from woodland soils compare
1133 well with the rates reported in the literature for restored forested wetlands in North
1134 America (Morse and Bernhardt 2013) and with the rates from northern hardwood forests
1135 in US (Kulkarni et al. 2014), using ^{15}N tracers at similar or lower application rates to
1136 ours. Our results are also comparable to the rates reported from central European forests,

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1137 under similar atmospheric N deposition rates, using the gas-flow soil core method
1138 (Butterbach-Bahl et al. 2002). For the grassland soils, the N₂ fluxes measured in the
1139 present study were significantly lower than previous applications of the ¹⁵N Gas-Flux
1140 method at high fertiliser application rates (Baily et al. 2012, Cuhel et al. 2010, Graham et
1141 al. 2013), whilst for the organic soils our rates were significantly lower than the ones
1142 reported by Tauchnitz et al. (2015) since their ¹⁵N tracer application rate (30 kg N ha⁻¹)
1143 was 300 times higher than ours. a comparison with the literature was not possible due to
1144 the lack of experimental data on N₂ fluxes. The N₂O fluxes were up to 200 times lower
1145 than the N₂ fluxes leading to low denitrification product ratios in all land use types, a
1146 result which is in line with the N₂O yields reported from ¹⁵N tracer studies in forest
1147 (Kulkarni et al. 2014, Morse and Bernhardt 2013) and grassland soils (Baily et al. 2012,
1148 Bergsma et al. 2001). It is likely that the denitrification product ratio in the grassland soils
1149 has been underestimated due to the extended incubation period (up to 20 hours), during
1150 which some of the denitrification derived N₂O may have diffused back into the soil and
1151 was further reduced to N₂. Therefore, we would recommend that in soils displaying high
1152 denitrification activity (e.g. improved grasslands) the incubation period should not exceed
1153 2 hours for a more accurate estimation of the N₂O/ N₂ + N₂O ratio. In the present study
1154 we have compared the in situ denitrification rates between three major land use types
1155 using an extended field incubation period to increase the probability of detecting a
1156 reliable ¹⁵N-N₂ signal, particularly under conditions of low denitrifier activity due to
1157 seasonality of denitrification and/or inherent capacity of soils (for example organic and
1158 deciduous forest soils). However, these rates should be considered as conservative since
1159 confounding issues such as subsoil diffusion and non-inhomogeneous labelling of the soil
1160 nitrate pool may in some cases have led to underestimations of the in situ denitrification
1161 rates.

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1163 ~~The significantly higher denitrification rates in grassland soils, compared to organic and~~
1164 ~~forest soils, observed under this study could be supported through the additional supply of~~
1165 ~~reactive nitrogen via fertilisation and the additional inputs of organic C and N through~~
1166 ~~grazing (Cowan et al. 2015, Rafique et al. 2012, van Beek et al. 2010). A relatively high~~
1167 ~~denitrification activity in poorly drained forest soils is usually sustained by the high soil~~
1168 ~~water filled pore space (WFPS) (Liu et al. 2013), while it may be limited by low~~
1169 ~~nitrification rates necessary for supplying the electron acceptors (nitrate) for~~
1170 ~~denitrification to occur (Sgouridis and Ullah 2014). Conversely, well drained forest soils~~
1171 ~~with organic N rich leaf litter may display high nitrification rates, but denitrification~~
1172 ~~activity is often limited by the low WFPS (Sgouridis and Ullah 2014). Organic soils are~~
1173 ~~naturally nutrient limited and their denitrification potential has been shown to be~~
1174 ~~primarily limited by the availability of nitrate (Francez et al. 2011, Hayden and Ross~~
1175 ~~2005, Sgouridis and Ullah 2014). It should be noted that N₂ fluxes below the detection~~
1176 ~~limit of 4 µg N m⁻² h⁻¹ were not used in denitrification rate calculations and this might~~
1177 ~~have led to slight overestimation of rates, particularly for the OS. It is beyond the scope of~~
1178 ~~this paper to discuss in detail the differences in denitrification rates between land use~~
1179 ~~types. The variation of denitrification across natural and semi natural land use types has~~
1180 ~~been investigated in these sites over two years using the adapted ¹⁵N Gas Flux method~~
1181 ~~and the results are presented in a separate publication (Sgouridis & Ullah, submitted).~~

