Response to Dr Reinhard Well's comments on the manuscript 'Application of the ^{15}N-Gas Flux method for measuring *in situ* N$_2$ and N$_2$O fluxes due to denitrification in natural and semi-natural terrestrial ecosystems and comparison with the acetylene inhibition technique.' (Manuscript ID = doi:10.5194/bgd-12-12653-2015)

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

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

Minor comments

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

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

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

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

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

3) Line 594-596 "The non-significant change of 15XN with incubation time suggested only one

denitrifying pool for both N2 and N2O, assuming negligible N2 production from anammox and codenitrification (Spott and Stange 2007)." Information on hybrid N2 or N2O can only be obtained from the comparison of 15XN and 15N atom fraction of extracted NO3. But since you did not analyse extracts, there is no evidence for or against hybrid fluxes. So I suggest to delete this sentence

Sentence deleted.

4) L 600-6003: Not clear to me for two reason: 15XN was measured in each gas sample , so

the decrease in 15XN was taken into account in the calculation, hence no bias from that. Also I don't see why low enrichment would lead to less dilution effect, since the relative change in the difference between 15XN and natural abundance is always the same irrespective of the initial enrichment So I suggest to delete this phrase.

Sentence deleted.

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

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

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

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

N2O reduction to N2 in topsoil would not be enhanced by subsoil diffusion. Do you mean

"extended enclosure time lead to lowering of N2O fluxes due to subsoil diffusion and enhanced N2O reduction to N2"?

65 **Sentence corrected as per the reviewer's instruction.**

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67 *7) L 666 please refer to Table S6 here*

68 *Suggest to reformulate "could potentially be explained by a delay in the de novo synthesis of*

69 *DENITRIFICTION ENZYMES AND THE FACT THAT THE N2O reductase enzyme is known to have a*
70 *slower expression than the preceding reduction enzymes (Knowles, 1982), leading to N2O*
71 *accumulation and lower N2 production after 2 hours of incubation." since the product ratio first*
72 *increases until T2 which could not be explained by a change in N2O reductase only.*

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74 **Sentence modified according to the reviewer's comment**

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

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78 **The unit ^{15}N at% has been added**

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80 *9) L 37-40*

81 *"Total denitrification 37 rates measured by the acetylene inhibition technique in the same land use*
82 *types correlated (r = 38 0.58) with the denitrification rates measured under the 15N Gas-Flux method*
83 *but were underestimated by a factor of 4 and this was partially attributed to the incomplete*
84 *inhibition of N2O reduction to N2 under a relatively high soil moisture content."*

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

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89 **Abstract modified as per the reviewer's suggestion**

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Application of the ^{15}N-Gas Flux method for measuring *in situ* N$_2$ and N$_2$O fluxes due to denitrification in natural and semi-natural terrestrial ecosystems and comparison with the acetylene inhibition technique.

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

Abstract

Soil denitrification is considered the most un-constrained process in the global N cycle due to uncertain *in situ* N_2 flux measurements, particularly in natural and semi-natural terrestrial ecosystems. ^{15}N tracer approaches can provide *in situ* measurements of both N_2 and N_2O simultaneously, but their use has been limited to fertilised agro-ecosystems due to the need for large ^{15}N additions in order to detect $^{15}N_2$ production against the high atmospheric N_2. For ^{15}N-N_2 analyses, we have used an 'in house' laboratory designed and manufactured N_2 preparation instrument which can be interfaced to any commercial continuous flow isotope ratio mass spectrometer (CF-IRMS). The N_2 prep unit has gas purification steps, a copper based reduction furnace, and allows the analysis of small gas injection volumes (4 μL) for ^{15}N-N_2 analysis. For the analysis of N_2O, an automated Tracegas Pre-concentrator (Isoprime Ltd) coupled to an IRMS was used to measure the ^{15}N-N_2O (4 mL gas injection volume). Consequently, the coefficient of variation for the determination of isotope ratios for N_2 in air and in standard N_2O (0.5 ppm) was better than 0.5 %. The ^{15}N Gas-Flux method was adapted for application in natural and semi-natural land use types (peatlands, forests and grasslands) by lowering the ^{15}N tracer application rate to 0.04 - 0.5 kg ^{15}N ha^{-1}. The minimum detectable flux rates were 4 μg N m^{-2} h^{-1} and 0.2 ng N m^{-2} h^{-1} for the N_2 and N_2O fluxes, respectively. Total denitrification rates measured by the acetylene inhibition technique in the same land use types correlated ($r = 0.58$) with the denitrification rates measured under the ^{15}N Gas-Flux method but were underestimated by a factor of 4 and this was partially attributed to the incomplete inhibition of N_2O reduction to N_2, under a relatively high soil moisture content, and/or the catalytic NO decomposition in the presence of acetylene. Even though relatively robust for *in situ* denitrification measurements, methodological uncertainties still exist in the estimation of N_2 and N_2O fluxes with the ^{15}N Gas-Flux method due to issues related to non-homogenous distribution of the added tracer and subsoil gas diffusion using open-bottom chambers,

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particularly during longer incubation duration. Despite these uncertainties, the ^{15}N Gas Flux

method constitutes a more reliable field technique for large scale quantification of N_2 and N_2O

fluxes in natural terrestrial ecosystems, thus significantly improving our ability to constrain

ecosystem N budgets.

158

1. Introduction

There has been a renewed interest recently in developing new or enhancing existing measurement approaches for improving our ability to constrain dinitrogen (N_2) fluxes due to denitrification in terrestrial ecosystems (Kulkarni et al. 2014, Lewicka-Szczebak et al. 2013, Wang et al. 2011, Yang et al. 2014). Denitrification, the reduction within soils of nitrogen oxides (NO_3^- and NO_2^-) to NO, N_2O and ultimately N_2 gas, constitutes the most important mechanism for the removal of reactive nitrogen (Nr) in terrestrial ecosystems (Galloway et al. 2008, Groffman 2012). Despite its importance, denitrification is considered the most un-constrained process in the global N cycle (Groffman 2012, Kulkarni et al. 2008) due to uncertainties in N_2 flux estimations that are likely leading to underestimations of denitrification rates at multiple scales (Butterbach-Bahl et al. 2013). Considering contemporary atmospheric N deposition rates globally including UK (Dore et al. 2012, Galloway et al. 2008, Payne 2014), the available Nr pool in soils may be greater than the capacity of denitrification for its removal with important consequences of chronic N enrichment of natural terrestrial ecosystems (Galloway et al. 2008, Limpens et al. 2003). Moreover, nitrous oxide (N_2O), an obligate intermediate of denitrification, is a potent greenhouse gas involved in the breakdown of stratospheric ozone (Ravishankara et al. 2009). Therefore, a reliable estimation of the relative magnitude of the major denitrification end products ($N_2 + N_2O$) in soils is crucial in evaluating the role of denitrification as an Nr sink (Kulkarni et al. 2008).

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N_2 comprises ~78 % of the atmosphere and thus it is extremely difficult to measure small N_2 fluxes from soil against this high background, particularly in natural terrestrial ecosystems (Groffman et al. 2006). Available methods for measuring both N_2 and N_2O are limited and can

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182 be categorised into the direct flux and ^{15}N isotope tracer methods (Kulkarni et al. 2014), whilst

183 micrometeorological approaches (Eddy covariance) are impossible in the N_2 rich atmosphere

184 (Felber et al. 2012). The gas-flow soil core method (Burgin and Groffman 2012, Butterbach-

185 Bahl et al. 2002, Scholefield et al. 1997, Wang et al. 2011) allows the direct measurement of

186 N_2 flux (without the addition of any substrate such as nitrate) from intact soil cores where the

187 soil atmosphere is replaced by a mixture of He/O_2. However, despite the high precision of the

188 technique, cores still need to be extracted from the field and conditioned over lengthy periods

189 of time for the complete removal of N_2 from the soil atmosphere. This method is therefore time

190 and resource intensive which limits its application to intensive temporal and large spatial scales

191 (Kulkarni et al. 2014). Moreover, the gas-flow soil core method cannot discriminate between

192 sources of N_2O thus overestimating the denitrification product ratio $N_2O/ (N_2 + N_2O)$

193 (Butterbach-Bahl et al. 2013, Morse et al. 2015). The acetylene inhibition technique (AIT) is

194 also a direct flux method that exploits the ability of acetylene (C_2H_2) at high concentrations (10

195 % v/v) to inhibit the reduction of N_2O to N_2 (Tiedje et al. 1989), thus total denitrification (N_2

196 + N_2O) is measured in C_2H_2 amended soil cores *in situ*, whilst N_2 flux is estimated indirectly

197 by difference from un-amended soil cores. Despite its simplicity and cost-effectiveness, the

198 AIT is becoming increasingly unpopular due its several limitations (Groffman et al. 2006), of

199 which the catalytic decomposition of NO in the presence of C_2H_2 under oxic or suboxic

200 conditions in the field (Bollmann and Conrad 1996, Nadeem et al. 2013) in particular, precludes

201 its use for reliable estimates of *in situ* denitrification rates (Felber et al. 2012).

