Denitrification as a Source of Nitric Oxide Emissions from incubated Soil Cores from a UK Grassland Soil

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Abstract

Agricultural soils are a major source of nitric oxide (NO) and nitrous oxide (N_2O), which are produced and consumed by biotic and abiotic soil processes. The dominant sources of NO and N_2O are microbial nitrification and denitrification. While N_2O emissions have been attributed to both processes, depending on the environmental conditions such as substrate availability, pH and water filled pore space (WFPS), NO emissions are thought to predominantly derive from nitrification. Although attributing gaseous emissions to specific processes is still difficult, recent findings challenge the latter of those assumptions. Using the gas-flow-soil-core method, i.e soil cores incubated under a He/O_2 atmosphere at constant surface gas flow, combined with ^{15}N labelled isotopic techniques, the present study investigated the role of denitrification on NO, N_2O and N_2 emissions in a UK grassland soil under high soil moisture and an aerobic headspace atmosphere. With the application of KNO3 and glucose to support denitrification, denitrification was the source of N loss of between 0.61 and 0.67% of the added N via NO emissions, 1.60 to 1.68% via N_2O and 0.03 to 0.05% via N_2 emissions. Overall, our study showed that denitrification has been overlooked as a source of NO emissions.

1. Introduction

Agricultural soils are the dominant source of nitrous oxide (N_2O), a potent greenhouse gas and a major cause of ozone layer depletion (IPCC, 2007; Ravishankara et al., 2009). Other gaseous forms of nitrogen (N) are lost from agricultural soils, such as N_2 which together with N_2O represents less N available for crop growth. Soils also act as a significant source of nitric oxide (NO), which catalyses the formation of ground level ozone, affecting human health and vegetation (Crutzen, 1981), and contributes to the formation of acid rain and

the eutrophication of semi-natural ecosystems. Microbial denitrification is often the dominant process generating N_2O, and as such, intense investigations (i.e. >1,000 published studies) have led to a good understanding of the abiotic factors regulating N_2O emissions via denitrification (Beaulieu et al., 2011). However, the role of this process on NO emissions remains largely unexplored, apart from a few studies (Wang et al., 2011; Wang et al., 2013), even though NO is an obligatory intermediate of N_2O formation in denitrification (Wolf and Russow, 2000; Russow et al., 2009).

Most experiments suggest that NO emitted from soils is mainly produced through nitrification (Skiba et al., 1997), whereas that produced from denitrification is further reduced to N_2O before it escapes to the soil surface (Skiba et al., 1997). This is attributed to high soil water content (it has been shown that at a WFPS above 70%, N_2O was produced solely by denitrification (Bateman and Baggs, 2005)), soil compaction and fine soil texture (sieved to <2 mm) creating low diffusivity for gases, which increases the residence time and the potential for further reduction when denitrification conditions dominate. Recent findings, however, challenge these assumptions. Using the gas-flow-soil-core technique, which has been proven to be a reliable tool for quantifying emissions from denitrification, Wang et al. (2013) observed significant NO fluxes from nitrate (NO_3^-) amended soils. Attributing these emissions specifically to denitrification has remained elusive due to methodological constraints to elucidate the underlying microbial production and consumption processes. Previous efforts to identify these processes have mostly relied on acetylene inhibition and isotope labelling techniques (Baggs, 2008).

Isotope analysis has emerged as a way to identify the source and thereby the processes from which N_2O is being produced (Arah, 1997). It is also known that microorganisms discriminate against the heavier molecule (e.g ^{15}N vs. ^{14}N), preferring to use the lighter

70 molecule which requires less energy to break the bonds (Kendall and Caldwell, 1998). This
71 should be considered when applying labelled substrate to investigate microbial processes.

72 The aim of this study was to explore the potential role of denitrification as a significant
73 source of NO emissions. We hypothesise that denitrification can be a major source of NO
74 emissions in a UK grassland soil under high moisture content. This study uses the gas-flow-
75 soil-core technique (Cárdenas et al., 2003), further developed to include NO
76 measurements, combined with isotopic analyses. A ^{15}N labelled substrate as well as an
77 unlabelled substrate at the same application ratio was used to determine whether there
78 was an effect of the labelled N on the investigated processes at a 5 atom% enrichment.
79 Additionally to adding potassium nitrate (KNO_3) as N source, glucose was added to supply a
80 readily available C source and thereby promote denitrification. During denitrification C is
81 used as electron donor and C availability is one factor controlling denitrification rates and
82 compared to other C-compounds, denitrification tends to be most stimulated after
83 addition of ethanol or glucose (Morley and Baggs, 2010).

84

85 **2. Materials and Methods**

86 *2.1. Soil preparation*

87 A clayey pelostagnogley soil of the Hallsworth series (Clayden and Hollis, 1984) (44% clay,
88 40% silt, 15% sand (w/w), Table 1) was collected on the 4th of November 2013 from a
89 typical grassland in SW England, located at Rothamsted Research, North Wyke, Devon, UK
90 (50°46'10''N, 3° 54'05''W). Spade-squares (20 x 20 cm to a depth of 15 cm) of soil were
91 taken from 12 locations along a 'W' line across a field of 600 m^2 size. After sampling, the
92 soil was air dried to ~30% H_2O (dry basis), roots and plant residue were removed and the

93 soil sieved to <2 mm and stored at 4°C for 5 days before packing into cores and starting the
94 incubation.