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1183 4.3. Comparison with the AIT

1184 The total denitrification rates measured with the C₂H₂ amended intact soil cores followed
1185 the same trend as the total denitrification (N₂ and N₂O fluxes combined) from the ¹⁵N

1186 Gas-Flux measurements, while they were on average 168 times lower than the
1187 denitrification potential measured in the same land use types in anaerobic soil slurries
1188 amended with acetylene and nitrate in a previous study (Sgouridis and Ullah 2014), thus
1189 reflecting lower *in situ* rates. ~~However, T~~the AIT denitrification rates were between 3 and
1190 5 times lower than the ¹⁵N Gas-Flux rates despite the fact that the AIT intact soil cores
1191 were capped at the bottom, thus not allowing any subsoil diffusion of the evolved gases
1192 due to denitrification. Therefore, the AIT rates should have been higher than the ¹⁵N Gas-
1193 Flux rates if serious underestimation was occurring due to subsoil diffusion in the open-
1194 bottom static chambers, which was not the case. Adding nitrate to the C₂H₂ amended
1195 cores is and would have been desirable for evaluating directly evaluating the priming
1196 effect of the added substrate on denitrification rates. Even though tThe ¹⁵N tracer
1197 addition to the static chambers corresponded to the amounts of N naturally deposited in
1198 these land use types either via management practices and/or atmospheric deposition, thus
1199 avoiding excessive N fertilisation of the sampling plots. ~~However,;~~ it cannot be
1200 conclusively argued that the same amount of applied nitrate would not have led to similar
1201 denitrification rates between the AIT and the ¹⁵N Gas-Flux methods, ~~which was an~~
1202 ~~indication that discrepancies due to overestimation of the soil NO₃⁻ pool ¹⁵N enrichment~~
1203 ~~most likely have not led to serious underestimation of the denitrification rates using the~~
1204 ~~“non-equilibrium equations”~~. Previous comparisons between the AIT and the ¹⁵N tracer
1205 method in field studies showed no significant difference between the two methods in
1206 measuring *in situ* total denitrification rates when tracer is applied at high fertilisation rates
1207 (50 - 200 kg N ha⁻¹) and relatively low soil moisture contents (WFPS: 40 - 60 %) (Aulakh
1208 et al. 1991, Mosier et al. 1986). Conversely, in laboratory incubations it was shown that
1209 the AIT significantly underestimated total denitrification compared to the ¹⁵N tracer
1210 approach (Yu et al. 2010) and the direct N₂ flux approach (Qin et al. 2012) due to the

