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

Nadine Loicka, Elizabeth R. Dixona, Diego Abalosb, Antonio Vallejoc, G. Peter Matthewsc,
Karen L. McGeoughd, Reinhard Welle, Catherine J. Watsond, Ronnie J. Laughlind, Laura M.
Cardenasab

a Rothamsted Research, North Wyke, Okehampton, Devon, EX20 2SB, UK
b Technical University of Madrid, Chemistry and Agricultural Analysis, Madrid, Spain
c School of Geography, Earth and Environmental Sciences, University of Plymouth, Davy Building, Drake Circus, Plymouth, Devon, PL4 8AA, UK
d Agri-Food and Biosciences Institute, Newforge Lane, Belfast, BT9 5PX, UK
e Thünen-Institut für Agrarklimaschutz, Bundesallee 50, 38116 Braunschweig, Germany

*corresponding author: E-mail address: laura.cardenas@rothamsted.ac.uk (phone: +44 (0)1837 883 500)

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Abstract

Agricultural soils are a major source of nitric oxide (NO) and nitrous oxide (N\textsubscript{2}O), which are produced and consumed by biotic and abiotic soil processes. The dominant sources of NO and N\textsubscript{2}O are microbial nitrification and denitrification. While N\textsubscript{2}O 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\textsubscript{2} atmosphere at constant surface gas flow, combined with \textsuperscript{15}N labelled isotopic techniques, the present study investigated the role of denitrification on NO, N\textsubscript{2}O and N\textsubscript{2} emissions in a UK grassland soil under high soil moisture and an aerobic headspace atmosphere. With the application of KNO\textsubscript{3} 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\textsubscript{2}O and 0.03 to 0.05\% via N\textsubscript{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\textsubscript{2}O), 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\textsubscript{2} which together with N\textsubscript{2}O 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 N₂O, and as such, intense investigations (i.e. >1,000
published studies) have led to a good understanding of the abiotic factors regulating N₂O
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 N₂O 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 N₂O 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%, N₂O 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 (NO₃⁻) 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 N₂O is being produced (Arah, 1997). It is also known that microorganisms
discriminate against the heavier molecule (e.g. ¹⁵N vs. ¹⁴N), preferring to use the lighter
molecule which requires less energy to break the bonds (Kendall and Caldwell, 1998). This should be considered when applying labelled substrate to investigate microbial processes. The aim of this study was to explore the potential role of denitrification as a significant source of NO emissions. We hypothesise that denitrification can be a major source of NO emissions in a UK grassland soil under high moisture content. This study uses the gas-flow-soil-core technique (Cárdenas et al., 2003), further developed to include NO measurements, combined with isotopic analyses. A $^{15}$N labelled substrate as well as an unlabelled substrate at the same application ratio was used to determine whether there was an effect of the labelled N on the investigated processes at a 5 atom% enrichment. Additionally to adding potassium nitrate (KNO$_3$) as N source, glucose was added to supply a readily available C source and thereby promote denitrification. During denitrification C is used as electron donor and C availability is one factor controlling denitrification rates and compared to other C-compounds, denitrification tends to be most stimulated after addition of ethanol or glucose (Morley and Baggs, 2010).

2. Materials and Methods

2.1. Soil preparation

A clayey pelostagnogley soil of the Hallsworth series (Clayden and Hollis, 1984) (44% clay, 40% silt, 15% sand (w/w), Table 1) was collected on the 4th of November 2013 from a typical grassland in SW England, located at Rothamsted Research, North Wyke, Devon, UK (50°46’10’’N, 3° 54’05’’W). Spade-squares (20 x 20 cm to a depth of 15 cm) of soil were taken from 12 locations along a ‘W’ line across a field of 600 m$^2$ size. After sampling, the soil was air dried to ~30% H$_2$O (dry basis), roots and plant residue were removed and the
soil sieved to <2 mm and stored at 4°C for 5 days before packing into cores and starting the incubation.