202

203 The ^{15}N Gas-Flux method (Mosier and Klemedtsson 1994) has the advantage of providing *in*

204 *situ* measurements of both N_2 and N_2O simultaneously, thus allowing its application over large

205 temporal and spatial scales. It requires the addition of a ^{15}N-labelled tracer in a soil enclosure

206 in the field which is subsequently covered by a chamber while the chamber headspace is

207 progressively enriched with ^{15}N-N_2 and ^{15}N-N_2O produced by denitrification (Stevens and

208 Laughlin 1998). Assuming that both N_2 and N_2O originate from the same uniformly labelled

209 soil NO_3^- pool (Stevens and Laughlin 2001), the true denitrification product ratio can be more

210 accurately estimated as opposed to the direct flux approaches (Bergsma et al. 2001). Field

211 applications of the ^{15}N Gas-Flux method so far have been limited to fertilised agro-ecosystems

212 (Baily et al. 2012, Cuhel et al. 2010, Graham et al. 2013) and more recently restored peatland

213 soils (Tauchnitz et al. 2015) with high ^{15}N tracer application rates (between 10 - 200 kg N ha$^-$

214 ^{1}), with the exception of Kulkarni et al. (2014) who have measured denitrification rates in

215 Northern hardwood forests of the US by adding tracer amounts of ^{15}N-labelled nitrate and

216 Morse and Bernhardt (2013) who applied the same technique in intact soil cores collected from

217 mature and restored forested wetlands in North Carolina, USA. These recent studies hold much

218 promise that the ^{15}N Gas-Flux method can be applied to a range of natural and semi-natural

219 terrestrial ecosystems allowing the quantification of the relative magnitude of N_2 and N_2O

220 fluxes due to denitrification from these under-represented ecosystems.

221

222 Natural and semi-natural terrestrial ecosystems in the UK (i.e. peatlands, heathlands, acid

223 grasslands, deciduous and coniferous forests), where there is no fertiliser use and the impact

224 from grazing and commercial forestry is minimal (Mills et al. 2013), along with improved and

225 unimproved grasslands (grazed and/or fertilised) constitute approximately 49 % and 85 % of

226 rural land use cover in England and Wales, respectively (Morton et al. 2011). Unlike arable

227 agriculture, these land use types have been poorly investigated for their role in Nr loss through

228 denitrification.

229

230   The major challenge in measuring ^{15}N-N$_2$ at near natural abundance levels is the possibility of

231   interference at m/z 30 (^{30}N$_2$) due to the reaction of oxygen in the ion source with N and the

232   formation of NO$^+$ ions that also have m/z 30 (Stevens et al. 1993). Commonly, this issue is

233   addressed in continuous flow isotope ratio mass spectrometers (CF-IRMS) with the inclusion

234   of a copper (Cu) oven for reducing O$_2$ in the gas sample (Russow et al. 1996). Recently, it has

235   been suggested that the interference at m/z 30 can be further reduced by including a molecular

236   sieve column in gas chromatograph IRMS (GC-IRMS) systems to not only separate N$_2$ and O$_2$

237   in the gas sample, but also to quantitatively remove O$_2$ and other trace gases such as carbon

238   monoxide (Lewicka-Szczebak et al. 2013, Yang et al. 2014). We hypothesise that the precision

239   for m/z 30 determination can be greatly improved by using a custom-built preparative unit for

240   the removal of H$_2$O, CO$_2$, N$_2$O, NO$^+$ and CO; a device which also permits the micro scale

241   injection of volumes of < 5 μL. These injection volumes are much smaller than have previously

242   been reported in the literature.

243

244   Studies that have compared the ^{15}N Gas-Flux method with the AIT in the field are rare and

245   have exclusively focused on highly fertilised agro-ecosystems with moderate to low soil

246   moisture contents (Aulakh et al. 1991, Mosier et al. 1986, Rolston et al. 1982). These studies

247   have measured comparable denitrification rates by both field techniques, although the

248   relatively low soil moisture contents have probably allowed greater diffusion of C$_2$H$_2$ to the

249   anaerobic microsites where denitrification occurs (Malone et al. 1998), whilst the high nitrate

250   application rates have probably favoured nitrate reduction over N$_2$O reduction (Dendooven and

251   Anderson 1995) resulting in high denitrification rates from the AIT. Conversely, laboratory

252   studies have shown that the AIT significantly underestimates total denitrification compared to

253   the ^{15}N tracer approach (Yu et al. 2010) and the direct N$_2$ flux approach (Qin et al. 2012) due

254   to the incomplete inhibition of N$_2$O reduction to N$_2$ by C$_2$H$_2$ in wet soils (Yu et al. 2010) or in

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soils with low nitrate content, where N_2O reduction is more energetically favourable (Qin et al. 2013, Qin et al. 2014). A comparison of the ^{15}N Gas-Flux method with the AIT under *in situ* conditions across a range of natural and semi-natural terrestrial ecosystems has not been attempted before. It can provide valuable insights in terms of the validity and applicability of the two field techniques for measuring denitrification rates across broad spatial and temporal scales.

The objectives of the present study were: (1) to determine the precision and suitability of our preparative-IRMS instrumentation for measuring ^{15}N-N_2 and ^{15}N-N_2O at low enrichment levels, (2) to adapt the ^{15}N Gas-Flux method for application across natural and semi-natural terrestrial ecosystems and (3) to compare the validity and applicability of the ^{15}N Gas-Flux method with the AIT for measuring *in situ* denitrification rates.

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2. Materials and methods

2.1. IRMS system

For N_2 gas isotopic analysis we used an Isoprime isotope ratio mass spectrometer (Isoprime Ltd, UK, Wythenshawe) coupled to an in house built N_2 preparative interface (Figure 1). Headspace gas (4 µL) was manually injected with a gas tight syringe (SGE Analytical science) into the preparative interface via an open split. Prior to its introduction into the IRMS, the sample was treated as follows: a) dried by passing through $Mg(ClO_4)_2$ (Elemental Microanalysis Ltd, Devon, UK), b) CO_2 removed with 0.7 - 1.2 mm Carbosorb (Elemental Microanalysis Ltd, Devon, UK), c) N_2O cryogenically trapped under liquid nitrogen, and d) O_2 removed over a copper-packed reduction furnace heated at 600°C. The N_2 was then directed towards the triple collectors of the isotope ratio mass spectrometer where m/z 28, m/z 29 and m/z 30 mass ions were measured. Mass/charge ratios for the m/z 28, m/z 29 and m/z 30 nitrogen ($^{28}N_2$, $^{29}N_2$ and $^{30}N_2$) were recorded for each sample at a trap current of 300 µAmps. Instrument stability checks were performed prior to each analysis by running a series of 10 reference pulses of N_2 (BOC special gases) until a standard deviation of $\delta^{15}N$ better than 0.05 ‰ was achieved. Additionally, 10 consecutive injections (4 µL) of atmospheric air were analysed prior to the analysis of actual samples. Precision of the instrument was better than $\delta^{15}N$ 0.08 ‰ in all quality control tests.

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Nitrous oxide was analysed using modified headspace methods described for the analysis of nitrogen gas above. Headspace gas (*ca*. 4 mL) was injected into a TraceGas™

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300   Preconcentrator coupled to an Isoprime^{TM} IRMS (GV instruments Ltd, UK) whereupon the

301   sample was directed through a series of chemical traps designed to remove H_2O and CO_2.

302   The N_2O was cryogenically trapped under liquid nitrogen. The waste was flushed out of

303   the instrument. The N_2O was further cryofocused in a second liquid nitrogen trap prior to

304   being introduced onto a 25 m x 0.32 mm Poraplot Q gas chromatography column

305   (Chrompack column, Varian, Surrey, U.K). The column separated N_2O from any residual

306   CO_2, and both entered the IRMS via an open split. The retention time between the first

307   eluting CO_2 ($< 2^{E-10}$ amplitude) and second eluting N_2O peak typically fell in the range

308   between 60 - 70 seconds to avoid isobaric interference of the CO_2 with the calculated ^{15}N.

309   The N_2O was directed towards the triple collectors of the isotope ratio mass spectrometer

310   where m/z 44, m/z 45 and m/z 46 mass ions were measured and recorded. Instrument

311   stability checks were performed prior to each analysis by running a series of 10 reference

312   pulses of N_2O (BOC special gases) until a standard deviation of $\delta^{15}N$ better than 0.05 ‰

313   was achieved. Prior to each sample batch analysis, trace gas N_2O measurements were made

314   on three 100 mL flasks containing atmospheric air collected from outside the stable isotope

315   laboratory. $\delta^{15}N$ precisions using the Trace gas Preconcentrator and Isoprime IRMS were

316   better than 0.3 ‰ respectively at 600 µAmp trap current.

317

318   2.2. Field application of the ^{15}N Gas-Flux and AIT techniques

319   *In situ* measurements of N_2 and N_2O were made using static chambers according to the ^{15}N

320   Gas-Flux method (Mosier and Klemedtsson 1994). Five plots were randomly established

321   in June 2013 in each of four study sites in the Ribble - Wyre River catchments (area 1145

322   km^2; NW England, 53°59'99" N, 2°41'79" W). The study sites were a heathland (R-HL), a

323   deciduous woodland (R-DW), an unimproved grassland (R-UG) and an improved

324 grassland (R-IG). In August 2013, four more study sites were tested in the Conwy River

325 catchment (area 345 km^2; N. Wales, 52°59'82" N, 3°46'06" W) following a similar sampling

326 design. These sites were an acid grassland (C-UG), an ombrotrophic peat bog (C-PB), a

327 mixed deciduous and coniferous woodland (C-MW) and an improved grassland (C-IG).

328 Further details on the location, land management status and major soil properties for all

329 study sites can be found in Sgouridis & Ullah (2014).

