Effect of soil saturation on denitrification in a grassland soil

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Abstract. Nitrous oxide (N_2O) is of major importance as a greenhouse gas and precursor of ozone (O_3) destruction in the stratosphere mostly produced in soils. The soil emitted N_2O is generally predominantly derived from denitrification and to a smaller extent, nitrification ~~in soils~~, both processes controlled by environmental factors and their interactions, and are influenced by agricultural management. Soil water content expressed as water filled pore space (WFPS) is a major controlling factor of emissions and its interaction with compaction, has not been studied at the micropore scale. A laboratory incubation was carried out at different saturation levels for a grassland soil and emissions of N_2O and N_2 were measured as well as the isotopocules of N_2O. We found that fluxes variability was larger in the less saturated soils probably due to nutrient distribution heterogeneity created from soil cracks and consequently nutrient hot spots. The results agreed with denitrification as the main source of fluxes at the highest saturations, but nitrification could have occurred at the lower saturation, even though moisture was still high (71% WFSP). The isotopocules data indicated isotopic similarities in the wettest treatments vs the two drier ones. The

results agreed with previous findings where it is clear there are 2 N-pools with different dynamics: added N producing intense denitrification, vs soil N resulting in less isotopic fractionation.

Keywords

Grassland, nitrous oxide,- isotopologues, isotopocule, greenhouse gases

1 Introduction

Nitrous oxide (N_2O) is of major importance as a greenhouse gas and precursor of ozone (O_3) destruction in the stratosphere (Crutzen, 1970). Agriculture is a major source of greenhouse gases (GHGs), such as carbon dioxide (CO_2), methane (CH_4) and also N_2O (IPCC, 2006). The application of organic and inorganic fertiliser N to agricultural soils enhances the production of N_2O (Baggs *et al.*, 2000). This soil emitted N_2O is predominantly derived from denitrification and to a smaller extent, nitrification in soils (Davidson and Verchot, 2000). Denitrification is a microbial process in which reduction of nitrate (NO_3^-) occurs to produce N_2O, and N_2 is the final product of this process, benign for the environment, but represents a loss of N in agricultural systems. Nitrification is an oxidative process in which ammonium (NH_4^+) is converted to NO_3^- (Davidson and Verchot, 2000). Both processes are controlled by environmental factors and their interactions, and are influenced by agricultural management (Firestone and Davidson, 1989). It is well recognised that soil water content expressed as water filled pore space (WFPS) is a major controlling factor and as Davidson (1991) illustrated, nitrification is a source of N_2O until WFPS values reach about 70%, after which denitrification dominates. In fact, Firestone and Davidson (1989) gave oxygen supply a ranking of 1 in importance as a controlling factor in fertilised soils, above C and N. At WFPS between 45 and 75% a mixture of nitrification and denitrification act as N_2O sources. Davidson also suggested that at WFPS values above 90% only N_2 is produced. Several studies have later proposed models to relate WFPS with emissions (Schmidt *et al.*, 2000; Dobbie and Smith, 2001; Parton *et al.*, 2001; del Prado *et al.*, 2006; Castellano *et al.*, 2010) but the "optimum" WFPS for N_2O emissions varies from soil to soil (Davidson, 1991). Soil structure could be influencing this effect and it has been identified to strongly interact with soil moisture (Ball *et al.*, 1999; van Groenigen *et al.*, 2005) through changes in

WFPS. Particularly soil compaction due to livestock treading and the use of heavy machinery affect soil structure and emissions as reported by studies relating bulk density to fluxes (Klefoth *et al.*, 2014b); and degrees of tillage to emissions (Ludwig *et al.*, 2011).

Compaction is known to affect the size of the larger pores (macropores) thereby reducing the soil air volume and therefore increasing the WFPS (for the same moisture content) (van der Weerden *et al.*, 2012). However, little is known about the effect of compaction on the smaller soil pores (micropores) and this could provide valuable information for understanding the simultaneous behaviour of the dynamics of water in the various pore sizes in soil. Such an understanding would lead to the development of better N_2O mitigation strategies via dealing with soil compaction issues.

The role of water in soils is closely linked to microbial activity but also relates to the degree of aeration and gas diffusivity in soils (Morley and Baggs, 2010). Water facilitates nutrient supply to microbes and restricts gas diffusion, thereby increasing the residence time of gases in soil, and the chance of further N_2O reduction before it can be released to the atmosphere. This is further aided by the restriction of the diffusion of atmospheric O_2 (Dobbie and Smith, 2001), increasing the potential for denitrification. As a consequence, counteracting effects (high microbial activity vs low diffusion) occur simultaneously making it difficult to predict net processes and corresponding outputs (Davidson, 1991). Detailed understanding of the sources of N_2O and the influence of physical factors, i.e. soil structure and its interaction with moisture, is a powerful ~~tool~~ basis for developing effective mitigation strategies.

Isotopocules of N_2O represent the isotopic substitution of the O and/or the two N atoms within the N_2O molecule. The isotopomers of N_2O, are those differing in the peripheral (β) and central N-positions (α) of the linear molecule (Toyoda and Yoshida, 1999) with the intramolecular ^{15}N site preference (SP; the difference between $\delta^{15}N^{\alpha}$ - $\delta^{15}N^{\beta}$) used to identify production processes at the level of microbial species or enzymes involved (Toyoda *et al.*, 2005; Ostrom, 2011). Moreover, $\delta^{18}O$, $\delta^{15}N$ and SP of emitted N_2O depend on the denitrification product ratio (N_2O / (N_2+N_2O)), and hence provide insight into the dynamics of N_2O reduction (Well and Flessa, 2009; Lewicka-Szczebak *et al.*,

2014; Lewicka-Szczebak *et al.*, 2015~~; Lewicka-Szczebak *et al.*, 2014~~). ~~Data reported in the literature provide values for these parameters in relation to the source process for N_2O.~~ Koster *et al.* (2013) for example recently reported $\delta^{15}N^{bulk}$ values of N_2O between –36.8‰ and –31.9‰ ~~in~~ under the conditions of their experiment, which are indicative of denitrification according to Perez *et al.* (2006) and Well and Flessa (2009) who proposed the range –54 to –10‰ relative to the substrate. Baggs (2008) summarised that values between –90 to –40‰ are indicative of nitrification. Determination of these values are normally carried out in pure culture studies or in conditions favouring either production or reduction of N_2O (Well and Flessa, 2009). The SP is however considered a better predictor of the N_2O source due to its independence from the substrate signature (Ostrom, 2011).

Simultaneous occurrence production and reduction of N_2O as in natural conditions presents a challenge for isotopic factors determination due to uncertainty on N_2 reduction and the co-existence of different microbial communities ~~resulting in other steps of denitrification happening as well~~ producing N_2O (Lewicka-Szczebak *et al.*, 2014). Recently, using data from the experiment ~~here~~ reported here, where soil was incubated under aerobic atmosphere and the complete denitrification process occurs, Lewicka-Szczebak *et al.* (2015) determined fractionation factors associated with N_2O production and reduction using a modelling approach. The analysis comprised measurements of the N_2O and N_2 fluxes combined with isotopocule data. Net isotope effects (η values) are variable to a certain extent as they result from a combination of several processes causing isotopic fractionation (Well et al., 2012). The results generally confirmed the range of values of η (net isotope effects) and $\eta^{18}O/\eta^{15}N$ ratios reported by previous studies for N_2O reduction for that part of the soil volume were denitrification was enhanced by the N+C amendment. This did not apply for the other part of the soil volume not reached by the N+C amendment, showing that the validity of published net isotope effects for soil conditions with low denitrification activity still needs to be evaluated.

Lewicka-Szczebak *et al.* (2015) observed a clear relationship between ^{15}N and ^{18}O isotope effects during N_2O production and denitrification rates. For N_2O reduction, differential isotope effects were observed for two distinct soil pools characterized by different product ratios N_2O / (N_2+N_2O).

For moderate product ratios (from 0.1 to 1.0) the range of isotope effects given by previous studies was confirmed and refined, whereas for very low product ratios (below 0.1) the net isotope effects were much smaller. In this paper, we present the results from the gas emissions measurements from soils collected from a long-term permanent grassland soil to assess the impact of different levels of soil saturation on N_2O and N_2 and CO_2 emissions after compaction. CO_2 emissions were measured in addition as an estimate of aerobic respiration and thus of O_2 consumption, which indicates denitrification is promoted. The measurements included the soil isotopomer ($^{15}N_\alpha$, $^{15}N_\beta$ and site preference) analysis of emitted N_2O, which in combination with the bulk ^{15}N and ^{18}O was used to distinguish between N_2O from bacterial denitrification and other processes (e.g. nitrification and fungal denitrification) (Lewicka-Szczebak, 201~~6~~7~~a~~).