2.2. Experimental setup

The incubation was carried out using the DENitrification System (DENIS), a specialized gas-flow-soil-core incubation system (Cárdenas et al., 2003). Twelve cores were packed with soil to a bulk density of 0.8 g cm\(^{-3}\) and a height of 75 mm into stainless steel vessels of 140 mm diameter. To ensure denitrification conditions, the soil moisture was adjusted to 85% WFPS, taking the later amendment into account. This WFPS was similar to those used in previous studies to promote denitrification processes (Meijide et al., 2010; Bergstermann et al., 2011). In order to measure N\(_2\) fluxes the native atmosphere was removed by flushing the soil cores from the bottom with a mixture of He:O\(_2\) (80:20) at 30 ml min\(^{-1}\) for 14 hours. Flow rates were then decreased to 12 ml min\(^{-1}\) and the flow re-directed over the surface of the soil core for three days before amendment application to measure baseline emissions. O\(_2\) was kept in the gas mixture at atmospheric levels as the objective was to investigate denitrification achieved by high WFPS instead of forcing anaerobic conditions by preventing any O\(_2\) diffusion.

The following treatments were applied to four replicate vessels: (a) labelled (\(^{15}\)N-labelled KNO\(_3\) at 5 atom% and glucose); (b) unlabelled (KNO\(_3\) and glucose); (c) control (water only).

The labelled and unlabelled treatments contained nitrogen at a rate equivalent to 75 kg N 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 soil), which is similar to previous studies (Meijide et al., 2010; Bergstermann et al., 2011). The amendment for each core was dissolved in 50 ml distilled water, and the controls also
received 50 ml distilled water each. The vessels were kept at 20°C during the whole incubation period, which lasted for 10 days after amendment application.

2.3. Gas analyses and data manipulation

Gas samples were taken every two hours for each vessel. Fluxes of N\(_2\)O and CO\(_2\) were quantified using a Perkin Elmer Clarus 500 gas chromatograph (Perkin Elmer Instruments, Beaconsfield, UK) equipped with an electron capture detector (ECD) for N\(_2\)O, and with a flame ionization detector (FID) and a methanizer for CO\(_2\). N\(_2\) emissions were measured by gas chromatography with a helium ionisation detector (VICI AG International, Schenkon, Switzerland) (Cárdenas et al., 2003), while NO concentrations were determined by chemiluminescence (Sievers NOA280i, GE Instruments, Colorado, USA). All gas concentrations were corrected for the surface area and flow rate going through the vessel (measured daily). Fluxes were calculated on a kg N or C ha\(^{-1}\) day\(^{-1}\) basis.

2.4. Isotopic analyses of N\(_2\)O

Gas sampling times for \(^{15}\)N analysis were pre-determined based on data from previous experiments (data not shown). Samples were taken just before (0 hours) and 4 hours after amendment application, then every 24 hours for the first week, followed by a final sample at day 10. This sampling strategy was decided on from previous experimental results to cover changes in isotopic signature before amendment application, as well as during the NO and N\(_2\)O peaks (4-5 h and 3-4 d, respectively), and after emissions returned to background levels. Samples were taken from the outlet line of each vessel using 12 ml exetainers (Labco) which had previously been flushed with He and evacuated. \(^{15}\)N
enrichment of N\textsubscript{2}O 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\textsubscript{4})\textsubscript{2}SO\textsubscript{4}) were prepared and used to generate 6.6 and 2.9 atom% N\textsubscript{2}O (Laughlin et al., 1997) which were used as reference and quality control standards.