330

331 In each plot a round PVC collar (basal area 0.05 m^2; chamber volume 4 L) was inserted

332 into the soil at c. 10 cm depth (15 cm for the R-HL and C-PB plots) 2 - 4 weeks before the

333 measurement date. The collars were open at the bottom to maintain natural drainage and

334 root growth during the measurements. The natural vegetation cover at the soil surface of

335 each installed collar remained unchanged. The PVC collars were fitted with a circular

336 groove of 25 mm depth to fit in an acrylic cylindrical cover (chamber) providing a gas-tight

337 seal when filled with water (Ullah and Moore 2011). The gas leak rate from the chamber

338 was determined in the laboratory by placing the sealed collar and chamber over a tray of

339 water, injecting CH_4 (10 ppm), and determining the change in CH_4 concentration within

340 the chamber headspace over time (Yang et al. 2011). The CH_4 concentration change within

341 24 hours was negligible with the relative standard deviation (RSD) being < 5 %. We did

342 not use a vent tube for pressure equilibration, as suggested by Hutchinson and Mosier

343 (1981), in our chamber design, which could have diluted the chamber headspace with

344 atmospheric N_2, as part of our effort to increase the probability of a detectable ^{15}N-N_2 signal

345 in the chamber headspace. Instead chambers were covered with reflective foil for

346 minimising temperature increase within the chamber headspace during the incubation

347 period (Ullah and Moore 2011). Labelled $K^{15}NO_3^-$ (98 at. % ^{15}N, Sigma-Aldrich) was

348 applied in each plot via ten injections of equal volume through a grid (4 x 6 cm) using

349　custom-made 10 cm long lumber needles (15 cm for the R-HL and C-PB plots) attached to

350　a plastic syringe (Ruetting et al. 2011). The ^{15}N tracer was delivered as the needle was

351　pushed into the soil from the surface up to 10 or 15 cm depth aiming to achieve as uniform

352　as possible labelling of the soil volume enclosed by the collar, as required by the ^{15}N gas

353　flux method (Mosier and Klemedtsson 1994). The volume and concentration of the labelled

354　K^{15}NO$_3^-$ tracer solution was determined from measurements of soil nitrate and moisture

355　content, as well as bulk density adjacent to each plot made during the installation of the

356　collars (Morse and Bernhardt 2013). Lower application rates (< 0.1 kg N ha^{-1}) were

357　administered to natural study sites (e.g. peat bog, heathland) and higher rates (< 1 kg N ha^{-1})

358　^{1}) administered to semi-natural (e.g. unimproved and improved grasslands). The tracer

359　solution (50 - 200 mL) was adjusted between 3 and 5 % of the ambient volumetric water

360　content (see Supplementary Table 1 for detailed data from each sampling plot). It should

361　be noted that no time was allowed for the equilibration of the added tracer solution in the

362　soil enclosure to avoid significant loss of the low amount of added nitrate via plant uptake.

363

364　Following the ^{15}N tracer application the collars were covered with the acrylic chamber

365　fitted with a rubber septum for gas sampling. Two sets of gas samples (20 mL each) were

366　collected with a gas tight syringe (SGE Analytical science) through the septum of the

367　chamber cover at T = 1h, T = 2h and T $\approx$ 20h after the tracer injection, while a T = 0h

368　sample was collected immediately after tracer injection above the plot surface before fitting

369　the chamber cover. The gas samples were transferred into pre-evacuated (<100 Pa) 12 mL

370　borosilicate glass vials with butyl rubber septa (Exetainer vial; Labco Ltd., High Wycombe,

371　United Kingdom) for storage under positive pressure and were analysed within 8 weeks

372　from collection without any significant change of the gas concentration (Laughlin and

373　Stevens 2003).

374

Adjacent to each PVC collar in each plot, two intact soil cores (50 mm I.D., 15 cm long)

were extracted from 10 cm depth leaving the top 5 cm void as a headspace volume. The

cores were capped on both ends with the top cap fitted with a rubber septum for gas

sampling. One set of cores was amended with pure C_2H_2 with 5 mL injected through the

septum directly in the middle of the soil core before 10 % of the headspace being also

replaced with pure C_2H_2. The second set of cores was not amended with C_2H_2 and both

cores were placed back in the ground where they came from. Gas samples (5 mL) were

collected with a gas tight syringe (SGE Analytical science) through the septa of the cores

at T = 1h and T = 2h after amendment with acetylene. The gas samples were transferred

into pre-evacuated (<100 Pa) 3 mL borosilicate glass vials with butyl rubber septa

(Exetainer vial; Labco Ltd., High Wycombe, United Kingdom) for storage under positive

pressure.

387

2.3. Flux calculations

The ^{15}N content of the N_2 in each 12 mL vial was determined using the IRMS system

described above and the ratios R29 ($^{29}N_2/^{28}N_2$) and R30 ($^{30}N_2/^{28}N_2$) were measured in both

enriched (T=1, 2 and 20 hours) and reference samples (T=0 hours). The inclusion of air

reference standards between every 10 samples indicated an upward drift for R30 over time,

potentially due to the formation of NO^+ in the ion source despite the inclusion of the Cu

reduction step (Lewicka-Szczebak et al. 2013). Subsequently, every sample batch was drift

corrected by fitting a linear regression through the air reference standards and calculating

an offset correction for both R29 and R30 (Yang et al. 2014). The minimum detectable

397 change (MDC) in R29 and R30 was defined with repeated manual analyses of air reference

398 standards (n=10) and was calculated using the following equation (Matson et al. 2009):

399 $$MDC = \mu_{pair\ diff} + (2\sigma_{pair\ diff})\qquad\qquad(1)$$

400 where μ is the mean difference of all possible unique pairs of air reference standards (n=45)

401 and σ is the standard deviation between sample pairs. The MDC for R29 was 7.7 x 10^{-7} and

402 for R30 was 6.1 x 10^{-7} and these values were used to determine if each time step sample

403 was significantly different from ambient reference samples (T=0 hours), and if not they

404 were excluded from the flux calculations.

405

406 For calculating the total N_2 flux from a uniformly labelled soil nitrate pool when both R29

407 and R30 are measured, the 'non-equilibrium' equations were applied as described by

408 Mulvaney (1984) for estimating first the ^{15}N fraction in the soil NO_3^- denitrifying pool

409 ($^{15}X_N$) as:

410 $$^{15}X_N = 2(\Delta R30/\Delta R29)/(1 + 2(\Delta R30/\Delta R29))\qquad\qquad(2)$$

411 where $\Delta R29$ and $\Delta R30$ is the difference between R29 and R30 respectively between

412 enriched (T=1, 2 and 20 hours) and reference samples (T=0 hours). Subsequently, the

413 $^{15}X_N$ allows the quantification of the fraction of the N_2 evolved from the ^{15}N-labelled pool

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

415 $$d = \frac{\Delta R30}{\left(^{15}X_N\right)^2}\qquad\qquad(3)$$

416 $$d = \frac{\Delta R29}{2\left(^{15}X_N\right)\left(1 - ^{15}X_N\right)^2}\qquad\qquad(4)$$

417

17

418   Using d and the concentration of $[N_2]$ (µg N) in the chamber headspace, the evolved N_2

419   from the soil pool was calculated:

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

421   The N_2 flux was then calculated using linear regression between the maximum evolved N_2

422   and the respective incubation time per plot surface area and was expressed in µg N m^{-2} h^{-1}

423   representing the total N_2 flux from the mixture of the ^{15}N-labelled tracer and the soil N at

424   natural abundance (Stevens and Laughlin 1998).

425

426   The ^{15}N content of the N_2O in the same 12 mL vials as well as the ratios R45 (^{45}N$_2$O /^{44}N$_2$O)

427   and R46 (^{46}N$_2$O /^{44}N$_2$O) were measured in both enriched (T=1, 2 and 20 hours) and

428   reference samples (T=0 hours). The application of the 'non-equilibrium' equations to N_2O

429   is analogous to N_2 after correcting for the naturally occurring oxygen isotopes (Bergsma et

430   al. 2001). Therefore, the ratios R45 and R46 were converted to ratios of R29 and R30

431   respectively by applying the following equations:

432    $R29 = R45 - R17$ (6)

433    $R30 = \left(R46 - (R29R17)\right) - R18$ (7)

434   where for $R17$ (^{17}O/^{16}O) the value 0.000373 was used and for $R18$ (^{18}O/^{16}O) the value

435   0.0020052 was used (Bergsma et al. 2001). There was no significant instrumental drift for

436   the ratios R45 and R46 over time. The MDC was defined, for the converted R29 and R30,

437   with repeated automatic analyses of 0.5 ppm N_2O standards (n=15) as 3.4 x 10^{-5} and 2.9 x

438   10^{-5} respectively. The second set of gas samples collected at the same time in the field were

439   analysed for total N_2O on a GC-µECD (7890A GC Agilent Technologies Ltd., Cheshire,

440   UK) and the concentration of $[N_2O]$ (µg N) was used in Eq. (5) to calculate the N_2O flux

441   due to denitrification of the mixture of the ^{15}N-labelled tracer and the soil N and expressed

442   in μg N-N$_2$O m^{-2} h^{-1}. Assuming that the N$_2$O originates from the same uniformly labelled

443   pool as N$_2$, the $^{15}X_N$ from N$_2$O was used to estimate d for N$_2$ using either R30 (Eq. 3) or R29

444   (Eq. 4), thus lowering the limit of detection for N$_2$ (Stevens and Laughlin 2001) and

445   allowing measurement of N$_2$ gas flux from natural terrestrial ecosystems at low ^{15}N-tracer

446   application rates.