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1211 incomplete inhibition of N₂O reduction to N₂ by C₂H₂ in wet soils (Yu et al. 2010) or in
1212 soils with low nitrate content (Qin et al. 2013, Qin et al. 2014). In our study, the soil
1213 WFPS ranged between 60 and 70 % in all land use types, with the exception of the C-WL
1214 MW site (mean WFPS 42 %), whilst the ¹⁵N-NO₃⁻ tracer application rate was low (< 1 kg
1215 N ha⁻¹). ~~was not added in the intact soil cores with or without acetylene treatment. Adding~~
1216 ~~nitrate to the C₂H₂-amended cores would have been desirable for evaluating directly the~~
1217 ~~priming effect of the added substrate on denitrification rates. However, the lack of tracer~~
1218 ~~addition in the AIT treatments is unlikely to be the cause of underestimation of~~
1219 ~~denitrification as the bulk N₂O flux (including all possible N₂O sources) was not different~~
1220 ~~between the ¹⁵N Gas-Flux chamber and the no-C₂H₂-amended intact soil core~~
1221 ~~measurements. If the ¹⁵N tracer addition in the static chambers, even at such low rate (< 1~~
1222 ~~kg ¹⁵N ha⁻¹), were to stimulate the denitrification activity, this might have been reflected~~
1223 ~~through high bulk N₂O flux from the chamber compared to the intact cores. Moreover, the~~
1224 disturbance of the soil structure during the extraction of the soil cores and the effect of the
1225 acetylene addition to microbial activity were not significant as it was suggested by the
1226 similar CO₂ production rates (Aulakh et al. 1991), representing soil respiration (Felber et
1227 al. 2012), in the static chambers and the C₂H₂ amended and un-amended intact soil cores.
1228 Therefore, we ~~conclude-could argue~~ that it is possible that the AIT underestimated total
1229 denitrification rates compared to the ¹⁵N Gas-Flux method due to the likely incomplete
1230 inhibition of N₂O reduction to N₂ under relatively high soil moisture contents, although
1231 the shorter incubation time (2h for the intact cores) may have limited the ability of C₂H₂
1232 to fully equilibrate within soil pore spaces. Other confounding factors such as the
1233 catalytic decomposition of NO inat the presence of C₂H₂ (Nadeem et al. 2013) may have
1234 also contributed to the lower denitrification rates measured by the AIT. ,but since NO was
1235 not measured in this study final conclusions cannot be drawn. This study has confirmed

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1236 some of the drawbacks of the AIT as a quantification method of in situ denitrification
1237 rates compared to the ¹⁵N Gas-Flux . However, more useful conclusions as to which
1238 method is more appropriate for measuring denitrification in the field can be drawn only if
1239 the AIT is compared to the ¹⁵N Gas Flux and the gas flow soil core methods under the
1240 same field conditions.

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1241
1242 ~~Consequently,~~ The estimation of the denitrification product ratio using the AIT method,
1243 from the un-amended cores (N₂O only) and the C₂H₂ amended cores (N₂ + N₂O), is
1244 grossly usually overestimated (Butterbach-Bahl et al. 2013) since the source of N₂O
1245 cannot be discriminated with the AIT, whilst the N₂ flux is underestimated due to the
1246 incomplete inhibition of N₂O reduction (Butterbach-Bahl et al. 2013). This was
1247 confirmed in the present study for all the land use types and even the maximum
1248 denitrification product ratio after 2 hours incubation in the case of the grassland soils (23
1249 %), was still significantly lower than the respective ratio from the AIT (50 %). Therefore,
1250 the much lower denitrification product ratio estimated from the ¹⁵N Gas-Flux
1251 measurements is significantly more reliable and the wider application of this field
1252 technique across a range of land use types can have important implications for evaluating
1253 the role of denitrification as a reactive nitrogen sink and as a source of N₂O emissions
1254 (Butterbach-Bahl et al. 2013, Kulkarni et al. 2008).

1256 5. Conclusion

1257 The analytical precision for both ¹⁵N-N₂ and ¹⁵N-N₂O analyses was greatly improved by
1258 using smaller sample volumes than previously reported, thus allowing us to quantify *in*
1259 *situ* N₂ and N₂O fluxes with low ¹⁵N enrichment under field conditions, which was

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1260 previously not possible. The ^{15}N Gas-Flux method was applied for the first time across a
1261 range of natural and semi-natural land use types at ^{15}N tracer application rates mimicking
1262 current estimates of atmospheric N deposition (natural systems) or grassland fertiliser
1263 application rates and yielded analytically valid flux rates for both N_2 and N_2O in all the
1264 land use types. A possible limitation of the adapted ^{15}N Gas-Flux method when applied at
1265 low ^{15}N enrichment levels is the uncertainty associated with the estimation of the soil NO_3^-
1266 pool enrichment and the possibility for subsoil diffusion of the evolved gases due to the
1267 extended enclosure period in cases of extended incubation (> 2 hr) that may result in the
1268 underestimation of denitrification rates. ~~However, the direct field comparison~~ Comparing
1269 ~~of the~~ ^{15}N Gas-Flux method with the AIT ~~suggested confirmed the drawbacks of the AIT~~
1270 ~~as a reliable quantification method that the AIT further underestimates of~~ in situ
1271 denitrification rates. ~~Moreover, the AIT method that also seriously overestimates the~~
1272 ~~denitrification product ratio compared to the~~ ^{15}N Gas-Flux method. ~~most likely due to the~~
1273 ~~incomplete inhibition of N_2O reduction to N_2 under relatively high soil moisture contents.~~
1274 ~~Consequently, the~~ ^{15}N Gas-Flux method, ~~as applied in our study, constitutes~~ holds much
1275 promise as a more reliable field technique for measuring *in situ* denitrification rates and its
1276 wider application across a range of terrestrial ecosystems can lead to its refinement and
1277 improvement and in the long term ~~and~~ can significantly ~~improve~~ contribute to our
1278 understanding of the role of denitrification as a reactive nitrogen sink.