We conducted measurements at defined saturation of pores size fractions as a prerequisite to model denitrification as a function of water status (Butterbach Bahl *et al.*, 2013 and Müller and Clough, 2014). We have under controlled conditions created a single compaction stress of 200 kPa (typical of soils compacted after grazing) in incremental layers using a uniaxial pneumatic piston to simulate a grazing pressure. We hypothesized that at high water saturation, spatial heterogeneity ~~in~~ of N emissions decreases due to more homogeneous distribution of the soil nutrients and/or anaerobic microsites. We also hypothesized that even at high soil moisture a mixture of nitrification and denitrification can occur. We also aimed to assess how these effects (spatial heterogeneity and source processes) occur in a relatively narrow range of moisture (70-100%). As far as we know there no other studies going to this level of detail. We aimed to understand changes in the ratio $N_2O/(N_2O+N_2)$ at the different moisture levels studied in a controlled manner on soil micro and macropores. Moreover, we used ~~and the behaviour and utility of~~ isotopocule values of N_2O to evaluate if the contribution of bacterial denitrification to the total N_2O flux was affected by moisture status ~~at the different moisture levels studied in a controlled manner on soil micro and macropores~~.

2 Materials and methods

2.1 Soil used in the study

An agricultural soil, under grassland management since at least 1838 (Barré *et al.*, 2010), was collected from a location adjacent to a long-term ley-arable experiment at Rothamsted Research in Hertfordshire (Highfield, see soil properties in Table 1 and further details in Rothamsted Research, 2006; Gregory *et al.*, 2010). The soil had been under permanent cut mixed-species (predominantly *Lolium* and *Trifolium*) vegetation. The soil was sampled as described in Gregory *et al.* (2010). Briefly it was sampled from the upper 150 mm of the profile, air dried in the laboratory, crumbled and sieved (<4 mm), mixed to make a bulk sample and equilibrated at a pre-determined water content (37 g 100 g^{-1}; Gregory *et al.*, 2010) in air-tight containers at 4° C for at least 48 hours.

1.2.Preparation of soil blocks

The equilibrated soil was then packed into twelve stainless steel blocks (145 mm diameter; h: 100 mm), each of which contained three cylindrical holes (i.d: 50 mm; h: 100 mm each). The cores were packed to a single compaction stress of 200 kPa in incremental layers using a uniaxial pneumatic piston. The three hole- blocks were used to facilitate the compression of the cores. The 200 kPa stress was analogous to a severe compaction event by a tractor (Gregory *et al.*, 2010) or livestock (Scholefield *et al.*, 1985). The total area of the upper surface of soil in each block was therefore 58.9 cm^2 (3 × 19.6 cm^2) and the target volume of soil was set to be 544.28 cm^3 (3 × 181.43 cm^3) with the objective of leaving a headspace of approximately 45 cm^3 (3 × 15 cm^3) for the subsequent experiment. The precise height of the soil (and hence the volume) was measured using the displacement measurement system of a DN10 Test Frame (Davenport-Nene, Wigston, Leicester, UK) with a precision of 0.001 mm.

2.3 Equilibration of soil cores at different saturations

The soil was equilibrated to four different initial saturation conditions or treatments (t0) which were based on the likely distribution of water between macropores and micropores. The first treatment was where both the macro- and micropores (and hence the total soil) was fully saturated; the second

157 treatment was where the macropores were half-saturated and the micropores remained fully saturated;
158 the third treatment was where the macropores were fully unsaturated and the micropores again
159 remained fully saturated; and the fourth treatment was where the macropores were fully unsaturated
160 and the micropores were half-saturated. These four treatments are hereafter referred to as SAT/sat;
161 HALFSAT/sat; UNSAT/sat and UNSAT/halfsat, respectively, where upper-case refers to the
162 saturation condition of the macropores and lower-case refers to the saturation condition of the
163 micropores. In order to set these initial saturation conditions, we referred to the gravimetric soil water
164 release characteristic for the soil, as given in Gregory *et al.* (2010) (see supplement 1). To achieve
165 target water contents during the incubation, the amount of liquid added with the C/N amendment (15
166 mL) was taken into account in the total volume of water added. For the SAT/sat and HALFSAT/sat
167 conditions, two sets of three replicate blocks were placed on two fine-grade sand tension tables
168 connected to a water reservoir. For the UNSAT/sat condition a set of three replicate blocks was placed
169 on a tension plate connected to a water reservoir, and the final set of three replicate blocks were placed
170 in pressure plate chambers connected to high-pressure air. All blocks were saturated on their
171 respective apparatus for 24 h, and were then equilibrated for 7 days at the adjusted target matric
172 potentials which were achieved by either lowering the water level in the reservoir (sand tables and
173 tension plate) or by increasing the air pressure (pressure chambers). At the end of equilibration period,
174 the blocks were removed carefully from the apparatus, wrapped in air-tight film, and maintained at 4
175 °C until the subsequent incubation.

176 **2.4 Incubation**

177 The study was carried out under controlled laboratory ~~controlled~~ conditions, using a
178 specialised laboratory denitrification (DENIS) incubation system (Cardenas *et al.*, 2003). Each block
179 containing three cores was placed in an individual incubation vessel of the automated laboratory
180 system in a randomised block design to avoid effect of vessel. The lids for the vessels containing
181 three holes were lined with the cores in the block to ensure that the solution to be applied later would
182 fall on top of each soil core. Stainless steel bulkheads fitted (size for ¼" tubing) on the lids had a

three-layered Teflon coated silicone septum (4 mm thick x 7 mm diameter) for supplying the amendment solution by using a gas tight hypodermic syringe. The bulkheads were covered with a stainless steel nut and only open when amendment was applied. The incubation experiment lasted 13 days. The incubation vessels with the soils were contained in a temperature controlled cabinet and the temperature set at 20°C. The incubation vessels were flushed from the bottom at a rate of 30 ml min^{-1} with a He/O_2 mixture (21% O_2, natural atmospheric concentration) for 24 h, or until the system and the soils atmosphere were emitting low background levels of both N_2 and N_2O (N_2 can get down to levels of 280 ppm much smaller than atmospheric values). Subsequently, the He/O_2 supply was reduced to 10 ml min^{-1} and directed across the soil surface and measurements of N_2O and N_2 carried out at approximately 2 hourly cycles to sample from all the 12 vessels. Emissions of CO_2 were simultaneously measured.

2.5 Application of amendment

An amendment solution equivalent to 75 kg N ha^{-1} and 400 kg C ha^{-1} was applied as a 5 ml aliquot a solution containing KNO_3 and glucose to each of the three cores in each vessel on day 0 of the incubation. Glucose is added to optimise conditions for denitrification to occur (Morley and Baggs, 2010). The aliquot was placed in a stainless steel container (volume 1.2 l) which had three holes drilled with bulkheads fitted, two to connect stainless steel tubing for flushing the vessel, and the third one to place a septum on a bulkhead to withdraw solution. Flushing was carried out with He for half an hour before the solution was required for application to the soil cores and continued during the application process to avoid atmospheric N_2 contamination (a total of one and a half hours). The amendment solution was manually withdrawn from the container with a glass syringe fitted with a three-way valve onto the soil surface; care was taken to minimise contamination from atmospheric N_2 entering the system. The syringe content was injected to the soil cores via the inlets on the lids consecutively in each lid (three cores) and all vessels, completing a total of 36 applications that lasted about 45 minutes. Incubation continued for twelve days, and the evolution of N_2O, N_2 and CO_2 was measured continuously. At the end of each incubation experiment, the soils were removed from the

incubation vessels for further analysis. The three cores in each incubation vessel were ~~pulled together~~pooled in one sample and subsamples taken and analysed for mineral N, total N and C and moisture status. ~~The results of the soil analysis for all cores are presented in Table 3..~~

2.6 Gas measurements

Gas samples were directed to the relevant analysers via an automated injection valve fitted with 2 loops to direct the sample to two gas chromatographs. Emissions of N_2O and CO_2 were measured by Gas Chromatography (GC), fitted with an Electron Capture Detector (ECD) and separation achieved by a stainless steel packed column (2 m long, 4 mm bore) filled with 'Porapak Q' (80–100 mesh) and using N_2 as the carrier gas. The detection limit for N_2O was equivalent to 2.3 g N ha^{-1} d^{-1}. The N_2 was measured by GC with a He Ionisation Detection (HID) and separation achieved by a PLOT column (30 m long 0.53 mm i.d.), with He as the carrier gas. The detection limit was 9.6 g N ha^{-1} d^{-1}. The response of the two GCs was assessed by measuring a range of concentrations for N_2O, CO_2 and N_2. Parent standards of the mixtures 10133 ppm N_2O + 1015.8 ppm N_2; 501 ppm N_2O + 253 ppm N_2 and 49.5 ppm N_2O + 100.6 ppm N_2 were diluted by means of Mass Flow controllers with He to give a range of concentrations of: for N_2O of up to 750 ppm and for N_2 1015 ppm. For CO_2 a parent standard of 30,100 ppm was diluted down to 1136 ppm (all standards were in He as the balance gas). Daily calibrations were carried out for N_2O and N_2 by using the low standard and doing repeated measurements. The temperature inside the refrigeration cabinet containing the incubation vessels was logged on an hourly basis and checked at the end of the incubation. The gas outflow rates were also measured and recorded daily, and subsequently used to calculate the flux.