447

448   Gas samples collected from the intact soil cores with or without acetylene amendment were

449   analysed for N$_2$O on a GC-μECD (7890A GC Agilent Technologies Ltd., Cheshire, UK)

450   and for CO$_2$ on a GC-FID (7890A GC Agilent Technologies Ltd., Cheshire, UK) and flux

451   rates were determined by linear regression between 0 and 2 hours. The instrument precision

452   was determined from repeated analyses of 6 ppm N$_2$O and 200 ppm CO$_2$ standards

453   respectively (n = 8) and the RSD was <1%.

454

455   2.4. Statistical analysis

456   Using factor analysis on selected soil physico-chemical properties, the samples from the 8

457   field sites were ordinated in three broad land use types: organic soils (C-PB, C-UG, R-HL);

458   forest soils (C-MW, R-DW) and grassland soils (C-IG, R-UG, R-IG) according to Sgouridis

459   and Ullah (2014). All subsequent statistical analyses were performed on the broad land use

460   types rather than individual field sites. The data were analysed for normality and

461   homogeneity of variance with the Kolmogorov-Smirnov test and the Levene statistic

462   respectively and logarithmic transformations were applied as necessary. One-Way

463   ANOVA combined with the Hochberg's GT2 *post hoc* test for unequal sample sizes or the

464   Games-Howell *post hoc* test for unequal variances was performed for comparing the

465   variance of the means between land use types for all gas fluxes. The non-parametric

466   Kruskal-Wallis test was used to compare mean flux rates between incubation time intervals.

467   Pearson correlation was used between log-transformed flux rates. Comparisons between

468   the ^{15}N Gas-Flux and AIT techniques were made with independent samples *t*-test. All

469   statistical analyses were performed using SPSS® 21.0 for Windows (IBM Corp., 2012,

470   Armonk, NY).

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3. Results

3.1. IRMS system evaluation

The precision of the IRMS systems was evaluated using repeated analyses of ambient air samples for N_2 (n=10) injected manually in one batch and repeated analyses of N_2O gas standard at natural abundance and 0.5 ppm concentration (n=15) using automated injections. The mean measured ratios of R29 and R30 for N_2 and of R45 and R46 for N_2O are shown in Table 1. Measurement precision was defined as the coefficient of variation (%) and it was lower for R29 compared to R30 and lower for R45 compared to R46, but still less than 0.5 % for all four measured ratios. We estimated the ^{15}N atom% abundance for both gases as per Yang et al. (2014) and the precision was less than 0.01 % for N_2 in air and 0.26 % for standard N_2O at natural abundance. The mean measured R30 (5.16 x 10^{-5}) was higher than the theoretical value of 1.35 x 10^{-5} for N_2 in ambient air suggesting some interference at *m/z* 30 potentially due to the formation of NO^+ ions in the ion source of the mass spectrometer despite the inclusion of the Cu reduction oven. The contribution of NO^+ ions (R30 measured - R30 theoretical) was 3.81 x 10^{-5}, whilst the ratio of R30 theoretical/ R30 measured was 0.26. Correcting the R30 ratio for the contribution of NO^+ ions results in a lower 'true' precision for the R30 (CV = 1.67 %).

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

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

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507 woodland and grassland soils (Table 2). Based on the soil nitrate content on the day of the

508 tracer amendments (Table 2), the estimated enrichment of the total soil nitrate pool was on

509 average between 13 and 25 ^{15}N at% (detailed data on the ^{15}N tracer application per field

510 site are shown in Supplementary Table 2).

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512 The ^{15}N fraction in the denitrifying pool ($^{15}X_N$), as calculated from the measured isotopic

513 ratios of the N_2O after 1 hour of incubation using Eq. (2), ranged between 65 and 93 ^{15}N

514 at%. The average change of the $^{15}X_N$ with incubation time, indicated by the slope shown in

515 Table 2, was not different from 0 in case of the organic (t-test; $t = 0.520$, $df = 18$, $p > 0.05$)

516 and grassland soils (t-test; $t = 0.047$, $df = 28$, $p > 0.05$), whilst it was significantly below 0

517 for the woodland soils (t-test; $t = 2.917$, $df = 18$, $p < 0.05$). Separating the woodland soils

518 to C-MW and R-DW sites, only the former displayed a significant negative slope of $^{15}X_N$

519 with incubation time (t-test; $t = 3.306$, $df = 8$, $p < 0.05$), suggesting N_2O production from a

520 second nitrate pool, possibly nitrate produced from the oxidation of NH_4^+ via nitrification,

521 in the C-MW. In cases where the $^{15}X_N$ could be calculated from the N_2 isotope ratio data

522 (woodland and grassland soils; data shown in Supplementary Table 3), this was not

523 significantly different from their respective $^{15}X_N$ calculated from the N_2O isotope ratio data

524 (t-test; $t\text{-}_{WL} = 0.929$, $df = 12$, $p > 0.05$; $t\text{-}_{GL} = 1.511$, $df = 20$, $p > 0.05$).

525

526 The mean evolved amount of N_2 and N_2O gases due to denitrification in each land use type

527 increased with increasing incubation time (Figure 2). The increase in the evolved N_2 was

528 statistically significant after 20 hours incubation in GL (ANOVA; $F = 19.8$, $p < 0.01$),

529 whilst due to the high variability among plots, shown by the large error bars at 20 hours

530 incubation in Figure 2a, it was not significant for the OS and WL soils. The amount of N_2O

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accumulated after 20 hours (Figure 2b) was significantly higher than in the previous time points for all land use types (ANOVA; $F_{OS} = 4.6$, $F_{WL} = 5.1$, $F_{GL} = 14.7$, $p < 0.05$). However, this pattern was not consistent in every sampling plot (data presented in Supplementary Tables 4 & 5), for example in C-MW highest N_2 accumulations were observed after the first or second hour of incubation, whilst in most cases the increase in N_2 and N_2O concentrations was not linear throughout the incubation period (Supplementary Tables 4 & 5). This suggested a complex temporal sequence of events, which was not consistent between plots among the different land use types, probably as a result of complex interactions between environmental controls of denitrification and the length of the incubation period (details below). Consequently, the N_2 flux rate decreased with increasing incubation time (Figure 3a) and this decrease was significant between each time interval in the OS (Kruskal-Wallis; $\chi^2 = 11.35$, $p = 0.003$), between 1 and 20 hours in the WL (Kruskal-Wallis; $\chi^2 = 10.78$, $p = 0.005$) and between 1 and 2 hours in the GL (Kruskal-Wallis; $\chi^2 = 10.10$, $p = 0.006$). Conversely, the N_2O flux rates increased between the first and second hour of incubation (Figure 3b), followed by a decrease after 20 hours, albeit the mean differences between time intervals were not statistically significant in any land use type (Kruskal-Wallis; $\chi^2_{OS} = 3.58$, $\chi^2_{WL} = 3.47$, $\chi^2_{GL} = 3.01$, $p > 0.05$).

The N_2 flux ranged between 2.4 and 416.6 μg N m^{-2} h^{-1} and was significantly different among land use types based on 20 hour incubation duration for comparison purposes (Table 3). The grassland soils showed on average 3 and 14 times higher denitrification rates than the woodland and organic soils respectively (Figure 4a). A similar pattern was observed for the N_2O flux due to denitrification (range: 0.003 - 20.8 μg N m^{-2} h^{-1}) with the grassland soils emitting on average 14 and 120 times more N_2O than the woodland and organic soils respectively (Figure 4b), whilst the N_2O flux was on average 20 to 200 times lower than

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556   the N_2 flux among land use types. Consequently, the denitrification product ratio $N_2O/ (N_2$

557    $+ N_2O)$ was low, ranging between 0.03 and 13 % and was highest in the GL and similar

558   between the WL and OS (Figure 4c). The change of the denitrification product ratio with

559   incubation time was evaluated in each sampling plot where both N_2 and N_2O fluxes were

560   available (data shown in Supplementary Table 6). Generally, there was no consistent

561   pattern between individual sampling plots with the exception of the grassland soils, where

562   the maximum product ratio was observed after 2 hours of incubation (ANOVA; $F = 6.11$,

563    $p < 0.05$). This was an indication of some reduction of the denitrification derived N_2O to

564   N_2 during the extended closure period (up to 20 hours) in the grassland soils.

565

566   3.3. Comparison with the AIT

567   The total denitrification rate measured from the C_2H_2 amended intact soil cores in the same

568   land use types ranged between 0.5 and 325.2 µg N m^{-2} h^{-1} and correlated positively with

569   the total denitrification rate (N_2 and N_2O fluxes combined) measured with the ^{15}N Gas-Flux

570   method (Pearson; $r = 0.581$, $n = 25$, $p < 0.01$) following a similar trend among land use

571   types, albeit only the OS being significantly lower than the grassland and woodland soils

572   (Table 3). The AIT denitrification rates were between 3 and 5 times lower than the total

573   denitrification from the ^{15}N Gas-Flux (Figure 5a) with the difference being significant in

574   woodland (t-test; $t = 3.914$, $df = 18$, $p < 0.01$) and grassland soils (t-test; $t = 3.521$, $df = 25$,

575    $p < 0.01$).

576

577   The total N_2O flux measured from the un-amended intact soil cores ranged between 0.15

578   and 86.6 µg N m^{-2} h^{-1} and was between 1 and 3 times lower than the total denitrification

579   rate from the C_2H_2 amended cores. There were no significant differences between bulk N_2O

fluxes measured with the static chambers and the un-amended intact soil cores (Figure 5b), which indicated that total N_2O emissions were comparable between the two field techniques. Consequently, estimating the denitrification product ratio from the un-amended and C_2H_2 amended intact soil cores resulted in significantly higher ratios compared to the [15]N Gas-Flux approach (Figure 5c), which were on average between 50 and 60 % and not significantly different among land use types (Table 3).