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1281 6. Acknowledgements

1282 The authors are grateful to Mr Edward Ritchie and Mr Richard Rhodes for granting us
1283 permission to access their land, as well as the National Trust in Conwy, the Abbeystead
1284 Estate in the Trough of Bowland and the Forestry Commission in Gisburn Forest for their

1285 guidance and advice. We are also thankful to Miss Ravindi Wanniarachchige at Keele
1286 University for her help during field sampling and laboratory analysis. Finally we are
1287 grateful to the two reviewers: an anonymous and Dr ~~Rienhardt~~Reinhard Well -for their
1288 comprehensive comments and suggestions-, which improved this manuscriptfor the
1289 improvement of the manuscript. This research was funded by the UK Natural Environment
1290 Research Council grant (NE/J011541/1) awarded to Keele University and supported by a
1291 ‘grant in kind’ from the NERC Life Sciences Mass Spectrometry Facility Steering
1292 Committee.

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1510 **Tables**

1511 **Table 1:** Measured ratios of R29 and R30 for N₂ in ambient air (n=10), ratios of R45 and
 1512 R46 in standard N₂O gas (0.5 ppm concentration, n=15) and ¹⁵N at% abundance calculated
 1513 from the respective ratios for both gases. SD; standard deviation, CV; coefficient of variation.

	R29 (N ₂)	R30 (N ₂)	R45 (N ₂ O)	R46 (N ₂ O)	¹⁵ N at% (N ₂)	¹⁵ N at% (N ₂ O)
Mean	7.38 10 ⁻³	5.16 10 ⁻⁵	8.00 10 ⁻³	2.21 10 ⁻³	3.71 10 ⁻¹	3.88 10 ⁻¹
SD	2.77 10 ⁻⁷	2.26 10 ⁻⁷	1.25 10 ⁻⁵	1.04 10 ⁻⁵	2.09 10 ⁻⁵	1.01 10 ⁻³
CV (%)	0.00	0.44	0.16	0.47	0.01	0.26

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1519 **Table 2:** The ambient soil nitrate pool, The areal application rate of the ¹⁵N tracer application
 1520 rate, the estimated enrichment of the total soil nitrate pool, the calculated ¹⁵X_N value from
 1521 N₂O and the slope of the ¹⁵X_N change with incubation time in the three land use types. Data
 1522 are means with standard errors in parentheses.

Land Use Type	Ambient NO ₃ ⁻ (kg N ha ⁻¹)	Tracer application rate (kg ¹⁵ N ha ⁻¹)	Enrichment of total soil NO ₃ ⁻ pool (%)	¹⁵ X _N (%)	¹⁵ X _N slope
Organic Soil (n=3)	0.53 (0.44)	0.04 (0.0215)	25 (11.8)	90 (1.5)	0.003 (0.0054)
Woodland (n=2)	3.86 (2.42)	0.62 (0.4196)	13 (0.7)	79 (8.3)	-0.007 (0.0025)
Grassland (n=3)	1.81 (0.96)	0.51 (0.199)	24 (5.1)	81 (8.4)	0.000 (0.0037)

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1524 **Table 3:** Comparison of mean flux rates and ratios between land use types for the two field
 1525 methods using One-Way ANOVA. All variables are log-transformed. *F*; *F* statistic, *P*;
 1526 probability level.