95 <Table 1: initial soil characteristics>

96

97 *2.2. Experimental setup*

98 The incubation was carried out using the DENItrification System (DENIS), a specialized gas-
99 flow-soil-core incubation system (Cárdenas et al., 2003). Twelve cores were packed with
100 soil to a bulk density of 0.8 g cm^{-3} and a height of 75 mm into stainless steel vessels of 140
101 mm diameter. To ensure denitrification conditions, the soil moisture was adjusted to 85%
102 WFPS, taking the later amendment into account. This WFPS was similar to those used in
103 previous studies to promote denitrification processes (Meijide et al., 2010; Bergstermann
104 et al., 2011). In order to measure N_2 fluxes the native atmosphere was removed by flushing
105 the soil cores from the bottom with a mixture of $He:O_2$ (80:20) at 30 ml min^{-1} for 14 hours
106 Flow rates were then decreased to 12 ml min^{-1} and the flow re-directed over the surface of
107 the soil core for three days before amendment application to measure baseline emissions.
108 O_2 was kept in the gas mixture at atmospheric levels as the objective was to investigate
109 denitrification achieved by high WFPS instead of forcing anaerobic conditions by
110 preventing any O_2 diffusion.

111 The following treatments were applied to four replicate vessels: (a) labelled (^{15}N-labelled
112 KNO_3 at 5 atom% and glucose); (b) unlabelled (KNO_3 and glucose); (c) control (water only).
113 The labelled and unlabelled treatments contained nitrogen at a rate equivalent to 75 kg N
114 ha^{-1} (i.e. 121.5 mg N kg^{-1} dry soil) and C as glucose at 400 kg C ha^{-1} (i.e. 648 mg C kg^{-1} dry
115 soil), which is similar to previous studies (Meijide et al., 2010; Bergstermann et al., 2011).
116 The amendment for each core was dissolved in 50 ml distilled water, and the controls also

117 received 50 ml distilled water each. The vessels were kept at 20°C during the whole
118 incubation period, which lasted for 10 days after amendment application.

119

120 *2.3. Gas analyses and data manipulation*

121 Gas samples were taken every two hours for each vessel. Fluxes of N_2O and CO_2 were
122 quantified using a Perkin Elmer Clarus 500 gas chromatograph (Perkin Elmer Instruments,
123 Beaconsfield, UK) equipped with an electron capture detector (ECD) for N_2O, and with a
124 flame ionization detector (FID) and a methanizer for CO_2. N_2 emissions were measured by
125 gas chromatography with a helium ionisation detector (VICI AG International, Schenkon,
126 Switzerland) (Cárdenas et al., 2003), while NO concentrations were determined by
127 chemiluminescence (Sievers NOA280i, GE Instruments, Colorado, USA). All gas
128 concentrations were corrected for the surface area and flow rate going through the vessel
129 (measured daily). Fluxes were calculated on a kg N or C ha^{-1} day^{-1} basis.

130

131 *2.4. Isotopic analyses of N_2O*

132 Gas sampling times for ^{15}N analysis were pre-determined based on data from previous
133 experiments (data not shown). Samples were taken just before (0 hours) and 4 hours after
134 amendment application, then every 24 hours for the first week, followed by a final sample
135 at day 10. This sampling strategy was decided on from previous experimental results to
136 cover changes in isotopic signature before amendment application, as well as during the
137 NO and N_2O peaks (4-5 h and 3-4 d, respectively), and after emissions returned to
138 background levels. Samples were taken from the outlet line of each vessel using 12 ml
139 exetainers (Labco) which had previously been flushed with He and evacuated. ^{15}N

enrichment of N_2O was measured using a TG2 trace gas analyser (Europa Scientific, now Sercon, Crewe, UK) and Gilson autosampler, interfaced to a Sercon 20-22 isotope ratio mass spectrometer (IRMS). Solutions of 6.6 and 2.9 atom% ammonium sulphate ($(NH_4)_2SO_4$) were prepared and used to generate 6.6 and 2.9 atom% N_2O (Laughlin et al., 1997) which were used as reference and quality control standards.

The process leading to the formation of the measured N_2O, i.e. whether it is produced by nitrification or denitrification, was determined by calculating how much of the N_2O was derived from NO_3^- as the parent molecule. When ^{15}N labelled NO_3^- is added, it is assumed that it completely mixes with the native soil NO_3^- pool to form a single uniformly labelled NO_3^- pool. The ^{15}N content of the N_2O was calculated from either ^{45}R or ^{46}R, with ^{45}R being the ratio of the ion currents (I) for mass 45 /44 ($^{45}R = {}^{45}I/{}^{44}I$) and ^{46}R for mass 46/44 ($^{46}R = {}^{46}I/{}^{44}I$). If the ^{15}N contents of the measured N_2O calculated from either ^{45}R or ^{46}R are equal, then the distribution of the ^{15}N atoms in the N_2O molecules is random, and therefore the N_2O originated from a single uniformly labelled NO_3^- pool (Stevens et al., 1997; Stevens and Laughlin, 1998). When the NO_3^- pool is labelled and the N_2O concentration is greater than the IRMS method detection limit (2 ppm), calculations of the fraction of N_2O derived from the denitrifying pool (d'_D) were performed. The sources of N_2O were then apportioned into d'_D and the fraction derived from the nitrifying pool ($d'_N = (1 - d'_D)$) and calculated as described in Arah (1997). In Arah's equation N_2O d'_D is the fraction of the emitted N_2O which is derived from the ^{15}N labelled, denitrifying NO_3^- pool. A N_2O d'_D value of unity (1.00) indicates that 100% of the N_2O emitted derived from the NO_3^- pool.