2.7 Measurement of N_2O isotopic signatures

Gas samples for isotopocule analysis were collected in 115 ml serum bottles sealed with grey butyl crimp-cap septa (Part No 611012, Altmann, Holzkirchen, Germany). The bottles were connected by a Teflon tube to the end of the chamber vents and were vented to the atmosphere through a needle, to maintain flow through the experimental system. Dual isotope and isotopocule signatures of N_2O, i.e. $\delta^{18}O$ of N_2O ($\delta^{18}O$-N_2O), average $\delta^{15}N$ ($\delta^{15}N^{bulk}$) and $\delta^{15}N$ from the central N-position ($\delta^{15}N^{\alpha}$) were

analysed after cryo-focussing by isotope ratio mass spectrometry as described previously (Well *et al.*, 2008). ^{15}N site preference (SP) was obtained as SP = 2 * ($\delta^{15}N^{\alpha}$ – $\delta^{15}N^{bulk}$). Dual isotope and isotopocule ratios of a sample (R_{sample}) were expressed as ‰ deviation from $^{15}N/^{14}N$ and $^{18}O/^{16}O$ ratios of the reference standard materials (R_{std}), atmospheric N_2 and standard mean ocean water (SMOW), respectively:

$$\delta X = (R_{sample}/R_{std} - 1) \times 1000 \quad [2]$$

where X = $^{15}N^{bulk}$, $^{15}N^{\alpha}$, $^{15}N^{\beta}$, or ^{18}O

2.8 Data analysis and additional measurements undertaken

The areas under the curves for the N_2O, CO_2 and N_2 data were calculated by using GenStat 11 (VSN International Ltd, Hemel Hempstead, Herts, UK). The resulting areas for the different treatments were analysed by applying analysis of variance (*ANOVA).* The isotopic ($^{15}N^{bulk}$, ^{18}O, and site preference (SP) differences between the four treatment for the different sampling dates were analysed by two-way ANOVA. We also used the Student's *t* test to check for changes in soil water content over the course of the experiments.

Calculation of the relative contribution of the N_2O derived from bacterial denitrification ($\%B_{DEN}$) was done according to Lewicka-Szczebak *et al.* (2015). The isotopic value of initially produced N_2O, *i.e.* prior to its partial reduction (δ_0) was determined using a Rayleigh model (Mariotti *et al.*, 1982), were δ_0 is calculated using the fractionation factor of N_2O reduction (η_{N2O-N2}) for SP and the fraction of residual N_2O (r_{N2O}) which is equal to the $N_2O/(N_2+N_2O)$ product ratio obtained from direct measurements of N_2 and N_2O flux. An endmember mixing model ~~is~~ was then used to calculate the percentage of bacterial N_2O in the total N_2O flux ($\%B_{DEN}$) from calculated δ_0 values and the SP and $\delta^{18}O$ endmember values of bacterial denitrification and fungal denitrification/nitrification. The range in endmember and η_{N2O-N2} values assumed (adopted from Lewicka-Szczebak, 201~~6~~7~~a~~) to calculated maximum and minimum estimates of $\%B_{DEN}$ is given in Table 4.

Because both, endmember values and η_{N2O-N2}values are not constant but subject to the given ranges, we calculated here several scenarios using combinations of maximum, minimum and average

endmember and η_{N2O-N2} values (Table 4) to illustrate the possible range of $\%B_{DEN}$ for each sample. For occasional cases where $\%B_{DEN} > 100\%$ the values were set to 100%.

At the same time as preparing the main soil blocks, a set of replicate samples was prepared in exactly the same manner, but in smaller cores (i.d: 50 mm; h: 25 mm). On these samples we analysed soil mineral N, total N and C and moisture at the start of the incubation. The same parameters were measured after incubation by doing destructive sampling from the cores. Mineral N (NO_3^-, NO_2^- and NH_4^+) was analysed after extraction with KCl by means of a segmented flow analyser using a colorimetric technique (Searle, 1984). Total C and N in the air dried soil were ~~analysed~~ determined using a thermal conductivity detector (TCD, Carlo Erba, model NA2000). Soil moisture was determined by gravimetric analysis after drying at 105°C.

3 Results

3.1 Soil composition

The results after moisture adjustment at the start of the experiment resulted in a range of WFPS of 100 to 71% for the 4 treatments (Table 2). The results from the end of the incubation also confirmed that there remained significant differences in soil moisture between the high moisture treatments (SAT/sat and HALFSAT/sat) and the two lower moisture treatments (Table 3; one-way ANOVA, $p<0.05$). Soil in the two wettest states lost statistically significant amounts of water (10% ($p=0.006$) and 4.4% ($p<0.001$) for SAT/sat and HALFSAT/sat, respectively) over the course of the 13-day incubation experiment. This was inevitable as there was no way to hold a high (near-saturation) matric potential once the soil was inside the DENIS assembly, and water would have begun to drain by gravitational forces out of the largest macropores (>30 µm). An additional factor was the continuous He/O_2 delivery over the soil surface which would have caused some drying. We accepted these as unavoidable features of the experimental set-up, but we ~~suggest~~ assume that the main response of the gaseous emissions occurred under the initial conditions, prior to the loss of water over subsequent days. Soil in the two drier conditions had no significant change in their water content over the experimental period ($p= 0.153$ and 0.051 for UNSAT/sat and UNSAT/halfsat, respectively). The

results of the initial soil composition were, for mineral N: 85.5 mg NO_3^--N kg^{-1} dry soil, 136.2 mg NH_4^+-N kg^{-1} dry soil. The mineral N contents of the soils at the end of the incubation are reported in Table 3 showing that NO_3^- was very small in treatments SAT/sat and HALFSAT/sat (~1 mg N kg^{-1} dry soil) compared to UNSAT/sat and UNSAT/halfsat (50-100 mg N kg^{-1} dry soil) at the end of the incubation. Therefore, there was a significant difference in soil NO_3^- between the former, high moisture treatments and the latter drier (UNSAT) treatments which were also significantly different between themselves ($p<0.001$ for both). The NH_4^+ content was similar in treatments SAT/sat, HALFSAT/sat and UNSAT/sat (~100 mg N kg^{-1} dry soil), but slightly lower in treatment UNSAT/halfsat (71.3 mg N kg^{-1} dry soil), however overall differences were not significant probably due to the large variability on the driest treatment ($p>0.05$).

3.2 Gaseous emissions of N_2O, CO_2 and N_2

~~The results for~~ All datasets of N_2O and N_2 emissions showed normal distribution (Fpr.<0.001). The treatments SAT/sat and HALFSAT/sat for all three gases, N_2O, CO_2 and N_2 showed fluxes that were well replicated for all the vessels (see Fig. 1), in contrast for UNSAT/sat and UNSAT/halfsat the emissions between the various replicated vessel in each treatment was not as consistent, leading to a larger within treatment variability in the magnitude and shape of the GHG fluxes measured. The cumulative fluxes also resulted in larger variability for the drier treatments (Table 3).

Nitrous oxide and nitrogen gas. The general trend was that the N_2O concentrations in the headspace increased shortly after the application of the amendment (Fig. 1). The duration of the N_2O peak for each replicate soil samples was about three days, except for UNSAT/halfsat in which one of the replicate soils exhibit a peak which lasted for about 5 days. The N_2O maximum in the SAT/sat and HALFSAT/sat treatments was of similar magnitude (~~ca. 5.5~~means of 5.5 and 6.5 kg N ha^{-1} d^{-1}, respectively) ~~and~~ but not those of UNSAT/sat and UNSAT/halfsat ~~also were comparable~~ (~~at around~~means of 7.1 and 11.9 kg N ha^{-1} d^{-1}, respectively). The N_2 concentrations always increased before the soil emitted N_2O reached the maximum. The lag between both N_2O and N_2 peak for all samples was only few hours. Peaks of N_2 generally lasted just over four days, except in

UNSAT/halfsat where one replicate lasted about 6 days (Fig. 1). Unlike in the N_2O data, there was larger within treatment variability in the replicates for all four treatments. The standard deviations of each mean (Table 3) also indicate the large variability in treatments UNSAT/sat and UNSAT/halfsat for both N_2O and N_2.

The product ratios, i.e. $N_2O/(N_2O+N_2)$ resulted in a peak just after amendment addition by ca. 0.73 (at 0.49 d), 0.65 (at 0.48 d), 0.99 (at 0.35 d) and 0.88 (at 0.42 d) for SAT/sat, HALFSAT/sat, UNSAT/sat and UNSAT/halfsat, respectively, and then decreases gradually until day 3 where it becomes nearly zero for the 2 wettest treatments, and stays stable for the driest treatments between 0.1-0.2 (see Table 5 where the daily means of these ratios are presented).