¹⁵ N Gas-Flux	<i>F</i>	<i>P</i>
Denitrification	19.4	< 0.001
N ₂ O emission	31.1	< 0.001
N ₂ O/ (N ₂ + N ₂ O)	7.4	< 0.01
Total bulk N ₂ O	19.4	< 0.001
CO ₂ production	19.8	< 0.001
AIT		
Denitrification	12.7	< 0.001
Total bulk N ₂ O	9.4	< 0.01
N ₂ O/ (N ₂ + N ₂ O)	0.3	> 0.05
CO ₂ production (un-amended cores)	11.2	< 0.001
CO ₂ production (C ₂ H ₂ amended cores)	11.7	< 0.001

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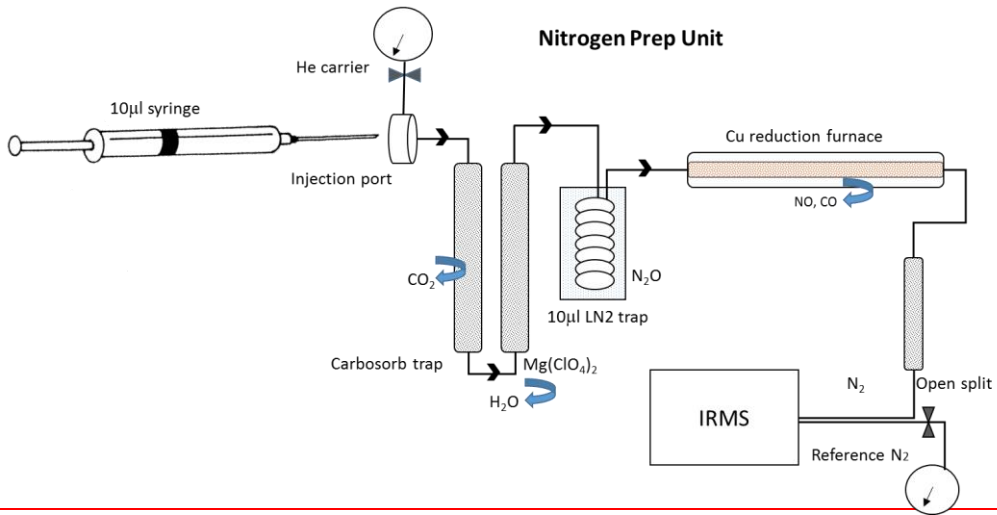
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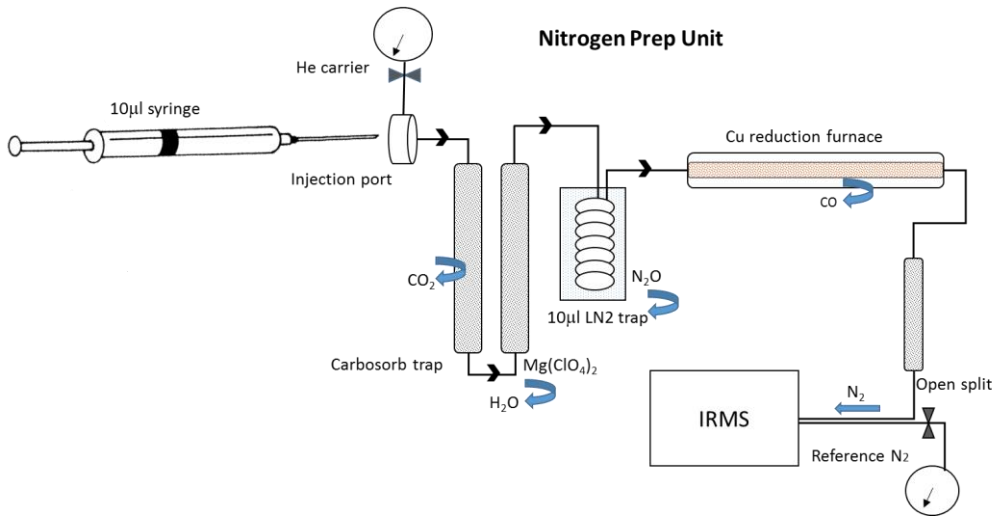
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Figure 1: Schematic of the ¹⁵N-N₂ analysis system