To determine the source of the measured N_2O, i.e. how much of it was derived from the amendment ($N_2O\_N_{amend}$) rather than the native soil N, the following equation was used for the labelled treatments (Senbayram et al., 2009):

$$N_2O\_N_{amend} = N_2O\_N_{total}\left(\frac{^{15}\mathrm{N}at\%ex_{sample}}{^{15}\mathrm{N}at\%ex_{fert}}\right) \quad (1)$$

where $N_2O\_N_{total}$ = total emissions of N_2O from the soil; $^{15}\mathrm{N}at\%ex_{sample}$ = ^{15}N atom% excess of the emitted N_2O (^{15}N atom% of the measured sample minus the mean natural ^{15}N abundance of background N_2O obtained in our experiment (0.366 atom %)); $^{15}\mathrm{N}at\%ex_{fert}$ = ^{15}N atom% excess of the applied amendment solution.

2.5. Soil analyses

Soil samples were taken at the beginning and end of the incubation to determine the initial and final moisture contents and the NH_4^+ and total oxidised N (TON: NO_3^- + NO_2^-) concentrations. Nitrite (NO_2^-) is generally thought to accumulate very rarely in nature, and it has been shown that NO_2^- is rapidly mineralised in soil (Paul and Clark, 1989; Burns et al., 1995, 1996). It is therefore assumed that NO_2^- concentrations in the soil samples are negligible, and TON is nearly exclusively made up of NO_3^-. For the final soil analyses, each core was divided in half to separate the top section from the bottom section. WFPS was calculated from soil moisture contents by drying a subsample (50 g) at 105°C overnight. Soil NH_4^+-N and TON were analysed by automated colorimetry from 2M KCl soil extracts using a Skalar SAN^{PLUS} Analyser (Skalar Analytical B.V., Breda, Netherlands) (Searle, 1984). ^{15}N abundance of NO_3^- and NH_4^+ was measured by quadrupole mass spectrometer (GAM 200, InProcess, Bremen, Germany) (as described by Stange et al. (2007) at the Thünen Institute of Climate Smart Agriculture (Brauschweig, Germany)). Briefly, NO_3^- was reduced to NO by Vanadium chloride ($V(III)Cl_3$) and NH_4^+ was oxidized to N_2 by Hypobromite (NaOBr). NO and N_2 were the gases measured.

187 *2.6. Statistical analysis*

188 Statistical analysis was performed using GenStat 16th edition (VSN International Ltd). Prior
189 to the statistical tests all data were analyzed to proof their normal distribution
190 (Kolmogorov-Smirnov test) and equality of variance (Levene test). Cumulative emissions of
191 NO, N_2O, N_2 and CO_2 were calculated from the area under the curve after linear
192 interpolation between sampling points. Differences in total emissions for each gas
193 measured between treatments as well as differences in soil characteristics between
194 treatments and between top and bottom of soil cores were assessed by ANOVA at $P < 0.05$.
195 Where treatment effects proved to be significant, Fisher's Least Significant Test (LSD) was
196 used as *post hoc* test to ascertain differences among treatment levels.

197

198 **3. Results**

199 *3.1. Gas emissions*

200 CO_2 fluxes showed constant emissions of 10 kg C ha^{-1} d^{-1} before and after the CO_2 peak (day
201 0-6) in all vessels. N_2 emissions increased at the moment the amendment was applied, but
202 decreased immediatelly after until day 3.5 when they reached background levels, before
203 increasing again. In order to show CO_2 and N_2 emissions attributed to amendment
204 application only, the fluxes were adjusted by subtracting background emissions. There
205 were no significant differences in fluxes, or cumulative emissions for any of the measured
206 gases between the labelled and unlabelled treatments (Table 2). Both treatments,
207 however, were significantly higher than the control for all gaseous emissions measured,
208 except for N_2.

209 Nitric oxide emissions peaked 14 hours after amendment application (Fig. 1), with
210 maximum average fluxes of 0.58 and 0.70 kg N ha^{-1} d^{-1}, for the labelled and unlabelled

211 treatment, respectively. Fluxes decreased afterwards resulting in values below 0.1 kg N ha^{-1}
212 d^{-1} 30 hours after amendment application. Fluxes then decreased further to below 0.05 kg
213 N ha^{-1} d^{-1}, before showing a linear increase over 5 days to values of around 0.1 kg N ha^{-1} d^{-1}
214 until the end of the experiment. Losses of N via NO emissions represented 0.61 and 0.67%
215 of the N added. The control treatment showed negligible fluxes of NO over the whole
216 experimental period.