The cumulative areas of the N_2O and N_2 peaks analysed by one-way ANOVA resulted in no significant differences between treatments for both N_2O and N_2 (Table 3). Due to the large variation in treatments UNSAT/sat and UNSAT/halfsat we carried out a pair wise analysis by using a weighted t-test (Cochran, 1957). This analysis resulted in treatment differences between SAT/sat and HALFSAT/sat, HALFSAT/sat and UNSAT/sat, SAT/sat and UNSAT/sat, but only at the 10% significance level ($P < 0.1$ for both N_2O and N_2). ~~It is possible that gases were trapped (particularly in the higher saturation treatments) due to low diffusion and thus possibly masked differences in N_2 and N_2O production since this fraction of gases was not detected (Harter et al. 2016).~~

The results ~~of total N emission (N_2O+N_2) (Table 3)~~ showed that total N emission (N_2O+N_2) (Table 3) ~~had a consistent decreasing trend, with decreasing~~decreased between the highest and lowest soil moistures i.e. from 63.4 for SAT/sat (100% WFPS) to 34.1 kg N ha^{-1} (71% WFPS) for UNSAT/halfsat. The maximum cumulative N_2O occurred at around 80% WFPS (Fig. 2) whereas the total N_2O+N_2 was largest at about 95% and for N_2 it was our upper treatment at 100% WFPS.

Carbon dioxide. The background CO_2 ~~values~~ fluxes (before amendment application, i.e. day -1 to day 0) were high at around 30 kg C ha^{-1} d^{-1} and variable (not shown). The CO_2 concentrations in the headspace increased within a few hours after amendment application. The maximum CO_2 flux was reached earlier in the drier treatments (about 1-2 days; ~70 kg C ha^{-1} d^{-1}) compared to the wettest

(3 days; ~40 kg C ha^{-1} d^{-1}) and former peaks were also sharper (Fig. 1). The cumulative CO_2 fluxes were significantly larger in the two drier unsaturated treatments (ca. 400-420 kg C ha^{-1}) when compared to the wetter more saturated treatment (ca. 280-290 kg C ha^{-1}, $P<0.05$) (Table 3).

3.3 Isotopocules of N_2O

The $\delta^{15}N^{bulk}$ of the soil emitted N_2O in our study differed significantly among the four treatments and between the seven sampling dates ($p<0.001$ for both); there was also a significant treatment*sampling date interaction ($p<0.001$). The maximum $\delta^{15}N^{bulk}$ generally occurred on day 3, except for SAT/sat on day 4 (Table 6).

The maximum $\delta^{18}O$-N_2O values were also found on day 3, except for SAT/sat which peaked at day 2 (Table 6). Overall, the $\delta^{18}O$-N_2O values varied significantly between treatment and sampling dates ($p<0.001$ for both), but there was no significant treatment*time interaction ($p>0.05$).

The site preference (SP) for the SAT/sat treatment had an initial maximum value on day 2 (6.3‰) which decreased thereafter in the period from day 3 to 5 to a mean SP values of the emitted N_2O of 2.0‰ on day 5, subsequently rising to 8.4‰ on day 12 of the experiment (Table 6). The HALFSAT/sat treatment had the highest initial SP values on day 2 and 3 (both 6.4‰), decreasing again to a value of 2.0‰, but now on day 4 followed by subsequent higher SP values of up to 9.2‰ on day 7 (Table 6). The two driest treatments (UNSAT/sat and UNSAT/halfsat) both had an initial maximum on day 3 (11.9‰ and 5.9‰, respectively), and in UNSAT/sat the SP value then decreased to day 7 (3.9‰), but in UNSAT/halfsat treatment after a marginal decrease on day 4 (5.4‰) it then increased throughout the experiment reaching 11.8‰ on day 12 (Table 6). The lowest SP values were generally on day 1 in all treatments. Overall, for all parameters, there was more similarity between the more saturated treatments SAT/sat and HALFSAT/sat, and between the two more dry and aerobic treatments UNSAT/sat and UNSAT/halfsat.

The N_2O / (N_2O + N_2) ratios vs SP for all treatments in the first two days (when N_2O was increasing and the N_2O / (N_2O + N_2) ratio was decreasing) shows a significant negative response of the SP when the ratio increased (Fig. 3). This behaviour suggests that when the emitted gaseous N is

dominated by N_2O (ratio close to 1) the SP values will be slightly negative with an intercept of -2‰ (Fig. 3), i.e. within the SP range of bacterial denitrification. With decreasing N_2O / (N_2O + N_2) ratio the SP values of soil emitted N_2O were increasing to values up to 8‰. This is in juxtaposition with the situation when the N emissions are dominated by N_2 or N_2O is low, where the SP values of soil emitted N_2O were much higher (Fig. 3), pointing to an overall product ratio related to an 'isotopic shift' of 10 to 12.5‰. We fitted 3 functions through this data including a second degree polynomial, a linear and logarithmic function. The fitted logarithmic function in Fig. 3, is in almost perfect agreement with Lewicka-Szczebak *et al.* (2014). Lewicka-Szczebak *et al.* (2014) data fits on the top left of Fig. 3 ~~(their values are for SP and ratio N_2O / (N_2O + N_2): 18.5, 0.18; 10.1, 0.19; 11, 0.28 and 13.4, 0.24, respectively)~~.

It has been reported that the combination of the isotopic signatures of N_2O potentially identifies the contribution of processes other than bacterial denitrification (Köster *et al*., 2015; Wu Di *et al.*, 2016; Deppe *et al*., 2017). The question arises to which extent the relationships between the $\delta^{18}O$ and $\delta^{15}N$bulk and between $\delta^{18}O$ and SP within the individual treatments denitrification dynamics. We checked this to evaluate the robustness of isotope effects during N_2O reduction as a prerequisite to calculate the percentage of bacterial denitrification in N_2O production~~so we have carried out similar analysis with our data. The~~ In our data, maximum $\delta^{18}O$ and SP values, were generally observed at or near the peak of N_2 emissions on days 2-3, independent of the moisture treatment (Table 6 and Fig. 3). $\delta^{15}N^{bulk}$ values of all treatments were mostly negative when N_2O fluxes started to increase (day 1, Fig. 1, Table 6), except for UNSAT/halfsat in which the lowest value was before amendment application, reaching their highest values between days 3 and 4 for when N_2O fluxes were back to the low initial values, and then decreased during the remaining period. $\delta^{18}O$ values increased about 10 - 20‰ after day 1 reaching maximum values on days 2 or 3 in all treatments, while SP increased in parallel, at least by 3‰ (SAT/sat) and up to 12‰(UNSAT/sat). While $\delta^{18}O$ exhibited a steady decreasing trend after day 3, SP behaved opposite to $\delta^{15}N^{bulk}$ with decreasing values while $\delta^{15}N^{bulk}$ was rising again after days 4 or 5.

We further explored the data by looking at the relationships between the $\delta^{18}O$ and $\delta^{15}N^{bulk}$ for all the treatments. The $\delta^{18}O$ vs $\delta^{15}N^{bulk}$ for all treatments is presented separating the data in three periods (Fig. 4): '-1', with $\delta^{18}O$ vs $\delta^{15}N^{bulk}$ values 1 day prior to the moisture adjustment (and N and C application); '1-2', with values in the first 2 days after the addition of water, N and C were added and N_2O emissions were generally increasing in all treatments; and, '3-12', the period in days after moisture adjustment and N and C addition when N_2O emissions generally decreased back to baseline soil emissions. There was a strong and significant relationship ($P<0.001$ and 0.05, respectively) between $\delta^{18}O$ vs $\delta^{15}N^{bulk}$ for the high moisture treatments ($R^2= 0.973$ and 0.923 for SAT/sat and HALFSAT/sat, respectively) at the beginning of the incubation ('1-2') when the N_2O emissions are still increasing, in contrast to those of the lower soil moisture treatments that were lower and not significant ($R^2= 0.294$ and 0.622, for UNSAT/sat and UNSAT/halfsat, respectively). The relationships between $\delta^{18}O$ vs $\delta^{15}N^{bulk}$ of emitted N_2O for the '3-12' period were significant for SAT/sat and HALFSAT/sat with R^2 values between 0.549 and 0.896 and P values <0.05 and 0.001, respectively (Fig. 4). Regressions were also significant for this period for the driest treatments ($P<0.001$). Interestingly, with decreasing soil moisture content (Fig. 4a to 4d) the regression lines of '1-2' and '3-12' day period got closer together in the graphs. Overall, the $\delta^{15}N^{bulk}$ isotopic distances between the two lines was larger for a given $\delta^{18}O$-N_2O value for SAT/sat and HALFSAT/sat (ca. 20‰) when compared to the UNSAT/sat and UNSAT/halfsat treatments (ca. 13‰) (Fig. 4). So it seems the $\delta^{15}N^{bulk}$ / $\delta^{18}O$-N_2O signatures are more similar for the drier soils than the two wettest treatments. In addition, Fig 4 exactly reflects the 2-pool dynamics with increasing $\delta^{15}N$ and $\delta^{18}O$ while the product ratio goes down (days 2,3), then only $\delta^{15}N$ continue increasing due to fractionation of the NO_3^- during exhaustion of pool 1 in the wet soil (days 3,4,5), finally as pool 1 is depleted and more and more comes from pool 2, the product ratio increases somewhat, and $\delta^{15}N$ decreases somewhat since pool 2 is less fractionated and also $\delta^{18}O$ decreases due to slightly increasing product ratio. Note that the turning points of $\delta^{18}O$ and product ratio (Table 3 and 4) for the wetter soils almost coincide.