The process leading to the formation of the measured N\textsubscript{2}O, i.e. whether it is produced by nitrification or denitrification, was determined by calculating how much of the N\textsubscript{2}O was derived from NO\textsubscript{3}\textsuperscript{−} as the parent molecule. When \(^{15}\)N labelled NO\textsubscript{3}\textsuperscript{−} is added, it is assumed that it completely mixes with the native soil NO\textsubscript{3}\textsuperscript{−} pool to form a single uniformly labelled NO\textsubscript{3}\textsuperscript{−} pool. The \(^{15}\)N content of the N\textsubscript{2}O 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\textsubscript{2}O calculated from either \(^{45}\)R or \(^{46}\)R are equal, then the distribution of the \(^{15}\)N atoms in the N\textsubscript{2}O molecules is random, and therefore the N\textsubscript{2}O originated from a single uniformly labelled NO\textsubscript{3}\textsuperscript{−} pool (Stevens et al., 1997; Stevens and Laughlin, 1998). When the NO\textsubscript{3}\textsuperscript{−} pool is labelled and the N\textsubscript{2}O concentration is greater than the IRMS method detection limit (2 ppm), calculations of the fraction of N\textsubscript{2}O derived from the denitrifying pool (\(d\textsubscript{\text{\textsuperscript{\text{\textsuperscript{D}}}D}}\)) were performed. The sources of N\textsubscript{2}O were then apportioned into \(d\textsubscript{\text{\textsuperscript{\text{\textsuperscript{D}}}D}}\) and the fraction derived from the nitrifying pool (\(d\textsubscript{\text{\textsuperscript{\text{\textsuperscript{N}}}N}} = (1 - d\textsubscript{\text{\textsuperscript{\text{\textsuperscript{D}}}D}}))\) and calculated as described in Arah (1997). In Arah’s equation N\textsubscript{2}O \(d\textsubscript{\text{\textsuperscript{\text{\textsuperscript{D}}}D}}\) is the fraction of the emitted N\textsubscript{2}O which is derived from the \(^{15}\)N labelled, denitrifying NO\textsubscript{3}\textsuperscript{−} pool. A N\textsubscript{2}O \(d\textsubscript{\text{\textsuperscript{\text{\textsuperscript{D}}}D}}\) value of unity (1.00) indicates that 100% of the N\textsubscript{2}O emitted derived from the NO\textsubscript{3}\textsuperscript{−} pool.

To determine the source of the measured N\textsubscript{2}O, i.e. how much of it was derived from the amendment (N\textsubscript{2}O\textsubscript{ amend}) rather than the native soil N, the following equation was used for the labelled treatments (Senbayram et al., 2009):
\[ N_2O_{\text{Amend}} = N_2O_{\text{Total}} \left( \frac{^{15}\text{Nat\%ex}_{\text{Sample}}}{^{15}\text{Nat\%ex}_{\text{Fert}}} \right) \] (1)

where \( N_2O_{\text{Total}} \) = total emissions of \( N_2O \) from the soil; \( ^{15}\text{Nat\%ex}_{\text{Sample}} \) = \( ^{15} \text{N} \) atom\% excess of the emitted \( N_2O \) \((^{15} \text{N} \) atom\% of the measured sample minus the mean natural \( ^{15} \text{N} \) abundance of background \( N_2O \) obtained in our experiment \((0.366 \text{ atom \%})\); \( ^{15}\text{Nat\%ex}_{\text{Fert}} \) = \( ^{15} \text{N} \) 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 \( \text{NH}_4^+ \) and total oxidised N (TON: \( \text{NO}_3^- + \text{NO}_2^- \)) concentrations. Nitrite (\( \text{NO}_2^- \)) is generally thought to accumulate very rarely in nature, and it has been shown that \( \text{NO}_2^- \) is rapidly mineralised in soil (Paul and Clark, 1989; Burns et al., 1995, 1996). It is therefore assumed that \( \text{NO}_2^- \) concentrations in the soil samples are negligible, and TON is nearly exclusively made up of \( \text{NO}_3^- \). 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 \((50 \text{ g})\) at 105°C overnight.

Soil \( \text{NH}_4^+ \)-N and TON were analysed by automated colorimetry from 2M KCl soil extracts using a Skalar SANPLUS Analyser (Skalar Analytical B.V., Breda, Netherlands) (Searle, 1984). \(^{15} \text{N} \) abundance of \( \text{NO}_3^- \) and \( \text{NH}_4^+ \) 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 (Braunschweig, Germany)). Briefly, \( \text{NO}_3^- \) was reduced to NO by Vanadium chloride \((\text{V(III)}\text{Cl}_3)\) and \( \text{NH}_4^+ \) was oxidized to \( N_2 \) by Hypobromite \((\text{NaOBr})\). NO and \( N_2 \) were the gases measured.
2.6. Statistical analysis

Statistical analysis was performed using GenStat 16th edition (VSN International Ltd). Prior to the statistical tests all data were analyzed to proof their normal distribution (Kolmogorov-Smirnov test) and equality of variance (Levene test). Cumulative emissions of NO, N₂O, N₂ and CO₂ were calculated from the area under the curve after linear interpolation between sampling points. Differences in total emissions for each gas measured between treatments as well as differences in soil characteristics between treatments and between top and bottom of soil cores were assessed by ANOVA at $P < 0.05$. Where treatment effects proved to be significant, Fisher’s Least Significant Test (LSD) was used as post hoc test to ascertain differences among treatment levels.