217 Similar to NO, emissions of N_2O increased immediately after amendment application. After
218 14 hours, N_2O showed a first maximum of 0.24 and 0.17 kg N ha^{-1} d^{-1} for the labelled and
219 unlabelled treatment, respectively (Fig. 1). In both treatments fluxes decreased over the
220 following 12 h by 0.02 kg N ha^{-1} d^{-1} before increasing again to a maximum of 0.45 and 0.44
221 kg N ha^{-1} d^{-1}, 3.3 and 3.8 days after amendment application, respectively. Total losses of
222 N_2O represented 1.60 and 1.68% of the N applied for the labelled and unlabelled
223 treatment, respectively. Again the control treatment maintained significantly lower fluxes
224 than the fertilized treatments over the whole experimental period.

225 Gaseous nitrogen (N_2) fluxes (Fig. 1) were very similar in all treatments, and showed a
226 decrease during the first 3.5 days of the experiment. After this initial phase, fluxes
227 increased again to maxima of 0.09, 0.08 and 0.05 kg N ha^{-1} d^{-1} for the unlabelled, labelled
228 and control treatment, respectively. Though not statistically different ($p=0.078$), both of
229 the amended treatments showed higher fluxes (maximum of 0.08 kg N ha^{-1} d^{-1}) than the
230 control (maximum of 0.05 kg N ha^{-1} d^{-1}), before decreasing again to the level they had
231 reached 3.5 days after amendment application. Total N_2-N losses attributed to the
232 amendment were 0.05% and 0.03% of the N applied, for the labelled and unlabelled
233 treatment, respectively.

234 Cumulative emissions over the course of the experiment (Table 2) show that about 2.5
235 times more N was lost via N_2O emissions than NO emissions, and total N losses via NO and
236 N_2O were over 40 times higher in the amended treatments than in the control.

237 Carbon dioxide fluxes (Fig. 1) increased immediately after amendment application,
238 reaching values of 27.3 kg C ha^{-1} d^{-1} for both labelled and unlabelled treatments 1.5 days
239 after amendment application, and 1.5 kg C ha^{-1} d^{-1} for the control 2 days after amendment
240 application. By day 4, CO_2 fluxes had decreased to values of 6 kg C ha^{-1} d^{-1} for both fertiliser
241 amended treatments, with further decreases to background levels. The control only
242 showed slightly elevated fluxes that decreased back to background levels by day 3. Above
243 background losses of CO_2 represented 22.0 and 23.2% of C added with the amendment for
244 the labelled and unlabelled treatments.

245 <Figure 1: Gaseous emissions over the course of the incubation>

246 <Figure 2: Evolution of gaseous emissions>

247 Figure 2 shows the average of the fluxes of all measured gases emitted from the fertiliser
248 amended treatments (mean of labelled and unlabelled). Emissions of NO, N_2O and CO_2
249 increased within the first 2 hours after amendment application. As expected from the
250 mechanistic pathway for denitrification, NO is the first gas to peak followed by N_2O, and
251 finally N_2. The sequence of emissions and processes can be described in 3 phases. Phase I
252 (day 0-1): NO peak and a first small N_2O peak; Phase II (day 1-4): main N_2O peak, maximum
253 CO_2; Phase III (day 4-10): N_2 peak, NO small gradual increase.

254 <Table 2: Cumulative emissions>

255

256 *3.2. Isotopic results*

257 The ^{15}N enrichment of the measured N_2O was equal whether it was calculated from ^{45}R or
258 ^{46}R, proving that N_2O originated from a single uniformly labelled NO_3^- pool (homogenuously
259 mixed labelled amendment with native soil NO_3^-). The N_2O d'_D values obtained from Arah's
260 equation, were not significantly different from unity (data not shown); therefore the
261 source of the N_2O was the uniformly mixed ^{15}N labelled NO_3^- pool.

262 The emitted N_2O of the labelled treatment was analysed for ^{15}N enrichment, and results
263 showed that up to day 5, around 85% of the emitted N_2O was derived from the
264 amendment and 15% originated from the native soil NO_3^-.

265

266 *3.3. Soil chemistry*

267 Total oxidised nitrogen (TON) (which is assumed to be nearly exclusively made up of NO_3^-)
268 was significantly higher in the top half than in the bottom half of the cores, and while there
269 was no significant difference between the labelled and unlabelled treatments, both had
270 significantly higher concentrations of TON and NH_4^+-N than the control (Table 3). The initial
271 soil TON content was about an eigth of the added N (15.1 vs 121.5 mg N kg dry soil^{-1}). At
272 the end of the incubation the amended treatments showed a 16 to 19 fold increase in TON
273 while the TON in the control increased 6 to 7 fold. The ^{15}N enrichment of TON was
274 significantly higher in the top (3.5803 ± 0.0496 atom%) than in the bottom (3.0708 ±
275 0.0536 atom%) half of the cores in the labelled treatment.

276 The soil NH_4^+-N concentrations were lower than TON concentrations at the end of the
277 incubation in all treatments, with slightly higher values in the bottom sections of the cores.
278 By the end of the incubation, NH_4^+ concentrations had increased from 9.2 mg N kg^{-1} dry soil
279 to around 13.2 and 15.0 mg N kg^{-1} at the top and bottom of the core respectively. The
280 enrichment of NH_4^+-N in the top (0.4624 ± 0.0164 atom%) was significantly different to

281 the bottom (0.3941 ± 0.0130 atom%) and to natural abundance, but the enrichment of the
282 NH_4^+-N at the bottom (though elevated) was not significantly higher than natural
283 abundance.