Similarly to Fig. 4, $\delta^{18}O$ vs the SP (Fig. 5) was analysed for the different phases of the experiment. Generally, the slopes (Table 7) for days 1-2 for the three wettest treatments were similar (~0.2-0.3) following the range of known reduction slopes and also had high and significant ($P<0.05$) regression coefficients (R^2= 0.65, 0.90 and 0.87 for SAT/sat, HALFSAT/Sat and UNSAT/sat, respectively). The slopes on days 3-5 were variable but slightly similar on days 7-12 (between 41 and 0.68) for the same three treatments. They were only significant for the 2 driest treatments ($P<0.05$). On days 7-12 SAT/sat and UNSAT/sat gave significant correlations ($P<0.001$ and 0.05, respectively). Figure 5 also shows the "map" for the values of SP and $\delta^{18}O$ from all treatments. Reduction lines (vectors) represent minimum and maximum routes of isotopocules values with increasing N_2O reduction to N_2 based on the reported range in the ratio between the isotope fractionation factors of N_2O reduction for SP and $\delta^{18}O$ (Lewicka-Szczebak et al., (2016~~7~~a). Most samples are located within the vectors (from Lewicka-Szczebak *et al.* 2016~~7~~a) area of N_2O production by bacterial denitrification with partial N_2O reduction to N_2 (within uppermost and lowermost N_2O reduction vectors representing the extreme values for the bacterial endmember and reduction slopes). Only a few values of the UNSAT/sat and UNSAT/halfsat treatments are located above that vector area and more close or within the vector area of mixing between bacterial denitrification and fungal denitrification/nitrification.

The estimated ranges of the proportion of emitted N_2O resulting from bacterial denitrification ($\%B_{DEN}$) were on day 1 and 2 after the amendment comparable in all four moisture treatments (Table 6). However, during day 3 to 12 the $\%B_{DEN}$ ranged from 78-100% in SAT/sat and 79-100% HALFSAT/Sat, which was generally higher than that estimated at 54-86% for UNSAT/halfsat treatment. The $\%B_{DEN}$ of the UNSAT/halfsat in that period was intermediate between SAT/sat and UNSAT/sat with range of range 60-100% (Table 6). The final values were similar to those on day -1 except for the UNSAT/sat treatment.

4 Discussion

4.1 N_2O and N_2 fluxes

The observed decrease in total N emissions with decreasing initial soil moisture reflects the effect of soil moisture as reported in previous studies (Well *et al.*, 2006). The differences when comparing the cumulative fluxes however, were only marginally ($p<0.1$) significant (Table 3) mostly due to large variability within replicates in the drier treatments (see Fig. 1b). Davidson *et al.* (1991) provided a WFPS threshold for determination of source process, with a value of 60% WFPS as the borderline between nitrification and denitrification as source processes for N_2O production. The WFPS in all treatments in our study was larger than 70%, above this 60% threshold, and referred to as the "optimum water content" for N_2O by Scheer *et al.* (2009), so we can be confident that denitrification was likely to have been the main source process in our experiment. In addition, Bateman *et al.* (2004) observed the largest N_2O fluxes at 70% WFPS on a silty loam soil, lower than the 80% value for the largest fluxes from the clay soil in our study (Fig. 2) suggesting that this optimum value could change with soil type. Further, the maximum total measured N lost (N_2O+N_2) in our study occurred at about 95% WFPS (Fig. 2), but not many studies report N_2 fluxes for comparison and we are still missing measurements of nitric oxide (NO) (Davidson *et al.*, 2000) and ammonia (NH_3) to account for the total N losses. It is however possible that the N_2O+N_2 fluxes in the SAT/sat treatment were underestimated due to low diffusivity in the water filled pores (Well *et al.*, 2001). ~~It is possible that~~ ~~g~~Gases would have been~~were~~ trapped (particularly in the higher saturation treatments) due to low diffusion and thus possibly masked differences in N_2 and N_2O production since this fraction of gases was not detected (Harter et al. 2016). It is worth mentioning that there was some drying during the incubation. The flow of the gas is very slow (10 ml/min) simulating a low wind speed so normally this would dry the soil in field conditions too. It would represent a rainfall event where the initial moisture differs between treatments but some drying occurs due to the wind flow. We believe however, that the effect of drying will be more relevant (and significant relative to the initial moisture) later in the incubation.

The smaller standard errors in both N_2O and N_2 data for the larger soil moisture levels (Table 3 and Fig. 1) could suggest that at high moisture contents nutrient distribution (N and C) on the top

of the core is more homogeneous making replicate cores to behave similarly. At the lower soil moisture for both N_2O and N_2, it is possible that some cracks appear on the soil surface causing downwards nutrient movement, resulting in heterogeneity in nutrient distribution on the surface and increasing variability between replicates, reflected in the larger standard errors of the fluxes. Laudone *et al.* (2011) studied, using a biophysical model, the positioning of the hot-spot zones away from the critical percolation path (described as 'where air first breaks through the structure as water is removed at increasing tensions') and found it slowed the increase and decline in emission of CO_2, N_2O and N_2. They found that hot-spot zones further away from the critical percolation path would reach the anaerobic conditions required for denitrification in shorter time, the products of the denitrification reactions take longer to migrate from the hot-spot zones to the critical percolation path and to reach the surface of the system. The model and its parameters can be used for modelling the effect of soil compaction and saturation on the emission of N_2O. They suggest that having determined biophysical parameters influencing N_2O production, it remains to determine whether soil structure, or simply saturation, is the determining factor when the biological parameters are constrained. Furthermore, Clough *et al.* (2013) indicate that microbial scale models need to be included on larger models linking microbial processes and nutrient cycling in order to consider spatial and temporal variation. Kulkarni *et al.* (2008) refers to "hot spots" and "hot moments" of denitrification as scale dependant and highlight the limitations for extrapolating fluxes to larger scales due to these inherent variabilities.

Well *et al.* (2003) found that under saturated conditions there was good agreement between laboratory and field measurements of denitrification, and attributed deviations, under unsaturated conditions, to spatial variability of anaerobic microsites and redox potential. Dealing with spatial variability when measuring N_2O fluxes in the field remains a challenge, but the uncertainty could be potentially reduced if water distribution is known. Our laboratory study suggests that soil N_2O and N_2 emission for higher moisture levels would be less variable than for drier soils and suggests that for the former a smaller number of spatially defined samples will be needed to get an accurate field estimate. This applied to a lesser extent to the CO_2 fluxes.

Our results, for the two highest water contents (SAT/sat and HALFSAT/sat), indicated that N_2O only contributed 20% of the total N emissions, as compared to 40-50% at the lowest water contents (UNSAT/sat and UNSAT/halfsat, Table 3). This was due to reduction to N_2 at the high moisture level, confirmed by the larger N_2 fluxes, favoured by low gas diffusion which increased the N_2O residence time and the chance of further transformation (Klefoth *et al.*, 2014a). We should also consider the potential underestimation of the fluxes in the highest saturation treatment due to restricted diffusion in the water filled pores (Well *et al.*, 2001). A total of 99% of the soil NO_3^- was consumed in the two high water treatments, whereas in the drier UNSAT/sat and UNSAT/halfsat treatments there still was 35% and 70% of the initial amount of NO_3^- left in the soil, at the end of the incubation, respectively (Table 3). The total amount of gas lost compared to the NO_3^- consumed was almost 3 times for the wetter treatments, and less than twice for the 2 drier ones. This agrees with denitrification as the dominant process source for N_2O with larger consumption of NO_3^- at the higher moisture and larger N_2 to N_2O ratios (5.7, 4.7 for SAT/sat and HALFSAT/sat, respectively), whereas at the lower moisture, ratios were lower (1.5 and 1.0 for UNSAT/sat and UNSAT/halfsat, respectively) (Davidson, 1991). This also indicates that with WFPS above the 60% threshold for N_2O production from denitrification, there was an increasing proportion of anaerobic microsites with increase in saturation controlling NO_3^- consumption and N_2/N_2O ratios in an almost linear manner. With WFPS values between 71-100 % and N_2/N_2O between 1.0 and 5.7, a regression can be estimated: $Y=0.1723\ X - 11.82$ ($R^2=0.8585$), where Y is N_2/N_2O and X is %WFPS. In summary, we propose that heterogeneous distribution of anaerobic microsites could have been the limiting factor for complete depletion of NO_3^- and conversion to N_2O in the two drier treatments. In addition, in the UNSAT/halfsat treatment there was a decrease in soil NH_4^+ at the end of the incubation (almost 50%; Table 3) suggesting nitrification could have been occurring at this water content which also agrees with the increase in NO_3^-, even though WFPS was relatively high (>71%) (Table 3). It is important to note that as we did not assess gross nitrification, the observed net nitrification based on lowering in NH_4^+ could underestimate gross nitrification since there might have been substantial N