3. Results

3.1. Gas emissions

CO₂ fluxes showed constant emissions of 10 kg C ha⁻¹ d⁻¹ before and after the CO₂ peak (day 0-6) in all vessels. N₂ emissions increased at the moment the amendment was applied, but decreased immediately after until day 3.5 when they reached background levels, before increasing again. In order to show CO₂ and N₂ emissions attributed to amendment application only, the fluxes were adjusted by subtracting background emissions. There were no significant differences in fluxes, or cumulative emissions for any of the measured gases between the labelled and unlabelled treatments (Table 2). Both treatments, however, were significantly higher than the control for all gaseous emissions measured, except for N₂.

Nitric oxide emissions peaked 14 hours after amendment application (Fig. 1), with maximum average fluxes of 0.58 and 0.70 kg N ha⁻¹ d⁻¹, for the labelled and unlabelled
treatment, respectively. Fluxes decreased afterwards resulting in values below 0.1 kg N ha\(^{-1}\) d\(^{-1}\) 30 hours after amendment application. Fluxes then decreased further to below 0.05 kg N ha\(^{-1}\) d\(^{-1}\), before showing a linear increase over 5 days to values of around 0.1 kg N ha\(^{-1}\) d\(^{-1}\) until the end of the experiment. Losses of N via NO emissions represented 0.61 and 0.67% of the N added. The control treatment showed negligible fluxes of NO over the whole experimental period.

Similar to NO, emissions of N\(_2\)O increased immediately after amendment application. After 14 hours, N\(_2\)O showed a first maximum of 0.24 and 0.17 kg N ha\(^{-1}\) d\(^{-1}\) for the labelled and unlabelled treatment, respectively (Fig. 1). In both treatments fluxes decreased over the following 12 h by 0.02 kg N ha\(^{-1}\) d\(^{-1}\) before increasing again to a maximum of 0.45 and 0.44 kg N ha\(^{-1}\) d\(^{-1}\), 3.3 and 3.8 days after amendment application, respectively. Total losses of N\(_2\)O represented 1.60 and 1.68% of the N applied for the labelled and unlabelled treatment, respectively. Again the control treatment maintained significantly lower fluxes than the fertilized treatments over the whole experimental period.

Gaseous nitrogen (N\(_2\)) fluxes (Fig. 1) were very similar in all treatments, and showed a decrease during the first 3.5 days of the experiment. After this initial phase, fluxes increased again to maxima of 0.09, 0.08 and 0.05 kg N ha\(^{-1}\) d\(^{-1}\) for the unlabelled, labelled and control treatment, respectively. Though not statistically different (p=0.078), both of the amended treatments showed higher fluxes (maximum of 0.08 kg N ha\(^{-1}\) d\(^{-1}\)) than the control (maximum of 0.05 kg N ha\(^{-1}\) d\(^{-1}\)), before decreasing again to the level they had reached 3.5 days after amendment application. Total N\(_2\)-N losses attributed to the amendment were 0.05% and 0.03% of the N applied, for the labelled and unlabelled treatment, respectively.
Cumulative emissions over the course of the experiment (Table 2) show that about 2.5 times more N was lost via N₂O emissions than NO emissions, and total N losses via NO and N₂O were over 40 times higher in the amended treatments than in the control.

Carbon dioxide fluxes (Fig. 1) increased immediately after amendment application, reaching values of 27.3 kg C ha⁻¹ d⁻¹ for both labelled and unlabelled treatments 1.5 days after amendment application, and 1.5 kg C ha⁻¹ d⁻¹ for the control 2 days after amendment application. By day 4, CO₂ fluxes had decreased to values of 6 kg C ha⁻¹ d⁻¹ for both fertiliser amended treatments, with further decreases to background levels. The control only showed slightly elevated fluxes that decreased back to background levels by day 3. Above background losses of CO₂ represented 22.0 and 23.2% of C added with the amendment for the labelled and unlabelled treatments.

Figure 2 shows the average of the fluxes of all measured gases emitted from the fertiliser amended treatments (mean of labelled and unlabelled). Emissions of NO, N₂O and CO₂ increased within the first 2 hours after amendment application. As expected from the mechanistic pathway for denitrification, NO is the first gas to peak followed by N₂O, and finally N₂. The sequence of emissions and processes can be described in 3 phases. Phase I (day 0-1): NO peak and a first small N₂O peak; Phase II (day 1-4): main N₂O peak, maximum CO₂; Phase III (day 4-10): N₂ peak, NO small gradual increase.