284 Soil moisture was 85% WFPS at the start of the incubation and was maintained for the
285 whole core at a similar level for all treatments throughout the experiment (top of cores
286 81.27 ± 1.319%, bottom of cores 88.90 ± 1.145). By the end of the experiment the WFPS
287 was significantly higher at the bottom of the core than the top with ~5% of the water
288 having been redistributed from the top to the bottom of the core.

289 <Table 3: Final soil data>

290

291 **4. Discussion**

292 *4.1. N_2O emissions*

293 Stable isotope ratios are determined by the isotope ratios of the precursor materials and
294 the preferential use of lighter isotopes by microorganisms (Holland and Turekian, 2010; Hu
295 et al., 2015). Results showed that using 5 atom% enriched KNO_3 had no influence on the
296 use of the native vs. enriched N-pool, providing confidence that the isotope analysis used
297 in this study was a good tool to further investigate the source process of the gaseous
298 emissions.

299 Data from the ^{15}N-labelled treatment indicate that 85% of N_2O was derived from the
300 exogenously applied NO_3^-, whereas only 15% was produced from the native soil NO_3^- pool
301 and/or NO_3^- formed by mineralisation. This source apportioning was maintained until day
302 5, after which N_2O emissions were negligible, and were similar to the initial apportioning of
303 the soil NO_3^-, with the native soil NO_3^- making up 11.1% of the total NO_3^-, while the

amendment represented 88.9%. This similarity suggests that the amendment NO_3^- was homogenuously mixed with the native soil NO_3^-. The amount of N_2O derived from the native soil NO_3 from the fertilizer amended treatments (0.18 kg N ha^{-1}) was higher than that emitted from the control (<0.01 kg N ha^{-1}, Fig. 2) also suggesting that the amendment (KNO_3 and C) and the native soil NO_3^- had mixed, becoming available to the microbial community.

The equation of Arah (1997) was used to determine the process leading to the formation of the measured N_2O for data collected during the first 5 days after amendment application; after this period, N_2O concentrations were too low to calculate d'_D values. The determined d'_D values for those first 5 days indicate that close to 100% of the emitted N_2O derived from denitrification of the NO_3^- pool.

Arah's equation assumes that nitrification and denitrification are the only source processes occurring. Our results, however, suggest that it is possible that some of the N_2O might have derived from dissimilatory nitrate reduction to ammonium (DNRA). In DNRA, NO_3^- is reduced to NH_4^+ under similar conditions as denitrification (Fazzolari et al., 1998) and is promoted at C:N ratios (glucose-C:NO_3^-) higher than 4 (Smith, 1982; Fazzolari et al., 1998). The increase in soil NH_4^+ in the N treatments and the increase in ^{15}N enrichment by 0.092atom% indicates that some of the added NO_3^- was transformed to NH_4^+. Although it has been argued that N_2O is produced by DNRA via NO_2^- reduction (Schmidt et al., 2011), the contribution of DNRA to N_2O production is still uncertain (Baggs, 2011). The C:N ratio following amendment in the current study was 5.3, and the formation of NH_4^+ from NO_3^- indicates the possibility that some of the N_2O was produced through DNRA.

4.2. NO emissions

328 Nitric oxide is an obligate intermediate of N_2O production through denitrification (e.g. Ye et
329 al. (1994)). However, if soil moisture content is high (WFPS > 80%), emission of NO is
330 generally considered to be non-detectable due to slow diffusion of NO from denitrifier-cells
331 to the soil atmosphere, and later to air (Russow et al., 2009), during which it is further
332 reduced to N_2O. Based on this assumption, most studies indicate that emitted NO is mainly
333 produced from hydroxylamine (NH_2OH) during nitrification by ammonium oxidisers, which
334 occurs at low soil moisture levels (Skiba et al., 1997). The control treatment did not show
335 any NO emissions. As both, control and N amended treatments, had similar initial soil NH_4^+
336 contents (9-13 mg N kg^{-1}), treatments should have had similar NO fluxes if nitrification of
337 NH_4^+ had been the only source of NO under our experimental conditions. As this is not the
338 case it can be assumed that nitrification did not contribute to initial NO emissions.

339 The increase observed with KNO_3 application in phase I (Fig. 2) indicates that NO came
340 from denitrification in our experiment. Several studies have measured NO fluxes under
341 anoxic/denitrifying conditions in the field or laboratory and have found increased NO
342 emissions after fertilisation or irrigation (e.g. Liu et al., 2010a; Liu et al., 2010b; Bakken et
343 al., 2012). However, to date only our study and those of Russow et al. (2009) and Wang et
344 al. (2011; 2013) have shown that significant NO emissions can be directly promoted by
345 denitrification in soils. Those previous studies confirmed NO as a free intermediate product
346 of denitrification, however, those findings were derived from experiments performed
347 under O_2 depleted atmospheres. The soil in our study had a high WFPS to create anaerobic
348 conditions, and therefore promote denitrification within the soil, the atmosphere above
349 the soil surface, however, was kept aerobic. To the best of our knowledge our study is the
350 first one showing high NO emissions derived from denitrification processes under an
351 aerobic atmosphere.