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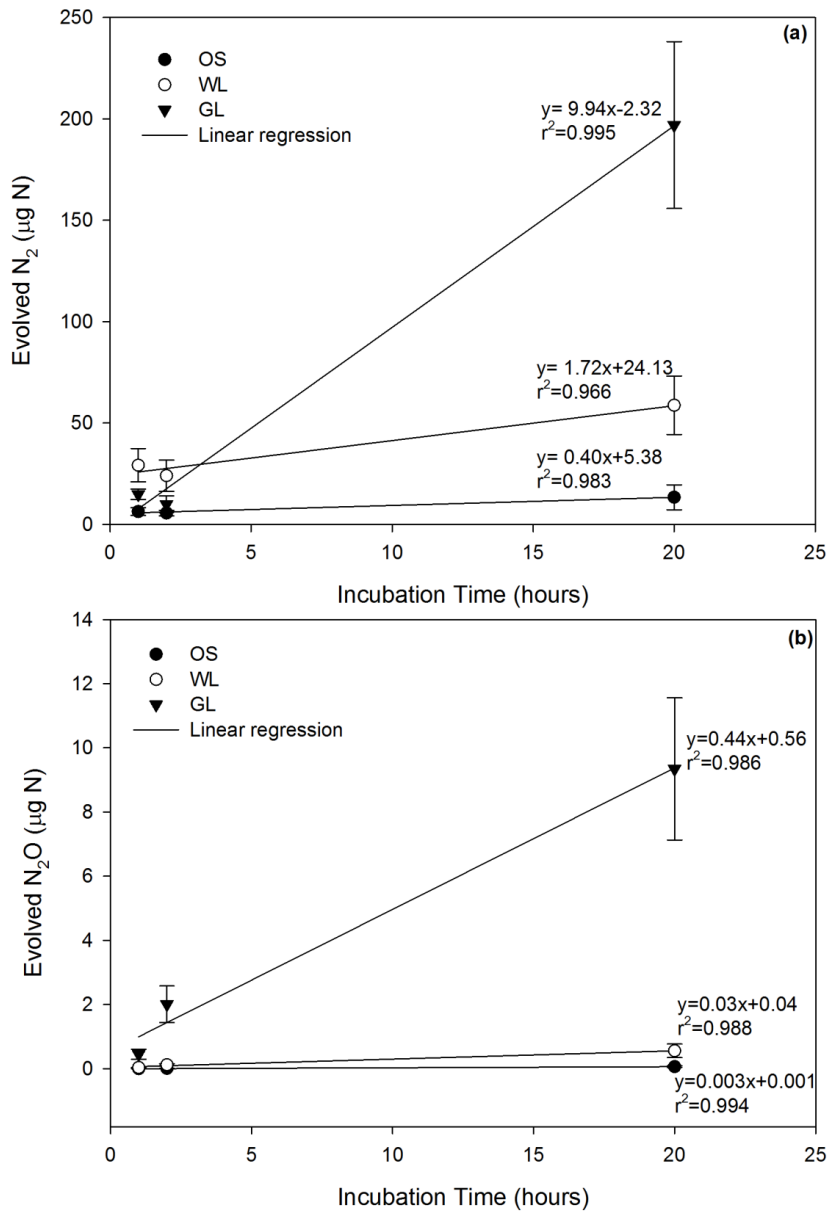
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