3.2. Isotopic results
The $^{15}$N enrichment of the measured N$_2$O was equal whether it was calculated from $^{45}$R or $^{46}$R, proving that N$_2$O originated from a single uniformly labelled NO$_3^-$ pool (homogeneously mixed labelled amendment with native soil NO$_3^-$). The N$_2$O $d_{15}$O values obtained from Arah’s equation, were not significantly different from unity (data not shown); therefore the source of the N$_2$O was the uniformly mixed $^{15}$N labelled NO$_3^-$ pool.

The emitted N$_2$O of the labelled treatment was analysed for $^{15}$N enrichment, and results showed that up to day 5, around 85% of the emitted N$_2$O was derived from the amendment and 15% originated from the native soil NO$_3^-$.

### 3.3. Soil chemistry

Total oxidised nitrogen (TON) (which is assumed to be nearly exclusively made up of NO$_3^-$) was significantly higher in the top half than in the bottom half of the cores, and while there was no significant difference between the labelled and unlabelled treatments, both had significantly higher concentrations of TON and NH$_4^+$-N than the control (Table 3). The initial soil TON content was about an eighth of the added N (15.1 vs 121.5 mg N kg dry soil$^{-1}$). At the end of the incubation the amended treatments showed a 16 to 19 fold increase in TON while the TON in the control increased 6 to 7 fold. The $^{15}$N enrichment of TON was significantly higher in the top (3.5803 ± 0.0496 atom%) than in the bottom (3.0708 ± 0.0536 atom%) half of the cores in the labelled treatment.

The soil NH$_4^+$-N concentrations were lower than TON concentrations at the end of the incubation in all treatments, with slightly higher values in the bottom sections of the cores. By the end of the incubation, NH$_4^+$ concentrations had increased from 9.2 mg N kg$^{-1}$ dry soil to around 13.2 and 15.0 mg N kg$^{-1}$ at the top and bottom of the core respectively. The enrichment of NH$_4^+$-N in the top (0.4624 ± 0.0164 atom%) was significantly different to
the bottom (0.3941 ± 0.0130 atom%) and to natural abundance, but the enrichment of the

NH₄⁺-N at the bottom (though elevated) was not significantly higher than natural

abundance.

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

<Table 3: Final soil data>

4. Discussion

4.1. N₂O emissions

Stable isotope ratios are determined by the isotope ratios of the precursor materials and
the preferential use of lighter isotopes by microorganisms (Holland and Turekian, 2010; Hu
et al., 2015). Results showed that using 5 atom% enriched KNO₃ had no influence on the
use of the native vs. enriched N-pool, providing confidence that the isotope analysis used
in this study was a good tool to further investigate the source process of the gaseous
emissions.

Data from the ¹⁵N-labelled treatment indicate that 85% of N₂O was derived from the
exogenously applied NO₃⁻, whereas only 15% was produced from the native soil NO₃⁻ pool
and/or NO₃⁻ formed by mineralisation. This source apportioning was maintained until day
5, after which N₂O emissions were negligible, and were similar to the initial apportioning of
the soil NO₃⁻, with the native soil NO₃⁻ making up 11.1% of the total NO₃⁻, while the
amendment represented 88.9%. This similarity suggests that the amendment NO$_3^-$ was homogeneously mixed with the native soil NO$_3^-$. The amount of N$_2$O 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$_2$O for data collected during the first 5 days after amendment application; after this period, N$_2$O 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$_2$O 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$_2$O 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 0.092 atom% indicates that some of the added NO$_3^-$ was transformed to NH$_4^+$. Although it has been argued that N$_2$O is produced by DNRA via NO$_2^-$ reduction (Schmidt et al., 2011), the contribution of DNRA to N$_2$O 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$_2$O was produced through DNRA.