352 During phase III (Fig. 2) of the experiment, NO emissions started to gradually increase
353 again. A possible explanation for this is that around day 5, at the point of the N_2 maximum,
354 the soil O_2 would have been depleted to its lowest levels, with rapid reduction of N_2O to N_2
355 as a result of anaerobic respiration. The CO_2 fluxes were back to background levels showing
356 aerobic respiration was back to pre-amendment application levels. The recovery of NO
357 after this point, and the lack of N_2O emissions suggest that the soil might be recovering
358 some aerobicity due to diffusion of the atmospheric oxygen from the headspace, and that
359 nitrification could have been the source of those later NO fluxes (day 5.5 to 10). The soil
360 NO_3^- increased during the incubation by about 125-130 mg N kg^{-1} dry soil (equivalent to
361 ~10 mg N kg^{-1} dry soil d^{-1}). This rate is similar to rates measured previously for the same soil
362 (unplublished data). This increase shows that mineralisation and nitrification occurred at
363 some point in the incubation and that the later increase in NO could have been the result
364 of these processes.

365

366 *4.3. N_2 emissions*

367 One indication of NO_3^- reduction by denitrification is the emission of N_2. The high N_2
368 concentrations in our experiment directly after amendment application were most likely
369 due to dissolved N_2 contained in the amendment solution being released into the vessel
370 and flushed out over the first few days, reducing the N_2 concentrations back to background
371 levels before the actual N_2 peak appeared after day 3.5. When N_2O was depleted in the
372 fertilizer treatments, N_2 increased slightly (Fig. 2), but concentrations were very low and
373 not significantly different from the control, indicating that the addition of water stimulated
374 production of N_2 in all treatments. Although there is scarce information regarding fluxes of
375 N_2 in agricultural soils in response to the application of C and N sources, the appearance of

376 the N_2 peak has also been observed 3-4 days after application of amendments in previous
377 experiments (Cardenas et al., 2007; Meijide et al., 2010; Bergstermann et al., 2011).

378 The relatively low N_2 emissions in comparison to high NO and N_2O emissions can be
379 explained by the physiology and metabolism of the denitrifying bacteria and the high soil
380 NO_3^- levels remaining at the end of the incubation. Energy yields from denitrification
381 reactions lessen in order of their appearance, with the reduction of of NO_3^- via NO_2^- to NO
382 being more energetically favourable than the reduction of NO to N_2O and of N_2O to N_2
383 (Koike and Hattori, 1975).

384

385 *4.4. Denitrification as the source process of emissions summarised*

386 The aim of this study was to investigate gaseous emissions from denitrification under an
387 atmosphere that still contained natural amounts of oxygen. To induce low oxygen
388 conditions in the soil, while the above atmosphere was kept at normal O_2 levels, the soil
389 cores had been set to a high WFPS and NO_3^- and a labile C source had been applied in
390 excess.

391 The apex of the peaks of the measured gases appear in the order that would be expected
392 from the denitrification pathway, i.e. NO_3^- is transformed to NO, which is then transformed
393 into N_2O and finally N_2. In our study NO was produced in the hours following NO_3^-
394 application (Fig.2, Phase I). These emissions start at the same time as those of N_2O, but
395 decline more rapidly (i.e. 2 *vs* 5 days after amendment application). The next gas to peak in
396 its emissions is N_2O (Fig.2, Phase II) followed by a small increase in N_2 (Fig.2, Phase III).

397 Overall, the results of this study indicate that denitrification played the most significant
398 role in gaseous emissions. Total denitrification (sum of NO, N_2O and N_2) is normally
399 affected by soil abiotic properties such as WFPS, NO_3^- and available C. A high soil WFPS

400 reduces O_2 diffusion to the pore space (Parton et al., 2001) which, in combination with
401 KNO_3 and C addition, promotes denitrifying conditions. The availability of C not only
402 supports the activity of denitrifiers *per se*, but also has the indirect effect of causing soil
403 microsite anaerobiosis, due to an increased respiratory demand for O_2. The high amount of
404 NO_3^-, which acts as an electron acceptor for denitrifiers, favoures the production of
405 gaseous N-oxides over other reduced forms such as N_2. Additionally, even though the
406 synergistic activities of microbial communities in soil can lead to complete denitrification of
407 NO_3^- to N_2, the earlier steps in the denitrification process are energetically more favourable
408 often resulting in N_2O consequently becoming the final denitrification product, especially if
409 NO_3^- is not limiting (Saggar et al., 2013).

410

411 **5. Conclusions**

412 This study shows that denitrification can be a major source of NO from soils at high water
413 content and under the presence of an easily available C source. Until now, most studies
414 indicated that NO produced in soils during denitrification was consumed by denitrifiers
415 forming N_2O or N_2. To the best of our knowledge, this study, on a UK grassland soil, is the
416 first showing high NO emissions derived from denitrification processes in a soil under high
417 WFPS (creating anaerobic soil conditions and promoting denitrification), but with aerobic
418 conditions above the soil surface. Our findings have several implications for an array of
419 research fields. For example, in simulation studies using process-based models, the
420 contribution of denitrification to NO emissions has been overlooked and needs to be taken
421 into account. Our results also show that NO was mainly produced when an external source
422 of NO_3^- was added to soils. N_2O fluxes, which appeared when NO fluxes had diminished,
423 were also affected by amendments. Complete denitrification from exogenous NO_3^- to N_2

did not occur, and consequently the N_2O:N_2 ratio increased with amendment addition. Further research combining molecular tools with isotopic analyses is needed to expand the findings of our study.