mineralisation during the incubation. However, under conditions favouring denitrification at high soil moisture the typical N_2O produced from nitrification is much lower compared to that from denitrification (Lewicka-Szczebak *et al.*, 2016~~7a~~) with the maximum reported values for the N_2O yield of nitrification of 1-3 % (e.g. Deppe *et al.*, 2017). If this is the case, nitrification fluxes could not have exceeded 1 kg N with NH_4^+ loss of < 30 kg * 3% ~1 kg N. This would have represented for the driest treatment, if conditions were suitable only for one day, that nitrification-derived N_2O would have been 6% of the total N_2O produced. Loss of NH_3 was not probable at such low pH (5.6). The corresponding rate of NO_3^- production using the initial and final soil contents and assuming other processes were less important in magnitude, would have been < 1 mg NO_3^--N kg dry $soil^{-1}$ d^{-1} which is a reasonable rate (Hatch *et al.*, 2002). The other three treatments lost similar amounts of soil NH_4^+ during the incubation (23-26%) which could have been due to some degree of nitrification at the start of the incubation before O_2 was depleted in the soil microsites or due to NH_4^+ immobilisation (Table 3) (Geisseler *et al.*, 2010).

~~The CO_2 released in all treatments supports the statement above in relation with the more aerobic status of UNSAT/sat and UNSAT/halfsat, because the cumulative CO_2 flux is roughly 1.5 times higher in the two drier treatments when compared to the wetter ones; but it could have also been the result of higher diffusion in the drier treatments.~~

A mass N balance, taking into account the initial and final soil NO_3^-, NH_4^+, added NO_3^- and the emitted N (as N_2O and N_2) results in unaccounted N-loss of 177.2, 177.6, 130.6 and 110.8 mg N kg^{-1} for SAT/sat, HALFSAT/sat, UNSAT/sat and UNSAT/halfsat, respectively, that could have been emitted as other N gases (such as NO), and some, immobilised in the microbial biomass. NO fluxes reported by Loick et al. (2016) for example, result in a ratio N_2O/NO of 0.4. In summary unaccounted-for N loss is two to three times the total measured gas loss (Table 3). In addition, in the SAT/sat treatment there was probably an underestimation of the produced N_2 and N_2O due to restricted diffusion at the high WFPS (e.g. Well *et al.*, 2001).

4.2 Isotopocule trends.

Trends of isotopocule values of emitted N_2O coincided with those of N_2 and N_2O fluxes. The results from the isotopocule data (Table 6 and Fig. 3) also indicated that generally there were more isotopic similarities between the two wettest treatments when compared to the two contrasting drier soil moisture treatments.

Isotopocule values of emitted N_2O reflect multiple processes where all signatures are affected by the admixture of several microbial processes, the extent of N_2O reduction to N_2 as well as the variability of the associated isotope effects (Lewicka-Szczebak *et al.*, 2015). Moreover, for $\delta^{18}O$ and $\delta^{15}N^{bulk}$ the precursor signatures are variable (Decock and Six, 2013), for $\delta^{18}O$ the O exchange with water can be also variable (Lewicka-Szczebak *et al.*, 201~~6~~7~~b~~). Since the number of influencing factors clearly exceeds the number of isotopocule values, unequivocal results can only be obtained if certain processes can be excluded or be determined independently, (Lewicka-Szczebak *et al.*, 2015; Lewicka-Szczebak, 201~~6~~7~~a~~). The two latter conditions were fulfilled in this study, i.e. N_2O fluxes were high and several order of magnitude above possible nitrification fluxes, since the N_2O – to- NO_3^- ratio yield of nitrification products rarely exceeds 1% (Well *et al.*, 2008; Zhu et al., 2012). Moreover, N_2 fluxes and thus N_2O reduction rates were exactly quantified.

The estimated values of % B_{DEN} indicate that in the period immediately after amendment application all moisture treatments were similar, reflecting that the microbial response to N and C added was the same and denitrification dominated. This was the same for the rest of the period for the wetter treatments. In the drier treatments, proportions decreased afterwards and were similar to values before amendment application, possibly due to recovery of more aerobic conditions that could have encouraged other processes to contribute. As N_2 was still produced in the driest treatment, (but in smaller amounts), this indicated ongoing denitrifying conditions and thus large contributions to the total N_2O flux from nitrification were not probable~~from nitrification were not probable~~, but some occurred as suggested by NH_4^+ consumption.

The trends observed reflect the dynamics resulting from the simultaneous application of NO_3^- and labile C (glucose) on the soil surface as described in previous studies (Meijide *et al.*,

573 2010; Bergstermann *et al.*, 2011) where the same soil was used, resulting in two locally distinct
574 NO_3^- pools with differing denitrification dynamics. In the soil volume reached by the NO_3^-/glucose
575 amendment, denitrification was initially intense with high N_2 and N_2O fluxes and rapid isotopic
576 enrichment of the NO_3^--N. When the NO_3^- and/or glucose of this first pool were exhausted, N_2 and
577 N_2O fluxes were much lower and dominated by the initial NO_3^- pool that was not reached by the
578 glucose/NO_3^- amendment and that is less fractionated due to its lower exhaustion by denitrification,
579 causing decreasing trends in $\delta^{15}N^{bulk}$ of emitted N_2O.

580 This is also reflected in Fig 4 where N_2O fluxes from both pools exhibited correlations (and
581 mostly significant) between $\delta^{15}N^{bulk}$ and $\delta^{18}O$ due to varying N_2O reduction, but $\delta^{15}N^{bulk}$ values in
582 days 1 and 2 - i.e. the phase when Pool 1 dominated - were distinct from the previous and later phase.

583 The fit of $^{15}N^{bulk}$ /^{18}O data to two distinct and distant regression lines can be attributed to
584 two facts: Firstly, in the wet treatment (Fig 4a, b) Pool 1 was probably completely exhausted and
585 there was little NO_3^- formation from nitrification (indicated by final NO_3^- values close to 0, Table 3)
586 whereas the drier treatment exhibited substantial NO_3^- formation and high residual NO_3^-. Hence,
587 there was probably still some N_2O from Pool 1 after day 2 in the dry treatment but not in the wetter
588 ones. Secondly, the product ratios after day 2 of the drier treatments were higher (0.13 to 0.44)
589 compared to the wetter treatments (0.001 to 0.09). Thus the isotope effect of N_2O reduction was
590 smaller in the drier treatments, leading to a smaller upshift of $\delta^{15}N^{bulk}$ and thus more negative values
591 after day 2, i.e. with values closer to days 1 +2.

592 This finding further confirms that $\delta^{15}N/\delta^{18}O$ patterns are useful to identify the presence of
593 several N pools, e.g. typically occurring after application of liquid organic fertilizers which has
594 been previously demonstrated using isotopocule patterns (Koster *et al.*, 2015).

595 Interestingly, the highest $\delta^{15}N^{bulk}$ and $\delta^{18}O$ values of the emitted N_2O were found in the soils
596 of the HALFSAT/sat treatment, although it may have been expected that the highest isotope values
597 from the N_2O would be found in the wettest soil (SAT/sat) because N_2O reduction to N_2 is favoured
598 under water-saturated conditions due to extended residence time of produced N_2O (Well et al., 2012).