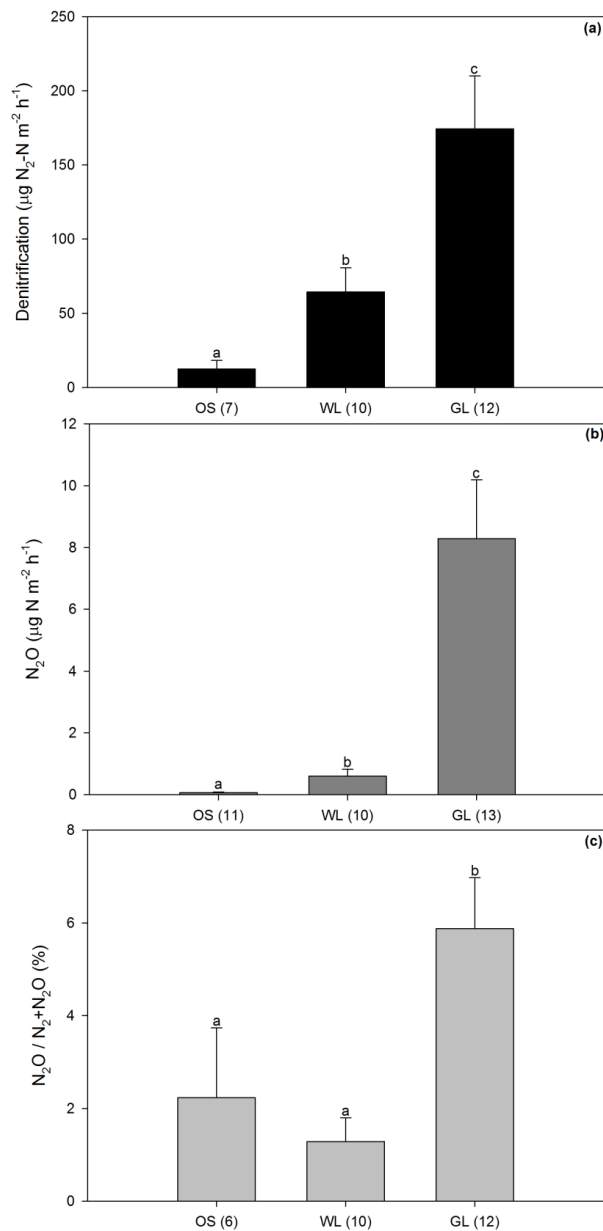
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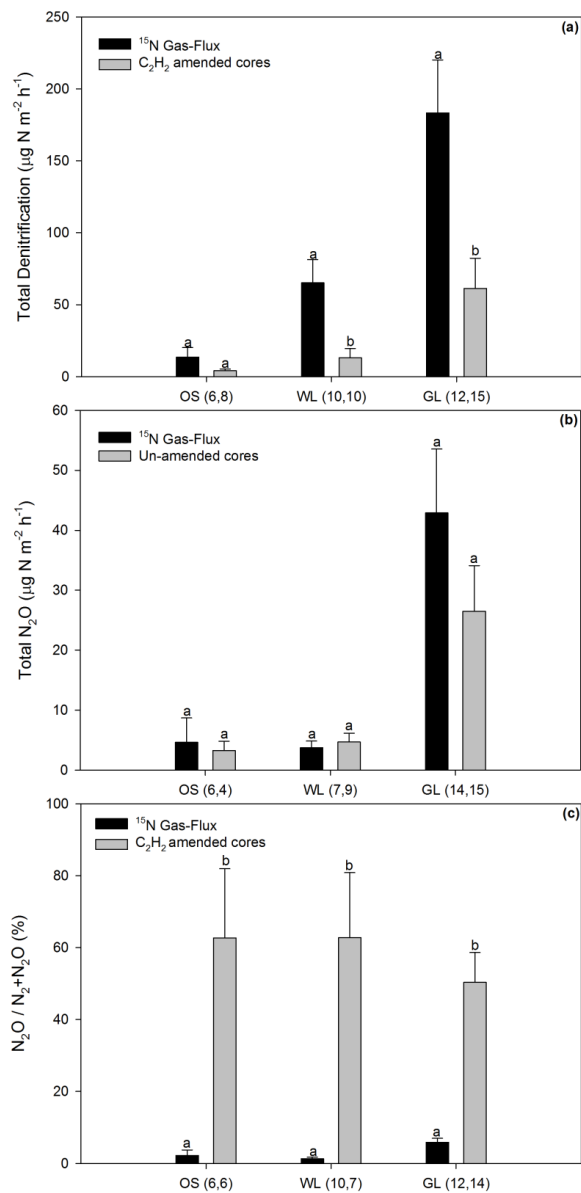