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552

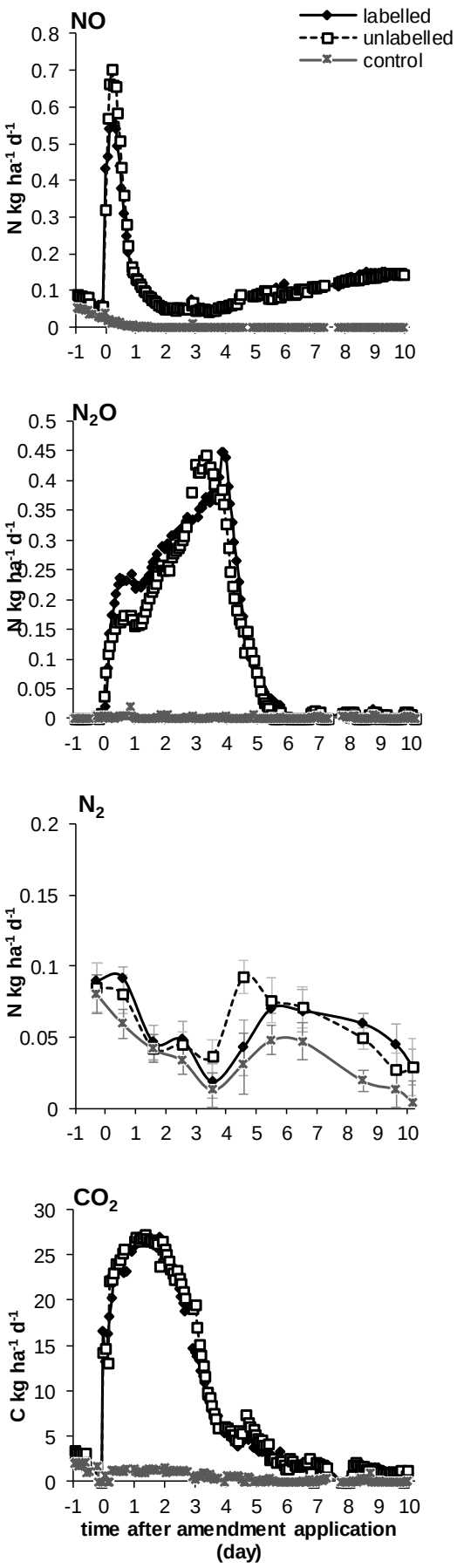
553 Figure 1: Gaseous emissions over the course of the incubation

554 (1 kg ha^{-1} d^{-1} = 4.17x10^{-4} mg cm^{-2} h^{-1})

555 *Phase I:* NO peak and N2O shows first peak. *Phase II*: NO emissions decrease. Main N_2O

556 peak, high CO_2 concentrations decrease. *Phase III*: NO emissions steadily increase again;

557 CO_2 and N_2O emissions decrease to background levels.


NO
labelled
unlabelled
control
N kg ha-1 d-1
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-1 0 1 2 3 4 5 6 7 8 9 10
N2O
N kg ha-1 d-1
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-1 0 1 2 3 4 5 6 7 8 9 10
N2
N kg ha-1 d-1
0.2
0.15
0.1
0.05
0
-1 0 1 2 3 4 5 6 7 8 9 10
CO2
C kg ha-1 d-1
30
25
20
15
10
5
0
-1 0 1 2 3 4 5 6 7 8 9 10
time after amendment application (day)

558

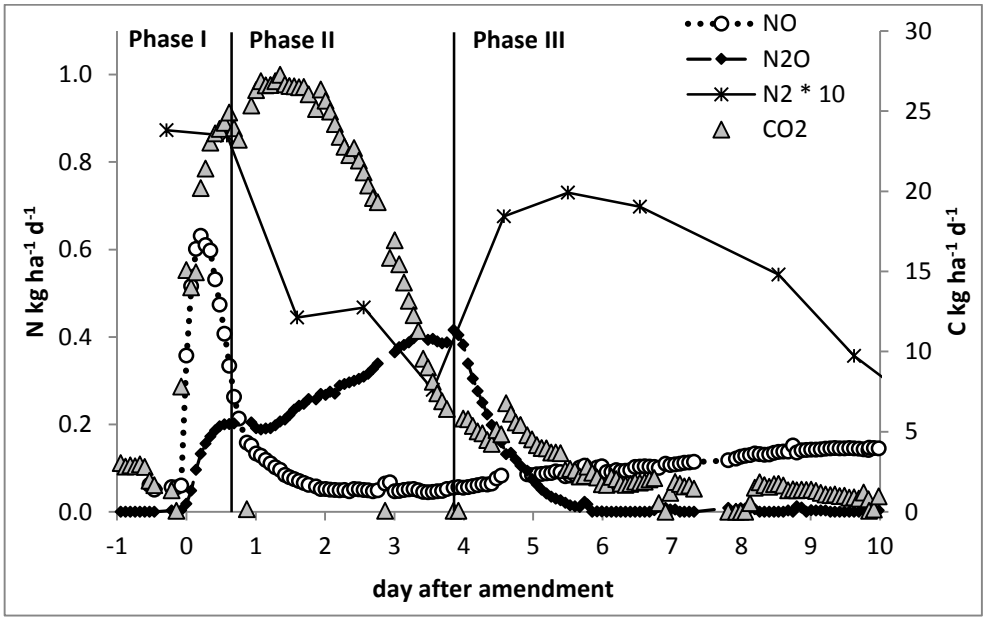
559 Figure 2: Evolution of gaseous emissions of NO, N_2O, N_2 and CO_2 from the amended