The mean CO_2 production rate was similar irrespective of whether it was measured in static chambers, in C_2H_2 amended or un-amended intact soil cores (Figure 6), indicating that soil respiration (including both microbial and plant respiration) was not affected by the measurement technique.

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4. Discussion

4.1. IRMS system evaluation

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

The TraceGas^{TM} Preconcentrator IRMS system used for ^{15}N-N$_2$O analysis displayed similar precision for the determination of R45 and R46 in standard N$_2$O gas at circa ambient

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concentration to a similar system used by Bergsma et al.(2001), while injecting only 4 mL of gas sample as opposed to 0.5 L used by Bergsma et al. (2001). When expressed in delta values ($\delta^{15}N$), the precision of our system was better than 0.05 ‰, which is significantly better than the respective precisions reported in Lewicka-Szczebak et al. (2013) and Yang et al. (2014), but comparable to Well et al. (1998). Therefore, the analytical precision achieved for both ^{15}N-N_2 and ^{15}N-N_2O analyses, using smaller gas sample volumes than previously reported, allowed us to quantify *in situ* N_2 and N_2O fluxes with low tracer addition under field conditions.

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

The average ^{15}N tracer application rate (0.04 - 0.5 kg ^{15}N ha^{-1} or 0.4 - 1.2 mg ^{15}N kg^{-1} dry soil) across land use types was one to two orders of magnitude lower than previous applications of the ^{15}N Gas-Flux method in highly fertilised agricultural systems (Baily et al. 2012, Bergsma et al. 2001, Cuhel et al. 2010, Graham et al. 2013) and in restored peatland soils (Tauchnitz et al. 2015). The estimated enrichment of the total soil NO_3^- pool was variable (2 – 40 ^{15}N at%, Supplementary Table 2) and this wide range was due to the fact that the tracer concentration was calculated based on the previous campaign's soil nitrate data, which in some cases did not reflect the soil nitrate content on the day of the tracer application a month later. It should be noted that the soil nitrate enrichment levels reported in this study correspond to the high end of the average soil NO_3^- pool enrichment (10 – 15 ^{15}N at%, Supplementary Table 2) for the period April 2013 to October 2014, which is presented in a separate publication (Sgouridis and Ullah 2015). To our knowledge, only Kulkarni et al. (2014) have applied the ^{15}N Gas-Flux method in the field with soil nitrate enrichment levels (5 ^{15}N at %) lower than in our study, but this had as a consequence poorly

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649 detected ^{15}N-N$_2$ fluxes. Nevertheless, for the organic soils the average tracer application

650 rate corresponded to current estimates of daily atmospheric N deposition (0.05 kg N ha^{-1} d^{-1}

651 ^{1}) in the UK (~ 15 - 20 kg N ha^{-1} y^{-1}) (Dore et al. 2012, Payne 2014), whilst for the grassland

652 soils the tracer application mimicked a daily fertiliser application rate of 0.5 kg N ha^{-1} d^{-1}.

653 Due to the inclusion of the NO$_3$$^-$-rich C-MW site in the woodland soils, tracer application

654 rates were higher than the daily atmospheric N deposition rates, but also reflecting internal

655 N cycling processes (e.g. nitrification) as an additional source of nitrate in these well-

656 drained forest soils. Therefore, the application of the ^{15}N tracer at these low rates should

657 not be expected to enrich the soil nitrate pool significantly, and potentially enhance the

658 denitrification activity, in excess of the amount of nitrogen normally deposited via natural

659 processes and common management practices.

660

661 The major assumptions of the ^{15}N Gas-Flux method and the associated 'non-equilibrium

662 equations' are that the denitrifying soil NO$_3$$^-$ pool is uniformly labelled with ^{15}N and that

663 the N$_2$ and N$_2$O originate from the same denitrifying pool (Stevens and Laughlin 1998).

664 The ^{15}N fraction in the denitrifying pool (^{15}X$_N$), calculated non-destructively from the

665 measured isotope ratios, ranged between 65 and 93 % and was well above the 10 %

666 threshold for the correct application of the 'non-equilibrium equations' (Lewicka-Szczebak

667 et al. 2013). However, the calculated ^{15}X$_N$ was higher than the estimated total soil NO$_3$$^-$

668 pool enrichment (range: 2 - 40 ^{15}N at %) suggesting non-homogeneous mixing of the added

669 tracer (98 ^{15}N at %) with the ambient soil nitrate at natural abundance despite our effort for

670 uniform tracer application with multiple injections across the investigated soil depth

671 (Ruetting et al. 2011). Wu et al. (2011) have optimised the number of injections and the

672 volume of tracer needed to achieve homogeneous labelling of a soil core (diameter 15 cm;

673 height 20 cm) and reported that 38 injections of 4 mL volume each were necessary. We

have used only 10 injections of 5- 20 mL volume (depending on the soil water content of each land use type) to minimise the disturbance of the soil matrix, particularly in the highly porous media such as peatland soils, and this was clearly sub-optimal for the homogenous labelling of the soil enclosure but probably a necessary compromise for large scale intensive measurements. We were not able to sample the soil within the chamber collars for directly estimating the $^{15}NO_3^-$ content of the soil pool due to time and budget constraints. However, in cases where destructive soil sampling was used to measure the soil nitrate pool enrichment (Kulkarni et al. 2014), the results were significantly different from the estimated enrichment due to sampling bias of the volume of soil affected by the tracer application. Non-uniform mixing of the ^{15}N label may lead to overestimation of the $^{15}X_N$ and underestimation of the denitrification flux rates (Boast et al. 1988). However, under field conditions, it is unlikely to achieve complete mixing of the added tracer with the ambient nitrate pool; and experimental studies (Mulvaney 1988, Mulvaney and Van den Heuvel 1988) have shown that the associated error is well-constrained and that accurate measurements can be made even with a less-uniformly labelled denitrifying pool. ~~The non-significant change of $^{15}X_N$ with incubation time suggested only one denitrifying pool for both N_2 and N_2O, assuming negligible N_2 production from anammox and co-denitrification (Spott and Stange 2007). Only Iin the case of the C-MW well-drained forest site, shown to exhibit the highest nitrification potential (Sgouridis and Ullah 2014), the slope of $^{15}X_N$ with time was negative suggesting dilution of the ^{15}N labelled soil NO_3^- pool by the oxidation of the ambient ammonium (nitrification). It is therefore possible that N_2 flux rates may be overestimated in C-MW, due to the underestimation of the $^{15}X_N$, but Bergsma et al. (1999) showed that temporal changes of the soil NO_3^- pool enrichment are negligible at ^{15}N enrichment levels similar to ours.~~

699   The larger volume of tracer per injection (>4 mL) in combination with the fewer number

700   of injections compared to Wu et al. (2011) may have created localised saturation effects

701   (saturated soil cylinders around the injection holes), even if the total soil moisture content

702   of the enclosure was not increased by more than 5%, which would require several hours to

703   equilibrate with the ambient soil moisture. We did not allow time for this soil moisture

704   equilibration to occur following the tracer injection to avoid significant loss of the added

705   nitrate via plant uptake (measurements occurring during the growth season). Therefore, it

706   is likely that in plots where denitrification activity may have been limited by soil moisture

707   (e.g. C-MW with mean WFPS 42 ± SE 0.76 %) the flux rates after 1 and 2 hours of

708   incubation may be overestimated due to moisture induced denitrification events.

709

710   Most studies using ^{15}N tracers and static chambers in highly fertilised systems typically

711   deploy their chambers between 1 and 2 hours (Baily et al. 2012, Cuhel et al. 2010,

712   Tauchnitz et al. 2015), but it has been shown that longer incubation periods (up to 24 or 48

713   hours) may be needed in case of low ^{15}N enrichment applications in intact soil cores (Morse

714   and Bernhardt 2013) and laboratory incubations (Yang et al. 2014) for a more precise and

715   accurate detectable ^{15}N-N_2 signal. However, it should be noted that in these cases the soil

716   cores or slurries were incubated in fully enclosed systems and were thus not affected by

717   potential bias from diffusion of evolved N_2 and N_2O to the subsoil (Clough et al. 2005).

718   The open-bottom, un-vented static chamber design used in this study in combination with

719   the extended incubation period up to 20 hours may have potentially allowed some loss of

720   the evolved N_2 and N_2O through downward subsoil diffusion and/or reduction of gas

721   exchanges at the soil-atmosphere interface due to ~~positive pressure build up in the chamber~~

722   ~~headspace~~decreasing concentration gradients (Healy et al. 1996). This could partly explain

723   the non-linear increase of the evolved N_2 and N_2O in the chamber headspace (Figures 2a &

724   b) and also the decrease of the N_2 flux rate with increasing incubation time (Figure 3a). The

725   N_2O flux rate increased up to 2 hours incubation followed by a decrease after 20 hours

726   consistently across land use types (Figure 3b), indicating that the extended enclosure period

727   lowered N_2O fluxes due to subsoil diffusion and enhanced N_2O reduction to N_2. and this

728   was an indication of potentially enhanced N_2O reduction due to both subsoil diffusion and

729   the increasing concentration of the N_2O in the topsoil. However, due to the high spatial

730   heterogeneity within each land use type, the mean N_2O flux rate was not significantly

731   different between the different incubation intervals. In other words, the non-linearity of

732   N_2O evolution had less effect on the flux rate estimation than the inherent spatial variability

733   within each land use type, which is in agreement with the findings of Chadwick et al.

734   (2014), who suggested that the spatial variability of N_2O fluxes far exceeds the bias due to

735   assumed linearity of fluxes.