599 However, $N_2O/(N_2+N_2O)$ ratios of the SAT/sat and SAT/halfsat treatments were not different (Table
600 5). Bol *et al.* (2004) also found that some estuarine soils under flooded conditions (akin to our
601 SAT/sat) showed some strong simultaneous depletions (rather than enrichments) of the emitted N_2O
602 $\delta^{15}N^{bulk}$ and $\delta^{18}O$ values. These authors suggested that this observation may have resulted from a flux
603 contribution of an 'isotopically' unidentified N_2O production pathway. Another explanation could be
604 complete consumption of some of the produced N_2O in isolated micro-niches in the SAT/sat treatment
605 due to inhibited diffusivity in the fully saturated pores space. N_2 formation in these isolated domains
606 would not affect the isotopocule values of emitted N_2O and this would thus result in lower apparent
607 isotope effects of N_2O reduction in water saturated environments as suggested by Well *et al.* (2012).
608 The SP values obtained were generally below 12‰ in agreement with reported ranges
609 attributed to bacterial denitrification: -2.5 to 1.8‰ (Sutka *et al.*, 2006); 3.1 to 8.9‰ (Well and
610 Flessa, 2009); -12.5 to 17.6‰ (Ostrom, 2011). The SP, believed to be a better predictor of the N_2O
611 source as it is independent of the substrate isotopic signature (Ostrom, 2011), has been suggested as
612 it can be used to estimate N_2O reduction to N_2 in cases when bacterial denitrification can be
613 assumed to dominate N_2O fluxes (Koster *et al.*, 2013; Lewicka-Szczebak *et al.*, 2015). There was a
614 strong correlation between the SP and N_2O / (N_2O+N_2) ratios on the first 2 days of the incubation
615 for all treatments up until the N_2O reached its maximum (Fig. 3) which reflects the accumulation of
616 $\delta^{15}N$ at the alpha position during ongoing N_2O reduction to N_2. Later on in the experiment beyond
617 day 3, this was not observed probably because in that period the product ratio remained almost
618 unchanged and very low (Table 6). Similar observations have been reported by Meijide *et al.* (2010)
619 and Bergstermann *et al.* (2011), as they also found a decrease in SP during the peak flux period in
620 total N_2+N_2O emissions, but only when the soil had been kept wet prior to the start of the
621 experiment (Bergstermann *et al.*, 2011). These results confirm from 2 independent studies
622 (Lewicka-Szczebak *et al.*, 2014) that there is a relationship between the product ratios and isotopic
623 signatures of the N_2O emitted. The $\delta^{18}O$ vs SP regressions indicate more similarity between the

three wettest treatments as well as high regression coefficients, suggesting this SP/$\delta^{18}O$ ratio could also be used to help identify patterns for emissions and their sources.

4.3 Link to modelling approaches.

Since isotopocule data could be compared to N_2 and N_2O fluxes, the variability of isotope effects of N_2O production and reduction to N_2 by denitrification could be determined from this data set (Lewicka-Szczebak *et al.*, 2015) and this included modelling the two pool dynamics discussed above. It was demonstrated that net isotope effects of N_2O reduction (η_{N2O-N2}) determined for both NO_3^- pools differed. Pool 1 representing amended soil and resulting in high fluxes but moderate product ratio, exhibited η_{N2O-N2} values and the characteristic $\eta^{18}O/\eta^{15}N$ ratios similar to those previously reported, whereas for Pool 2 characterized by lower fluxes and very low product ratio, the net isotope effects were much smaller and the $\eta^{18}O/\eta^{15}N$ ratios, previously accepted as typical for N_2O reduction processes (i.e., higher than 2), were not valid. The question arises, if the poor coincidence of Pool 2 isotopologue fluxes with previous N_2O reduction studies reflects the variability of isotope effects of N_2O reduction or if the contribution of other processes like fungal denitrification could explain this (Lewicka-Szczabak *et al.*, 2017). The latter explanation is evaluated in section 4.3

~~The question arises, if the poor coincidence of Pool 2 isotopocule fluxes with previous N_2O reduction studies reflects the variability of isotope effects of N_2O reduction or if the contribution of other processes like fungal denitrification could explain this.~~

~~-~~Liu *et al.* (2016) noted that on the catchment scale potential N_2O emission rates were related to hydroxylamine and NO_3^-, but not NH_4^+ content in soil. Zou *et al.* (2014) found high SP (15.0 to 20.1‰) values at WFPS of 73 to 89% suggesting that fungal denitrification and bacterial nitrification contributed to N_2O production to a degree equivalent to that of bacterial denitrification.

To verify the contribution of fungal denitrification and/or hydroxylamine oxidation we can first look at the $\eta SP_{N2O-NO3}$ values calculated in the previous modelling study applied on the same dataset, (Table 1, the final modelling Step, Lewicka-Szczebak *et al.*, 2015). For Pool 1 there are no

significant differences between the values of various treatments, SP_0 ranges from (-1.8±4.9) to (+0.1±2.5). Pool 1 emission was mostly active in days 1-2, hence these values confirm the bacterial dominance in the emission at the beginning of incubation, which originates mainly from the amendment addition and represent similar pathway for all treatments. However, for the Pool 2 emission we could observe a significant difference when compared the two wet treatments (SAT/sat and HALFSAT/sat: (-5.6±7.0)) with the UNSAT/sat treatment (+3.8±5.8). This represents the emission from unamended soil which was dominating after the third day of the incubation and indicates higher nitrification contribution for the drier treatment.

4.4 Contribution of bacterial denitrification.

An endmember mixing approach has been previously used to estimate the fraction of bacterial N_2O ($\%B_{DEN}$), but without independent estimates of N_2O reduction (Zou *et al.*, 2014), but due to the unknown isotopic shift by N_2O reduction, the ranges of minimum and maximum estimates were large, showing that limited information is obtained without N_2 flux measurement.

In an incubation study with two arable soils, Koster *et al.* (2013) used $N_2O/(N_2+N_2O)$ ratios and isotopocule values of gaseous fluxes to calculate SP of N_2O production (referred to as SP_0), which is equivalent to SP_0 using the Rayleigh model and published values of η_{N2O-N2}. The endmember mixing approach based on SP_0 was then used to estimate fungal denitrification and/or hydroxylamine oxidation giving indications for a substantial contribution in a clay soil, but not in a loamy soil. Here we presented for the first time an extensive data set with large range in product ratios and moisture to calculate the contribution of bacterial denitrification ($\%B_{DEN}$) of emitted N_2O from SP_0. The uncertainty of this approach arises from three factors, (i) from the range of SP_0 endmember values for bacterial denitrification of -11 to 0 per mil and 30 to 37 for hydroxylamine oxidation/fungal denitrification, (ii) from the range of net isotope effect values of N_2O reduction (η_{N2O-N2}) for SP which vary from -2 to -8 per mil (Lewicka-Szczebak *et al.*, 2015), and iii) system condition (open vs. closed) taken to estimate the net isotope effect (Wu *et al.*, 2016).

675 The observation that $\%B_{DEN}$ of emitted N_2O was generally high (63-100%) in the wettest
676 treatment (SAT/sat) was not unexpected. However interestingly $\%B_{DEN}$ in the HALFSAT/sat
677 treatment was very similar (71-98%), pointing to the role of the wetter areas of the soil
678 microaggregates contributing to high $\%B_{DEN}$ values. The slightly lower values, i.e. down 60% in
679 UNSAT/sat $\%B_{DEN}$ range of 60-100%, suggest that the majority of N_2O derived from bacterial
680 denitrification still results from the wetter microaggregates of the soils, despite the fact that the
681 macropores are now more aerobic. Only, when the micropores become partially wet, as in the
682 UNSAT/halfsat treatment, do the more aerobic soil conditions allow a higher contribution of
683 nitrification/fungal denitrification ranging from 0 - 46% (1 - $\%B_{DEN}$, Table 6) on days 3-12 (Zhu *et*
684 *al.*, 2013). Differences in the contribution of nitrification/fungal denitrification between the flux
685 phases when different NO_3^- pools were presumably dominating are only indicated in the driest
686 treatment, since 1-$\%B_{DEN}$ was higher after day 2 (14 to 46%) compared to days 1+2 (0 to 33 %).
687 This larger share of nitrification/fungal denitrification can be attributed to the increasing
688 contribution from Pool 2 to the total flux as indicated by the modeling of higher SP_0 for Pool 2 (see
689 previous section and Lewicka-Szczebak *et al.* (2015). In addition, indication for elevated
690 contribution of processes other than bacterial denitrification were only evident in the drier
691 treatments during phases before and after N_2, N_2O fluxes were strongly enhanced by glucose
692 amendment. The data supply no clue whether the other processes were suppressed during the anoxia
693 induced by glucose decomposition or just masked by the vast glucose-induced bacterial N_2O fluxes.
694

695 ## 5 Conclusions

696 The results from this study demonstrated that at high soil moisture levels, there was less variability
697 in N fluxes between replicates, potentially decreasing the importance of soil hot spots in emissions
698 at these moisture levels. At high moisture there also was complete depletion of nitrate confirming
699 denitrification as the main pathway for N_2O emissions, and due to less diffusion of the produced
700 N_2O, the potential for further reduction to N_2 increased. Under less saturation, but still relatively

high soil moisture, nitrification occurred. Isotopic similarities were observed between similar saturation levels and patterns of $\delta^{15}N/\delta^{18}O$ and $SP/\delta^{18}O$ are suggested as indicators of source processes.

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Figures

Figure 1. Mean of the three replicates for N_2O, N_2 and CO_2 emissions from a. SAT/sat treatment; b. HALFSAT/sat; c. UNSAT/sat; d. UNSAT/halfsat. Grey lines correspond to the standard error of the means.

Figure 2 Total N emissions (N_2O+N_2)-N, N_2O and N_2 vs WFPS. Fitted functions through each dataset are also shown.