560 treatments; N_2 flux from the amended treatment is multiplied by ten, to improve visibility

561 on the graph. CO_2 and N_2 emissions are baseline corrected to show amendment effects

562 only. (1 kg ha^{-1} d^{-1} = 4.17x10^{-4} mg cm^{-2} h^{-1})

563



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565

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Table 1. Soil characteristics (before amendment application). Mean ± standard error (n = 3).

Parameter	*Amount*
pH water [1:2.5]	5.6 ± 0.27
Available Magnesium (mg kg^{-1} dry soil)	100.4 ± 4.81
Available Phosphorus (mg kg^{-1} dry soil)	10.4 ± 1.10
Available Potassium (mg kg^{-1} dry soil)	97.5 ± 12.83
Available Sulphate (mg kg^{-1} dry soil)	51.7 ± 0.62
Total N (% w/w)	0.5 ± 0.01
Total Oxidised N (mg kg^{-1} dry soil)	15.1 ± 0.07
Ammonium N (mg kg^{-1} dry soil)	9.2 ± 0.09
Organic Matter (% w/w)	11.7 ± 0.29

567

568

Table 2. Cumulative emissions of NO, N_2O, N_2 as kg N ha^{-1} and CO_2 as kg C ha^{-1} over the time of the respective peaks. N_2 and CO_2 emissions are baseline subtracted. Different letters indicate a significant difference between treatments for each measured gas (n = 4, p < 0.05).

Gas	*Labelled (^{15}N-KNO_3+C)*	*Unlabelled (KNO_3+C)*	*Control*
NO	0.46 ± 0.02 ^{A}	0.50 ± 0.02 ^{A}	0.03 ± 0.03 ^{B}
N_2O	1.20 ± 0.28 ^{A}	1.26 ± 0.08 ^{A}	0.01 ± 0.01 ^{B}
N_2	0.30 ± 0.03 ^{A}	0.33 ± 0.07 ^{A}	0.14 ± 0.06 ^{A}
CO_2	87.89 ± 3.73 ^{A}	92.68 ± 2.68 ^{A}	5.50 ± 3.39 ^{B}

569

Table 3. Total Oxidized N (TON) and ammonium (NH_4^+) at the end of the experiment. Different letters indicate significant differences between treatments for each layer [Top (A/B) or Bottom (X/Y)]; * indicates significant differences between the Top and Bottom layer within a single treatment (TON and NH_4^+: n = 4, p < 0.05, p < 0.05).

Parameter	Layer	*Labelled (^{15}N-KNO_3+C)*	*Unlabelled (KNO_3+C)*	*Control*
TON (mg N kg^{-1} dry soil)	Top	271.8 ± 17.32 $*^{A}$	292.6 ± 17.09 $*^{A}$	90.5 ± 3.61 $*^{B}$
	Bottom	246.0 ± 21.37 $*^{X}$	239.5 ± 14.85 $*^{X}$	108.3 ± 5.22 $*^{Y}$
NH_4^+ (mg N kg^{-1} dry soil)	Top	13.4 ± 1.66 $*^{A}$	13.0 ± 1.25 $*^{A}$	8.5 ± 0.55 $*^{B}$
	Bottom	15.2 ± 2.42 $*^{X}$	14.9 ± 2.11 $*^{X}$	9.5 ± 0.77 $*^{Y}$

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Table 4. Total Oxidized N (TON) and ammonium (NH_4^+) at the end of the experiment. Different letters indicate significant differences between treatments for each layer [Top (A/B) or Bottom (X/Y)]; * indicates significant differences between the Top and Bottom layer within a single treatment (TON and NH_4^+: $n = 4$, $p < 0.05$, $p < 0.05$).

Parameter	Layer	*Labelled* *(^{15}N-KNO_3+C)*	*Unlabelled* *(KNO_3+C)*	*Control*
TON (mg N kg^{-1} dry soil)	Top	271.8 ± 17.32 $*^A$	292.6 ± 17.09 $*^A$	90.5 ± 3.61 $*^B$
	Bottom	246.0 ± 21.37 $*^X$	239.5 ± 14.85 $*^X$	108.3 ± 5.22 $*^Y$
NH_4^+ (mg N kg^{-1} dry soil)	Top	13.4 ± 1.66 $*^A$	13.0 ± 1.25 $*^A$	8.5 ± 0.55 $*^B$
	Bottom	15.2 ± 2.42 $*^X$	14.9 ± 2.11 $*^X$	9.5 ± 0.77 $*^Y$

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