736

737   The lack of a consistent pattern of N_2 flux rate change with incubation time among the

738   different land use types suggested a more complex temporal variability of N_2 fluxes that

739   apart from the duration of incubation could have also been affected by the distribution of

740   the added nitrate tracer. In the OS sites with the lowest average nitrate content (Table 2)

741   and the highest water filled pore space (Mean WFPS: C-PB = 70 ± SE 3.21 %; C-UG = 66

742   ± SE 1.58 %; R-HL = 69 ± SE 2.00 %), non-homogeneous tracer distribution ($^{15}X_N = 90$

743   ^{15}N at%) could have led to the creation of hotspots of denitrification activity due to substrate

744   availability resulting in potentially overestimated flux rates in the first or even the second

745   hour of incubation. However, analytical uncertainty due to fluxes being close to the limit

746   of detection could not be ruled out. Conversely, in the soil moisture limited forest site (C-

747   MW), the injection of even 50 mL of tracer solution could have led to an increased moisture

748   induced denitrification activity within the first 1 – 2 hours of incubation, until the added

749 water started to equilibrate with the ambient soil moisture. Therefore the N_2 flux rate in C-

750 MW may be significantly overestimated after 1 hour of incubation. In the grassland sites

751 and the R-DW forest site with intermediate soil moistures (Mean WFPS: R-DW = 65 ± SE

752 1.79 %; R-UG = 64 ± SE 1.41 %; C-IG = 60 ± SE 1.45 %; R-IG = 61± SE 2.46 %) and

753 nitrate content, the tracer injection is unlikely to have significantly affected the

754 denitrification rate when all the conditions (i.e. soil moisture and substrate availability)

755 were favourable, and therefore flux rates estimated after one hour of incubation should be

756 more reliable as long as the bias from analytical uncertainty was low. In these sites

757 denitrification rates estimated after one or 20 hours of incubation were not significantly

758 different (Figure 3a), suggesting a quasi-linear N_2 evolution throughout the incubation

759 period (at least in 37.5% of the sampling plots, see Supplementary Table 4). However, the

760 N_2 flux rates were significantly lower after 2 hours of incubation, whereas the N_2O flux

761 rates were maximum at 2 hours of incubation consequently leading to an increased product

762 ratio $N_2O/ (N_2 + N_2O)$ (Supplementary Table 6). This observation could potentially be

763 explained by a delay in the *de novo* synthesis of denitrification enzymes and the fact that

764 the N_2O reductase enzyme,is known to have a slower expression than the preceding

765 reduction enzymes (Knowles, 1982), leading to N_2O accumulation and lower N_2 production

766 after 2 hours of incubation. After 20 hours incubation, the decrease in the product ratio

767 could be explained by a higher reduction rate of N_2O to N_2 due to probably higher N_2O

768 reductase activity but also slower soil-atmosphere exchange of N_2O due to the decreasing

769 concentration gradient (Healy et al. 1996).

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771 It has been shown that the N_2 flux estimation with the [15]N Gas Flux method is sensitive to

772 the incubation time interval and the homogeneity of the tracer distribution due to the

773 combination of several antagonistic effects such as decreasing gas diffusion gradients and

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soil moisture and substrate availability effects due to the added tracer solution. The uncertainty in the estimated in situ N_2 fluxes can be significantly reduced if additional effort is made to increase the homogeneity of the tracer application by increasing the number of injections while reducing the volume of the applied tracer (particularly in soils where denitrification is limited by moisture). Moreover, allowing the equilibration of the added tracer solution with the ambient soil water before gas sampling commences and by closely monitoring the linear evolution of the produced gases with more frequent gas sampling at shorter equal incubation intervals could help in identifying the appropriate length of incubation, thus avoiding potential over-estimation of denitrification in nitrate and moisture limited ecosystems and potential under-estimation due to subsoil diffusion of evolved gases. The detailed uncertainty analysis of the N_2 and N_2O flux estimation presented in this study complements the large scale application of the ^{15}N Gas Flux method in the same land use types between April 2013 and October 2014 for estimating annual rates of denitrification and N_2O emission, which is presented in Sgouridis and Ullah (2015).

The minimum detectable N_2 and N_2O fluxes depend on the precision of the IRMS systems, the soil NO_3^- pool enrichment and the incubation parameters, such as the dimensions of the static chamber and the incubation time (Bergsma et al. 2001, Stevens and Laughlin 2001). For our chamber design, an incubation time of up to 20 hours (which integrates the equilibration of the added tracer solution within the soil enclosure), and using the estimated MDC values (for both N_2 and N_2O) for calculating a $^{15}X_N$ value of 0.660 ^{15}N at%, the minimum detectable flux rates were 4 μg N m^{-2} h^{-1} and 0.2 ng N m^{-2} h^{-1} for the N_2 and N_2O fluxes respectively. These were significantly better than the minimum rates (175 - 900 μg N_2-N m^{-2} h^{-1} and 0.04 - 0.21 μg N_2O-N m^{-2} h^{-1}) reported by Bergsma et al. (2001), Kulkarni et al (2014) and Tauchnitz et al (2015), using similar field ^{15}N tracer approaches, and

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799 comparable to the minimum rates measured by a high precision ^{15}N gas flux approach in a

800 laboratory soil incubation (Yang et al. 2014) and the gas-flow soil core method (8 μg N_2-

801 $N m^{-2} h^{-1}$ and < 1 μg N_2O-$N m^{-2} h^{-1}$) by Wang et al. (2011). Our N_2 fluxes from woodland

802 soils compare well with the rates reported in the literature for restored forested wetlands in

803 North America (Morse and Bernhardt 2013) and with the rates from northern hardwood

804 forests in US (Kulkarni et al. 2014), using ^{15}N tracers at similar or lower application rates

805 to ours. Our results are also comparable to the rates reported from central European forests,

806 under similar atmospheric N deposition rates, using the gas-flow soil core method

807 (Butterbach-Bahl et al. 2002). For the grassland soils, the N_2 fluxes measured in the present

808 study were significantly lower than previous applications of the ^{15}N Gas-Flux method at

809 high fertiliser application rates (Baily et al. 2012, Cuhel et al. 2010, Graham et al. 2013),

810 whilst for the organic soils our rates were significantly lower than the ones reported by

811 Tauchnitz et al. (2015) since their ^{15}N tracer application rate (30 kg $N ha^{-1}$) was 300 times

812 higher than ours. The N_2O fluxes were up to 200 times lower than the N_2 fluxes leading to

813 low denitrification product ratios in all land use types, a result which is in line with the N_2O

814 yields reported from ^{15}N tracer studies in forest (Kulkarni et al. 2014, Morse and Bernhardt

815 2013) and grassland soils (Baily et al. 2012, Bergsma et al. 2001). In the present study we

816 have compared the in situ denitrification rates between three major land use types using an

817 extended field incubation period to increase the probability of detecting a reliable ^{15}N-N_2

818 signal, particularly under conditions of low denitrifier activity due to seasonality of

819 denitrification and/or inherent capacity of soils (for example organic and deciduous forest

820 soils). However, these rates should be considered conservative since confounding issues

821 such as subsoil diffusion and non-homogeneous labelling of the soil nitrate pool may in

822 some cases have led to underestimations of the in situ denitrification rates.

823

824   4.3. Comparison with the AIT

825   The total denitrification rates measured with the C_2H_2 amended intact soil cores followed

826   the same trend as the total denitrification (N_2 and N_2O fluxes combined) from the ^{15}N Gas-

827   Flux measurements, while they were on average 168 times lower than the denitrification

828   potential measured in the same land use types in anaerobic soil slurries amended with

829   acetylene and nitrate in a previous study (Sgouridis and Ullah 2014), thus reflecting lower

830   in situ rates. The AIT denitrification rates were between 3 and 5 times lower than the ^{15}N

831   Gas-Flux rates despite the fact that the AIT intact soil cores were capped at the bottom,

832   thus not allowing any subsoil diffusion of the evolved gases due to denitrification.

833   Therefore, the AIT rates should have been higher than the ^{15}N Gas-Flux rates if serious

834   underestimation was occurring due to subsoil diffusion in the open-bottom static chambers,

835   which was not the case. Adding nitrate to the C_2H_2 amended cores would have been

836   desirable for directly evaluating the priming effect of the added substrate on denitrification

837   rates. The ^{15}N tracer addition to the static chambers corresponded to the amounts of N

838   naturally deposited in these land use types either via management practices and/or

839   atmospheric deposition, thus avoiding excessive N fertilisation of the sampling plots.

840   However, it cannot be conclusively argued that the same amount of applied nitrate would

841   not have led to similar denitrification rates between the AIT and the ^{15}N Gas-Flux methods.

842   Previous comparisons between the AIT and the ^{15}N tracer method in field studies showed

843   no significant difference between the two methods in measuring *in situ* total denitrification

844   rates when tracer is applied at high fertilisation rates (50 - 200 kg N ha^{-1}) and relatively low

845   soil moisture contents (WFPS: 40 - 60 %) (Aulakh et al. 1991, Mosier et al. 1986).