4.2. NO emissions
Nitric oxide is an obligate intermediate of N₂O production through denitrification (e.g. Ye et al. (1994)). However, if soil moisture content is high (WFPS > 80%), emission of NO is generally considered to be non-detectable due to slow diffusion of NO from denitrifier-cells to the soil atmosphere, and later to air (Russow et al., 2009), during which it is further reduced to N₂O. Based on this assumption, most studies indicate that emitted NO is mainly produced from hydroxylamine (NH₂OH) during nitrification by ammonium oxidisers, which occurs at low soil moisture levels (Skiba et al., 1997). The control treatment did not show any NO emissions. As both, control and N amended treatments, had similar initial soil NH₄⁺ contents (9-13 mg N kg⁻¹), treatments should have had similar NO fluxes if nitrification of NH₄⁺ had been the only source of NO under our experimental conditions. As this is not the case it can be assumed that nitrification did not contribute to initial NO emissions.

The increase observed with KNO₃ application in phase I (Fig. 2) indicates that NO came from denitrification in our experiment. Several studies have measured NO fluxes under anoxic/denitrifying conditions in the field or laboratory and have found increased NO emissions after fertilisation or irrigation (e.g. Liu et al., 2010a; Liu et al., 2010b; Bakken et al., 2012). However, to date only our study and those of Russow et al. (2009) and Wang et al. (2011; 2013) have shown that significant NO emissions can be directly promoted by denitrification in soils. Those previous studies confirmed NO as a free intermediate product of denitrification, however, those findings were derived from experiments performed under O₂ depleted atmospheres. The soil in our study had a high WFPS to create anaerobic conditions, and therefore promote denitrification within the soil, the atmosphere above the soil surface, however, was kept aerobic. To the best of our knowledge our study is the first one showing high NO emissions derived from denitrification processes under an aerobic atmosphere.
During phase III (Fig. 2) of the experiment, NO emissions started to gradually increase again. A possible explanation for this is that around day 5, at the point of the N$_2$ maximum, the soil O$_2$ would have been depleted to its lowest levels, with rapid reduction of N$_2$O to N$_2$ as a result of anaerobic respiration. The CO$_2$ fluxes were back to background levels showing aerobic respiration was back to pre-amendment application levels. The recovery of NO after this point, and the lack of N$_2$O emissions suggest that the soil might be recovering some aerobicity due to diffusion of the atmospheric oxygen from the headspace, and that nitrification could have been the source of those later NO fluxes (day 5.5 to 10). The soil NO$_3^-$ increased during the incubation by about 125-130 mg N kg$^{-1}$ dry soil (equivalent to ~10 mg N kg$^{-1}$ dry soil d$^{-1}$). This rate is similar to rates measured previously for the same soil (unpublished data). This increase shows that mineralisation and nitrification occurred at some point in the incubation and that the later increase in NO could have been the result of these processes.

4.3. \( \text{N}_2 \) emissions

One indication of NO$_3^-$ reduction by denitrification is the emission of N$_2$. The high N$_2$ concentrations in our experiment directly after amendment application were most likely due to dissolved N$_2$ contained in the amendment solution being released into the vessel and flushed out over the first few days, reducing the N$_2$ concentrations back to background levels before the actual N$_2$ peak appeared after day 3.5. When N$_2$O was depleted in the fertilizer treatments, N$_2$ increased slightly (Fig. 2), but concentrations were very low and not significantly different from the control, indicating that the addition of water stimulated production of N$_2$ in all treatments. Although there is scarce information regarding fluxes of N$_2$ in agricultural soils in response to the application of C and N sources, the appearance of
the N₂ peak has also been observed 3-4 days after application of amendments in previous experiments (Cardenas et al., 2007; Meijide et al., 2010; Bergstermann et al., 2011).

The relatively low N₂ emissions in comparison to high NO and N₂O emissions can be explained by the physiology and metabolism of the denitrifying bacteria and the high soil NO₃⁻ levels remaining at the end of the incubation. Energy yields from denitrification reactions lessen in order of their appearance, with the reduction of NO₃⁻ via NO₂⁻ to NO being more energetically favourable than the reduction of NO to N₂O and of N₂O to N₂ (Koike and Hattori, 1975).

4.4. Denitrification as the source process of emissions summarised

The aim of this study was to investigate gaseous emissions from denitrification under an atmosphere that still contained natural amounts of oxygen. To induce low oxygen conditions in the soil, while the above atmosphere was kept at normal O₂ levels, the soil cores had been set to a high WFPS and NO₃⁻ and a labile C source had been applied in excess.