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 1553 **Figure 2:** Evolved (a) N₂ and (b) N₂O gas measured between 1, 2 and 20 hours incubation
 1554 time points using the ¹⁵N Gas-Flux method in the organic soil (OS), woodland (WL) and
 1555 grassland (GL) land use types. Data points are means and the error bars represent standard
 1556 errors.



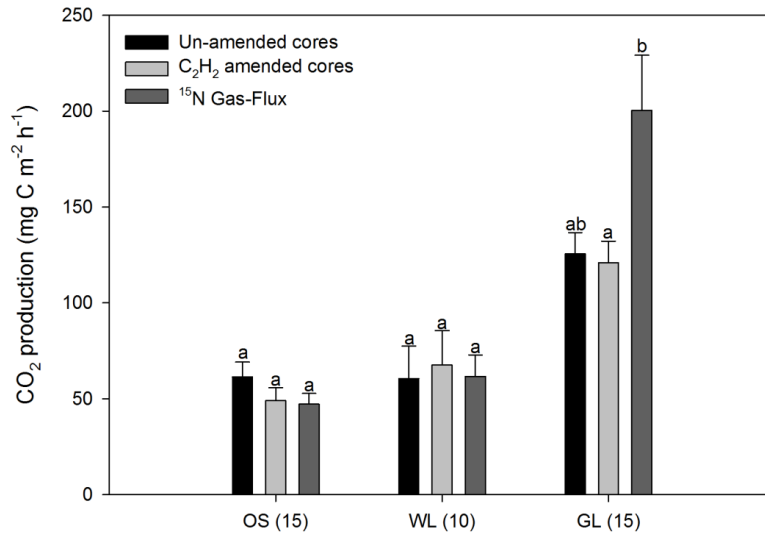
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1558 **Figure 3:** Mean rates of: (a) N_2 flux, (b) N_2O emission due to denitrification and (c) the
 1559 denitrification product ratio $\text{N}_2\text{O} / (\text{N}_2 + \text{N}_2\text{O})$ in the three land use types (OS; organic soils,
 1560 WL; woodland and GL; grassland). Same lower case letters indicate no significant
 1561 differences ($p > 0.05$) between land use types according to One-way ANOVA and the
 1562 Games-Howell *post hoc* test. The sample size (n) is given in parenthesis for each land use
 1563 type on the x-axis. Error bars represent standard errors.



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1565 **Figure 4:** (a) Mean total denitrification measured with the ¹⁵N Gas-Flux method and the AIT,
 1566 (b) Mean bulk N₂O emission measured in the static chambers of the ¹⁵N Gas-Flux method
 1567 and in un-amended intact soil cores and (c) the denitrification product ratio N₂O/ (N₂ + N₂O)
 1568 with the ¹⁵N Gas-Flux method and the AIT in the three land use types (OS; organic soils,
 1569 WL; woodland and GL; grassland). Same lower case letters indicate no significant
 1570 differences ($p > 0.05$) between measurement methods according to independent samples t-
 1571 test. The sample size (n) is given in parenthesis for each land use type and each method on
 1572 the x-axis. Error bars represent standard errors.



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1574 **Figure 5:** Mean CO₂ production measured in the static chambers of the ¹⁵N Gas-Flux
 1575 method, in un-amended and C₂H₂ amended intact soil cores in the three land use types (OS;
 1576 organic soils, WL; woodland and GL; grassland). Same lower case letters indicate no
 1577 significant differences ($p > 0.05$) between measurement methods according to independent
 1578 samples t-test. The sample size (n) is given in parenthesis for each land use type on the x-
 1579 axis. Error bars represent standard errors.

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