846   Conversely, in laboratory incubations it was shown that the AIT significantly

847   underestimated total denitrification compared to the ^{15}N tracer approach (Yu et al. 2010)

848   and the direct N_2 flux approach (Qin et al. 2012) due to the incomplete inhibition of N_2O

35

849 reduction to N_2 by C_2H_2 in wet soils (Yu et al. 2010) or in soils with low nitrate content

850 (Qin et al. 2013, Qin et al. 2014). In our study, the soil WFPS ranged between 60 and 70

851 % in all land use types, with the exception of the C-MW site (mean WFPS 42 %), whilst

852 the ^{15}N-NO_3^- tracer application rate was low (< 1 kg N ha^{-1}). Moreover, the disturbance of

853 the soil structure during the extraction of the soil cores and the effect of the acetylene

854 addition to microbial activity were not significant as it was suggested by the similar CO_2

855 production rates (Aulakh et al. 1991), representing soil respiration (Felber et al. 2012), in

856 the static chambers and the C_2H_2 amended and un-amended intact soil cores. Therefore, we

857 could argue that it is possible that the AIT underestimated total denitrification rates

858 compared to the ^{15}N Gas-Flux method due to the likely incomplete inhibition of N_2O

859 reduction to N_2 under relatively high soil moisture contents, although the shorter incubation

860 time (2h for the intact cores) may have limited the ability of C_2H_2 to fully equilibrate within

861 soil pore spaces. Other confounding factors such as the catalytic decomposition of NO in

862 the presence of C_2H_2 (Bollmann and Conrad 1996, Nadeem et al. 2013) may have also

863 contributed to the lower denitrification rates measured by the AIT. This study has

864 confirmed some of the drawbacks of the AIT as a quantification method of in situ

865 denitrification rates compared to the ^{15}N Gas-Flux.

866

867 The estimation of the denitrification product ratio using the AIT method, from the un-

868 amended cores (N_2O only) and the C_2H_2 amended cores (N_2 + N_2O), is usually

869 overestimated since the source of N_2O cannot be discriminated with the AIT, whilst the N_2

870 flux is underestimated due to the incomplete inhibition of N_2O reduction (Butterbach-Bahl

871 et al. 2013). This was confirmed in the present study for all the land use types and even the

872 maximum denitrification product ratio after 2 hours incubation in the case of the grassland

873 soils (23 %), was still significantly lower than the respective ratio from the AIT (50 %).

874   Therefore, the much lower denitrification product ratio estimated from the ^{15}N Gas-Flux

875   measurements is significantly more reliable and the wider application of this field technique

876   across a range of land use types can have important implications for evaluating the role of

877   denitrification as a reactive nitrogen sink and as a source of N_2O emissions (Butterbach-

878   Bahl et al. 2013, Kulkarni et al. 2008).

879

880   **5. Conclusion**

881   The improved analytical precision for both ^{15}N-N_2 and ^{15}N-N_2O analyses allowed us to

882   quantify in situ N_2 and N_2O fluxes with low ^{15}N tracer addition under field conditions in

883   natural and semi-natural land use types for the first time. The estimation of N_2 fluxes was

884   sensitive to the incubation time interval and the homogeneity of the tracer distribution due

885   to the combination of several antagonistic effects such as decreasing gas diffusion gradients

886   over time and soil moisture and substrate priming effects due to the added nitrate tracer

887   solution. The spatial variability of N_2O fluxes superseded any bias associated with non-

888   linear fluxes due to the extended incubation period. The uncertainty in the estimated N_2 and

889   N_2O fluxes can be significantly reduced by increasing the homogeneity of the tracer

890   application and by closely monitoring the linear evolution of the produced gases with more

891   frequent gas sampling at shorter equal incubation intervals to avoid under or over estimation

892   of denitrification. Comparing the ^{15}N Gas-Flux method with the AIT confirmed the

893   drawbacks of the AIT as a reliable quantification method of in situ denitrification rates.

894   Moreover, the AIT method overestimated the denitrification product ratio compared to the

895   ^{15}N Gas-Flux method. The ^{15}N Gas-Flux method holds much promise as a more reliable

896   field technique for measuring in situ denitrification rates and its wider application across a

897   range of terrestrial ecosystems can lead to its refinement and improvement and in the long

term can significantly contribute to our understanding of the role of denitrification as a reactive nitrogen sink.

6. Acknowledgements

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Tables

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1098 **Table 1:** Measured ratios of R29 and R30 for N_2 in ambient air (n=10), ratios of R45 and R46

1099 in standard N_2O gas (0.5 ppm concentration, n=15) and ^{15}N at% abundance calculated from the

1100 respective ratios for both gases. SD; standard deviation, CV; coefficient of variation.

	R29 (N_2)	R30 (N_2)	R45 (N_2O)	R46 (N_2O)	^{15}N at% (N_2)	^{15}N at% (N_2O)
Mean	$7.38 \ 10^{-3}$	$5.16 \ 10^{-5}$	$8.00 \ 10^{-3}$	$2.21 \ 10^{-3}$	$3.71 \ 10^{-1}$	$3.88 \ 10^{-1}$
SD	$2.77 \ 10^{-7}$	$2.26 \ 10^{-7}$	$1.25 \ 10^{-5}$	$1.04 \ 10^{-5}$	$2.09 \ 10^{-5}$	$1.01 \ 10^{-3}$
CV (%)	0.00	0.44	0.16	0.47	0.01	0.26

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1106 **Table 2:** The ambient soil nitrate pool, the ^{15}N tracer application rate, the estimated enrichment

1107 of the total soil nitrate pool, the calculated $^{15}X_N$ value from N_2O and the slope of the $^{15}X_N$

1108 change with incubation time in the three land use types. Data are means with standard errors in

1109 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}N at %)	$^{15}X_N$ (%)	$^{15}X_N$ slope
Organic Soil (n=3)	0.53 (0.44)	0.04 (0.02)	25 (11.8)	90 (1.5)	0.003 (0.0054)
Woodland (n=2)	3.86 (2.42)	0.62 (0.41)	13 (0.7)	79 (8.3)	-0.007 (0.0025)

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Grassland (n=3)	1.81 (0.96)	0.51 (0.19)	24 (5.1)	81 (8.4)	0.000 (0.0037)

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

^{15}N Gas-Flux	F	P
Denitrification	19.4	< 0.001
N_2O emission	31.1	< 0.001
$N_2O/ (N_2 + N_2O)$	7.4	< 0.01
Total bulk N_2O	19.4	< 0.001
CO_2 production	19.8	< 0.001
AIT		
Denitrification	12.7	< 0.001
Total bulk N_2O	9.4	< 0.01
$N_2O/ (N_2 + N_2O)$	0.3	> 0.05
CO_2 production (un-amended cores)	11.2	< 0.001
CO_2 production (C_2H_2 amended cores)	11.7	< 0.001

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Figures

Nitrogen Prep Unit

He carrier

10µl syringe

Injection port

Cu reduction furnace

CO

CO_2

N_2O

10µl LN2 trap

Carbosorb trap $Mg(ClO_4)_2$

H_2O

IRMS

N_2

Open split

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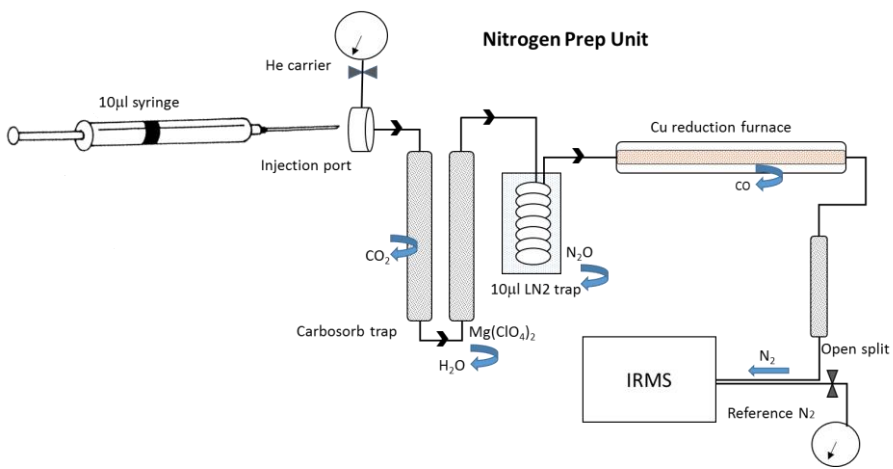
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Figure 1: Schematic of the ^{15}N-N_2 analysis system