The apex of the peaks of the measured gases appear in the order that would be expected from the denitrification pathway, i.e. NO₃⁻ is transformed to NO, which is then transformed into N₂O and finally N₂. In our study NO was produced in the hours following NO₃⁻ application (Fig. 2, Phase I). These emissions start at the same time as those of N₂O, but decline more rapidly (i.e. 2 vs 5 days after amendment application). The next gas to peak in its emissions is N₂O (Fig. 2, Phase II) followed by a small increase in N₂ (Fig. 2, Phase III).

Overall, the results of this study indicate that denitrification played the most significant role in gaseous emissions. Total denitrification (sum of NO, N₂O and N₂) is normally affected by soil abiotic properties such as WFPS, NO₃⁻ and available C. A high soil WFPS
reduce O$_2$ diffusion to the pore space (Parton et al., 2001) which, in combination with
KNO$_3$ and C addition, promotes denitrifying conditions. The availability of C not only
supports the activity of denitrifiers *per se*, but also has the indirect effect of causing soil
microsite anaerobiosis, due to an increased respiratory demand for O$_2$. The high amount of
NO$_3^-$, which acts as an electron acceptor for denitrifiers, favours the production of
gaseous N-oxides over other reduced forms such as N$_2$. Additionally, even though the
synergistic activities of microbial communities in soil can lead to complete denitrification of
NO$_3^-$ to N$_2$, the earlier steps in the denitrification process are energetically more favourable
often resulting in N$_2$O consequently becoming the final denitrification product, especially if
NO$_3^-$ is not limiting (Saggar et al., 2013).

5. Conclusions

This study shows that denitrification can be a major source of NO from soils at high water
content and under the presence of an easily available C source. Until now, most studies
indicated that NO produced in soils during denitrification was consumed by denitrifiers
forming N$_2$O or N$_2$. To the best of our knowledge, this study, on a UK grassland soil, is the
first showing high NO emissions derived from denitrification processes in a soil under high
WFPS (creating anaerobic soil conditions and promoting denitrification), but with aerobic
conditions above the soil surface. Our findings have several implications for an array of
research fields. For example, in simulation studies using process-based models, the
contribution of denitrification to NO emissions has been overlooked and needs to be taken
into account. Our results also show that NO was mainly produced when an external source
of NO$_3^-$ was added to soils. N$_2$O fluxes, which appeared when NO fluxes had diminished,
were also affected by amendments. Complete denitrification from exogenous NO$_3^-$ to N$_2$
did not occur, and consequently the N$_2$O: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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Figure 1: Gaseous emissions over the course of the incubation

\[1 \text{ kg ha}^{-1} \text{ d}^{-1} = 4.17 \times 10^{-4} \text{ mg cm}^{-2} \text{ h}^{-1}\]

**Phase I:** NO peak and N\(_2\)O shows first peak. **Phase II:** NO emissions decrease. Main N\(_2\)O peak, high CO\(_2\) concentrations decrease. **Phase III:** NO emissions steadily increase again; CO\(_2\) and N\(_2\)O emissions decrease to background levels.
Figure 2: Evolution of gaseous emissions of NO, N$_2$O, N$_2$ and CO$_2$ from the amended treatments; N$_2$ flux from the amended treatment is multiplied by ten, to improve visibility on the graph. CO$_2$ and N$_2$ emissions are baseline corrected to show amendment effects only. (1 kg ha$^{-1}$ d$^{-1}$ = 4.17x10$^{-4}$ mg cm$^{-2}$ h$^{-1}$)
### Table 1. Soil characteristics (before amendment application). Mean ± standard error ($n = 3$).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH water [1:2.5]</td>
<td>5.6 ± 0.27</td>
</tr>
<tr>
<td>Available Magnesium (mg kg⁻¹ dry soil)</td>
<td>100.4 ± 4.81</td>
</tr>
<tr>
<td>Available Phosphorus (mg kg⁻¹ dry soil)</td>
<td>10.4 ± 1.10</td>
</tr>
<tr>
<td>Available Potassium (mg kg⁻¹ dry soil)</td>
<td>97.5 ± 12.83</td>
</tr>
<tr>
<td>Available Sulphate (mg kg⁻¹ dry soil)</td>
<td>51.7 ± 0.62</td>
</tr>
<tr>
<td>Total N (% w/w)</td>
<td>0.5 ± 0.01</td>
</tr>
<tr>
<td>Total Oxidised N (mg kg⁻¹ dry soil)</td>
<td>15.1 ± 0.07</td>
</tr>
<tr>
<td>Ammonium N (mg kg⁻¹ dry soil)</td>
<td>9.2 ± 0.09</td>
</tr>
<tr>
<td>Organic Matter (% w/w)</td>
<td>11.7 ± 0.29</td>
</tr>
</tbody>
</table>

### Table 2. Cumulative emissions of NO, N₂O, N₂ as kg N ha⁻¹ and CO₂ as kg C ha⁻¹ over the time of the respective peaks. N₂ and CO₂ emissions are baseline subtracted. Different letters indicate a significant difference between treatments for each measured gas ($n = 4$, $p < 0.05$).

<table>
<thead>
<tr>
<th>Gas</th>
<th>Labelled ($^{15}$N-KNO₃+C)</th>
<th>Unlabelled (KNO₃+C)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO</td>
<td>0.46 ± 0.02 A</td>
<td>0.50 ± 0.02 A</td>
<td>0.03 ± 0.03 B</td>
</tr>
<tr>
<td>N₂O</td>
<td>1.20 ± 0.28 A</td>
<td>1.26 ± 0.08 A</td>
<td>0.01 ± 0.01 B</td>
</tr>
<tr>
<td>N₂</td>
<td>0.30 ± 0.03 A</td>
<td>0.33 ± 0.07 A</td>
<td>0.14 ± 0.06 A</td>
</tr>
<tr>
<td>CO₂</td>
<td>87.89 ± 3.73 A</td>
<td>92.68 ± 2.68 A</td>
<td>5.50 ± 3.39 B</td>
</tr>
</tbody>
</table>

### Table 3. Total Oxidized N (TON) and ammonium (NH₄⁺) 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 NH₄⁺; $n = 4$, $p < 0.05$, $p < 0.05$).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Layer</th>
<th>Labelled ($^{15}$N-KNO₃+C)</th>
<th>Unlabelled (KNO₃+C)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>TON (mg N kg⁻¹ dry soil)</td>
<td>Top</td>
<td>271.8 ± 17.32 *A</td>
<td>292.6 ± 17.09 *A</td>
<td>90.5 ± 3.61 *B</td>
</tr>
<tr>
<td></td>
<td>Bottom</td>
<td>246.0 ± 21.37 *X</td>
<td>239.5 ± 14.85 *X</td>
<td>108.3 ± 5.22 *Y</td>
</tr>
<tr>
<td>NH₄⁺ (mg N kg⁻¹ dry soil)</td>
<td>Top</td>
<td>13.4 ± 1.66 *A</td>
<td>13.0 ± 1.25 *A</td>
<td>8.5 ± 0.55 *B</td>
</tr>
<tr>
<td></td>
<td>Bottom</td>
<td>15.2 ± 2.42 *X</td>
<td>14.9 ± 2.11 *X</td>
<td>9.5 ± 0.77 *Y</td>
</tr>
</tbody>
</table>
Table 4. Total Oxidized N (TON) and ammonium (NH₄⁺) 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 NH₄⁺; n = 4, p < 0.05, p < 0.05).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Layer</th>
<th>Labelled (¹⁵N-KNO₃+C)</th>
<th>Unlabelled (KNO₃+C)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>TON (mg N kg⁻¹ dry soil)</td>
<td>Top</td>
<td>271.8 ± 17.32 *ᴬ</td>
<td>292.6 ± 17.09 *ᴬ</td>
<td>90.5 ± 3.61 *ᴮ</td>
</tr>
<tr>
<td></td>
<td>Bottom</td>
<td>246.0 ± 21.37 *ₓ</td>
<td>239.5 ± 14.85 *ₓ</td>
<td>108.3 ± 5.22 *ᵧ</td>
</tr>
<tr>
<td>NH₄⁺ (mg N kg⁻¹ dry soil)</td>
<td>Top</td>
<td>13.4 ± 1.66 *ᴬ</td>
<td>13.0 ± 1.25 *ᴬ</td>
<td>8.5 ± 0.55 *ᴮ</td>
</tr>
<tr>
<td></td>
<td>Bottom</td>
<td>15.2 ± 2.42 *ₓ</td>
<td>14.9 ± 2.11 *ₓ</td>
<td>9.5 ± 0.77 *ᵧ</td>
</tr>
</tbody>
</table>