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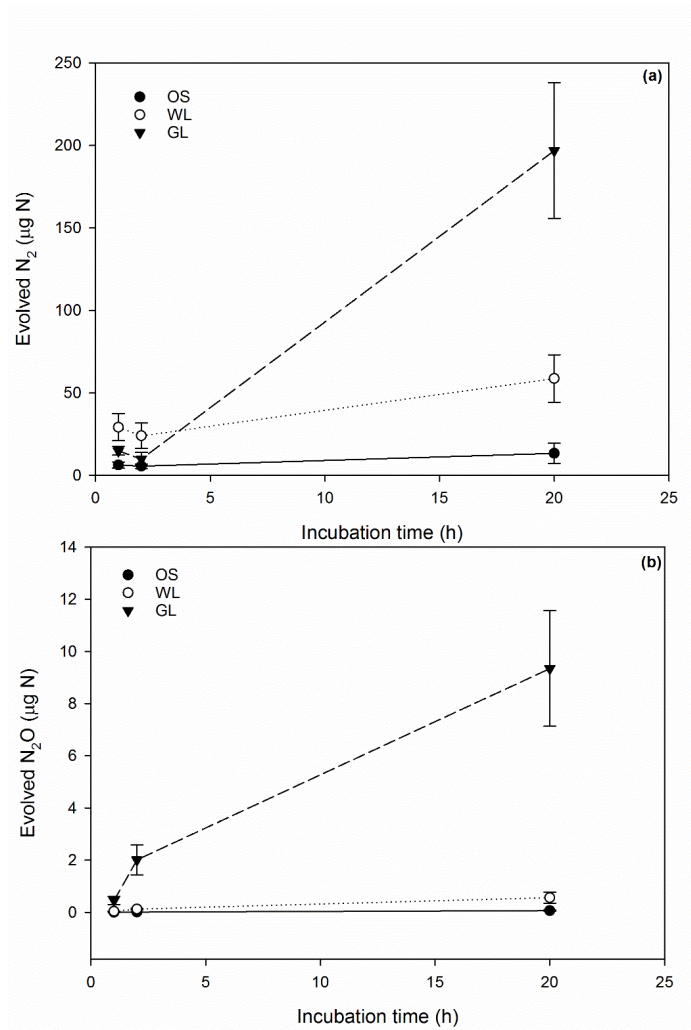
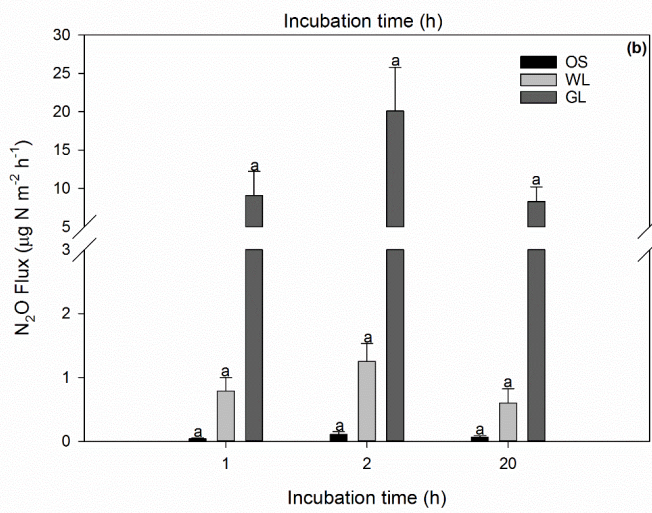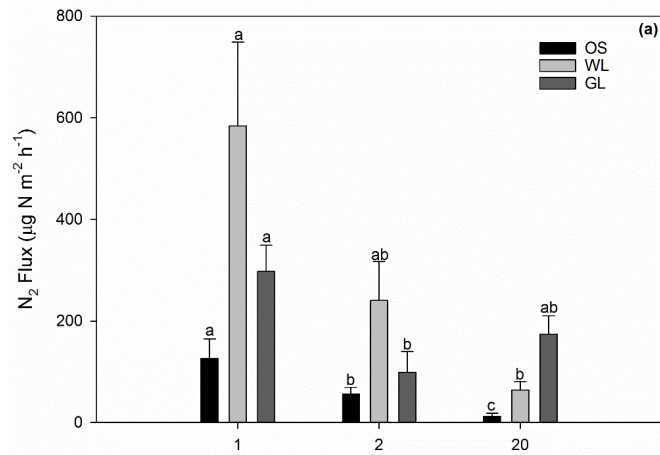

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Figure 2: Evolved (a) N_2 and (b) N_2O gas measured between 1, 2 and 20 hours incubation time intervals using the ^{15}N Gas-Flux method in the organic soil (OS), woodland (WL) and grassland (GL) land use types. Data points are means and the error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

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

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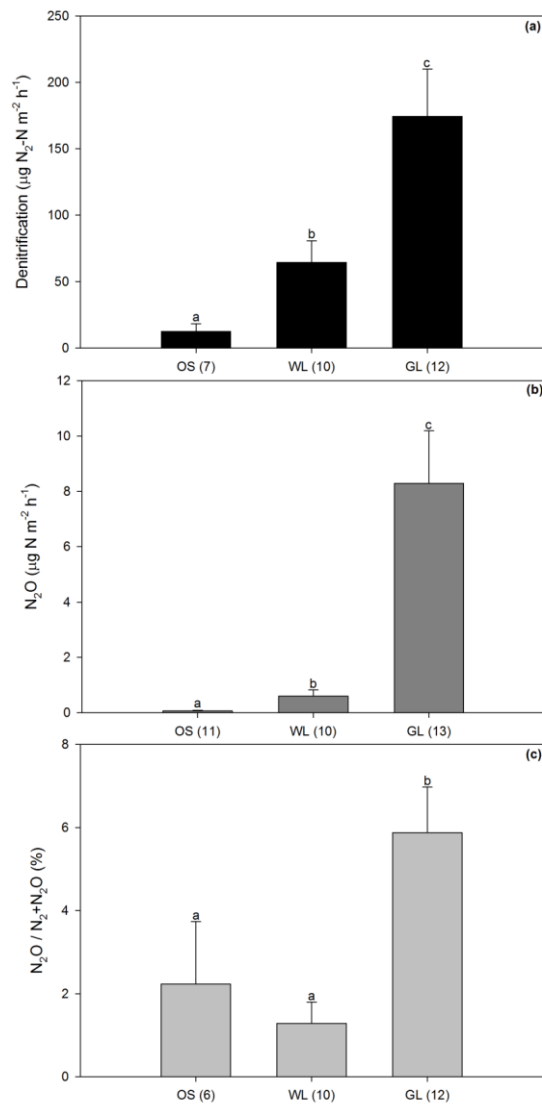
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Figure 4: Mean rates of: (a) N_2 flux, (b) N_2O emission due to denitrification and (c) the
denitrification product ratio $N_2O/ (N_2 + N_2O)$ in the three land use types (OS; organic soils,
WL; woodland and GL; grassland). Same lower case letters indicate no significant differences
($p > 0.05$) between land use types according to One-way ANOVA and the Games-Howell *post
hoc* test. The sample size (n) is given in parenthesis for each land use type on the x-axis. Error
bars represent standard errors.
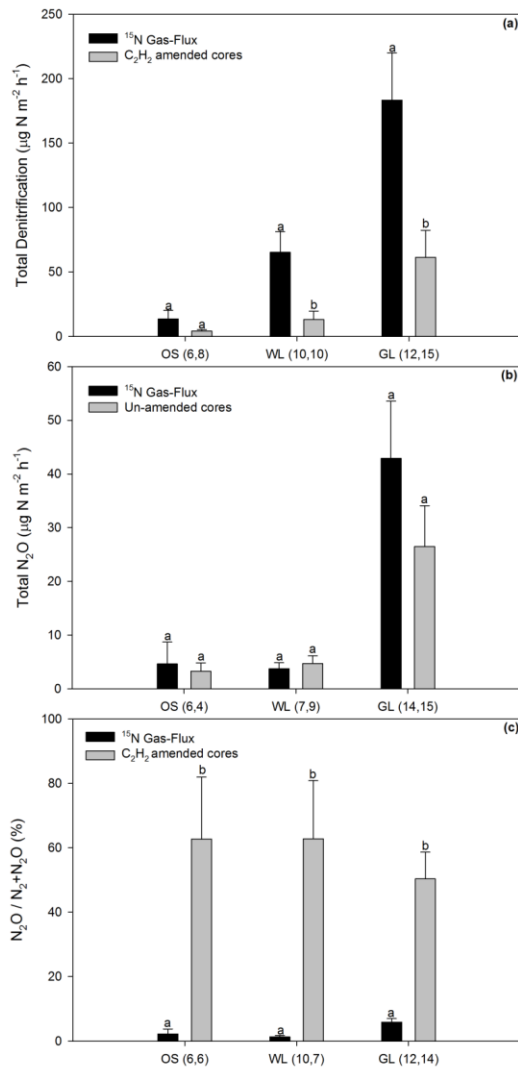
1158

Figure 5: (a) Mean total denitrification measured with the ^{15}N Gas-Flux method and the AIT,
(b) Mean bulk N_2O emission measured in the static chambers of the ^{15}N Gas-Flux method and
in un-amended intact soil cores and (c) the denitrification product ratio $N_2O/ (N_2 + N_2O)$ with
the ^{15}N Gas-Flux method and the AIT in the three land use types (OS; organic soils, WL;
woodland and GL; grassland). Same lower case letters indicate no significant differences ($p >$
0.05) between measurement methods according to independent samples t-test. The sample size
(n) is given in parenthesis for each land use type and each method on the x-axis. Error bars
represent standard errors.
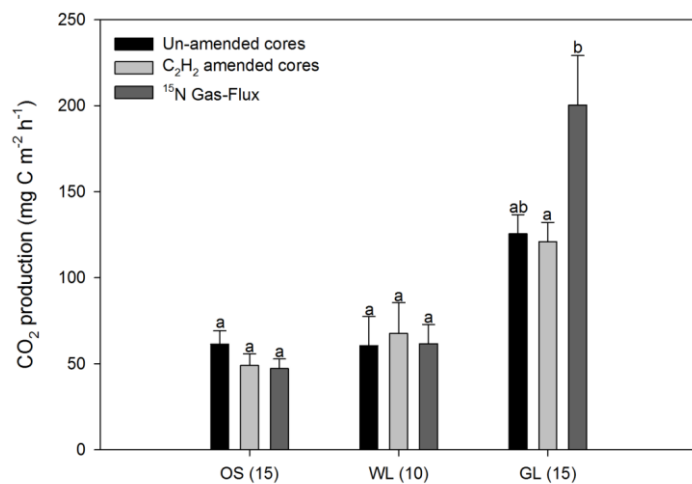
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Figure 6: Mean CO_2 production measured in the static chambers of the [15]N Gas-Flux method, in un-amended and C_2H_2 amended intact soil cores in the three land use types (OS; organic soils, WL; woodland and GL; grassland). Same lower case letters indicate no significant differences ($p > 0.05$) between measurement methods according to independent samples t-test. The sample size (n) is given in parenthesis for each land use type on the x-axis. Error bars represent standard errors.

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