Figure 3 Ratio N_2O / (N_2O + N_2) vs. Site Preference (SP) for all for treatments in the first two days. A logarithmic function was fitted through the data, the corresponding equation and correlation coefficient are given.

Figure 4 $\delta^{18}O$ vs $\delta^{15}N_{bulk}$ in all treatments for three periods (day -1 in diamond symbol, days 1-2 in square symbol and days 3-12 in triangle symbol, respectively) in the experiment: a. SAT/sat treatment; b. HALFSAT/sat; c. UNSAT/sat; d. UNSAT/halfsat. Equations of fitted functions and correlation coefficients are shown. Correlations are unadjusted, the P value tests if the slope is different from zero.

Figure 5 Site Preference vs $\delta^{18}O$ in all treatments for three periods (day -1, days 1-2 and days 3-12) in the experiment: a. SAT/sat treatment; b. HALFSAT/sat; c. UNSAT/sat; d. UNSAT/halfsat. Equations of fitted functions and correlation coefficients are in Table 7 for 1-2, 3-5 and 7-12 (5-12 for c.). Endmember areas for nitrification, N; bacterial denitrification, D; fungal denitrification, FD and nitrifier denitrification, ND and corresponding vectors or reduction lines (black solid lines) are from Lewicka-Szczebak et al., (201~~6~~7~~a~~), and represent minimum and maximum routes of isotopocule values with increasing N_2O reduction to N_2 based on the reported range in the ratio between the isotope fractionation factors of N_2O reduction for SP and $\delta^{18}O$ (Lewicka-Szczebak et al., 201~~6~~7~~a~~).

Tables

Table 1 Soil properties of the soil used in the experiment

Table 2 The four saturation conditions used for the soil in the experiment

739 **Table 3** Contents of soil moisture, NO_3^-, NH_4^+ and C:N ratio and cumulative fluxes of N_2O and N_2

740 and CO_2 from all treatments at the end of the incubation.

741 **Table 4** Scenarios with different combinations of $\delta^{18}O$ and SP endmember values and $\eta N_2O\text{-}N_2$

742 values to calculate maximum and minimum estimates of $\%B_{DEN}$ (minimum, maximum and average

743 values adopted from Lewicka-Szczebak *et al.*, (2016).

744 **Table 5** Ratios N_2O / (N_2O + N_2) for all treatments

745 **Table 6** The temporal trends in $\delta^{15}N_{bulk}$, $\delta^{18}O$, $\delta^{15}N_{\alpha}$, SP and $\%B_{DEN}$ for all experimental treatments

746 **Table 7** Equations of fitted functions and correlation coefficients corresponding to Figure 5 for Site

747 Preference vs $\delta^{18}O$ in all treatments for three periods.

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Table 1. Highfield soil properties

Property	Units	Highfield
Location		Rothamsted Research Herts.
Grid reference	GB National Grid	TL129130
	Longitude	00°21'48"W
	Latitude	51°48'18"N
Soil type	SSEW[a] group[c]	Paleo-argillic brown earth
	SSEW[a] series[d]	Batcombe
	FAO[bc]	Chromic Luvisol
Landuse		Grass; unfertilised; cut
pH		5.63
Sand (2000-63 µm)	g g^{-1} dry soil	0.179
Silt (63-2 µm)	g g^{-1} dry soil	0.487
Clay (<2 µm)	g g^{-1} dry soil	0.333
Texture	SSEW[a] class[c]	Silty clay loam
Particle density	g cm^{-3}	2.436
Organic matter	g g^{-1} dry soil	0.089
Water content for packing	g g^{-1} dry soil	0.37

[a]Soil Survey of England and Wales classification system
[b]United Nations Food and Agriculture Organisation World Reference Base for Soil Resources classification system (approximation)
[c]Avery (1980)
[d]Clayden & Hollis (1984)

Table 2. The four saturation conditions set for the Highfield soil.

Saturation condition	SAT/sat	HALFSAT/sat	UNSAT/sat	UNSAT/halfsat
Macropores	Saturated	Half-saturated	Unsaturated	Unsaturated
Micropores	Saturated	Saturated	Saturated	Half-saturated
As prepared:				
Matric potential, -kPa	4.1	12.3	27.3	136.9
Water content, g 100 g^{-1}	47.7	42.5	37.2	29.4
Water content, cm^{-3} 100 cm^{-3}	61.1	54.4	47.7	37.3
Water-filled pore space, %	98	91	82	68
Threshold pore size saturated, µm	73	24	11	2
Final, following amendment:				
Matric potential, -kPa	0	8.6	20.0	78.1
Water content, g 100 g^{-1}	49.8	44.6	39.3	31.5
Water content, cm^{-3} 100 cm^{-3}	63.8	57.1	50.4	40.0
Water-filled pore space, %	100	94	85	71
Threshold pore size saturated, µm	all	35	15	4

Table 3. Contents of soil moisture, NO_3^-, NH_4^+ and C:N ratio and cumulative fluxes of N_2O and N_2 and CO_2 from all treatments at the end of the incubation. Values in brackets are standard deviation of the mean of three values (emissions are expressed per area and soil weight basis).

Treatment	% Mean moisture	NO_3^-, mg N kg^{-1} dry soil	NH_4^+, mg N kg^{-1} dry soil	Total C, %	Total N, %	N_2O, kg N ha^{-1}	N_2O, mg N kg^{-1} dry soil	N_2, kg N ha^{-1}	N_2, mg N kg^{-1} dry soil	Total emitted N, kg N ha^{-1}	CO_2, kg C ha^{-1}
SAT/sat	39.8 (1.3)	1.1 (0.4)	104.3 (1.1)	3.61 (0.04)	0.35 (0.004)	9.4 (1.1)	7.8 (0.9)	54.0 (14.0)	44.8 (11.6)	63.4	289.2 (30.4)
HALFSAT/sat	40.2 (0.2)	0.8 (1.0)	104.2 (6.8)	3.64 (0.08)	0.36 (0.004)	10.9 (0.4)	9.0 (0.3)	51.7 (9.0)	42.8 (7.4)	62.6	283.0 (35.5)
UNSAT/sat	36.5 (2.1)	51.2 (37.4)	100.8 (5.7)	3.64 (0.10)	0.36 (0.007)	23.7 (11.0)	20.0 (9.5)	36.0 (28.5)	30.2 (23.7)	59.7	417.6 (57.1)
UNSAT/halfsat	34.3 (1.1)	100.6 (16.1)	71.3 (33.6)	3.53 (0.08)	0.36 (0.01)	16.8 (15.8)	14.0 (13.1)	17.2 (19.4)	14.3 (16.1)	34.1	399.7 (40.6)

Table 4: Scenarios with different combinations of $d^{18}O$ and Site Preference (SP) endmember values and η_{N2O-N2} values to calculate maximum and minimum estimates of %Bden (minimum, maximum and average values adopted from Lewicka-Szczabak et al., 2017~~6a~~).

	SP0BD	SP0FDN	ηSP	$\eta^{18}O$
model (min endmember plus η)	-11	30	-2	-12
model (max endmember plus η)	0	37	-8	-12
model (max endmember)	0	37	-5.4	-12
model (min endmember)	-11	30	-5.4	-12
model (max η)	-5	33	-8	-12
model (min η)	-5	33	-2	-12

984 Table 5. Ratios N_2O / (N_2O + N_2) for all treatments
985

	SAT/sat		**HALFSAT/sat**		**UNSAT/halfsat**		**UNSAT/sat**	
Days	mean	s.e.	mean	s.e.	mean	s.e.	mean	s.e.
-1	0.276	0.043	0.222	0.009	0.849	0.043	0.408	0.076
0	0.630	0.022	0.538	0.038	0.763	0.053	0.861	0.043
1	0.371	0.025	0.360	0.019	0.622	0.018	0.644	0.031
2	0.096	0.016	0.139	0.015	0.425	0.005	0.296	0.020
3	0.004	0.002	0.015	0.006	0.439	0.052	0.256	0.025
4	0.017	0.002	0.008	0.001	0.475	0.049	0.232	0.012
5	0.019	0.003	0.012	0.001	0.503	0.037	0.174	0.010
6	0.068	0.008	0.020	0.001	0.459	0.052	0.135	0.010
7	0.085	0.008	0.047	0.003	0.333	0.057	0.127	0.003
8	0.106	0.004	0.066	0.002	0.277	0.006	0.122	0.002
9	0.089	0.003	0.053	0.005	0.265	0.006	0.122	0.005
10	0.060	0.003	0.090	0.014	0.428	0.086	0.118	0.006
11	0.063	0.002	0.053	0.002	0.414	0.051	0.125	0.005

986
987

Table 6. The temporal trends in $\delta^{15}N_{bulk}$, $\delta^{18}O$, $\delta^{15}N_{\alpha}$, Site Preference (SP) and $\%B_{DEN}$ for all experimental treatments (values in brackets are the standard deviation of the mean)

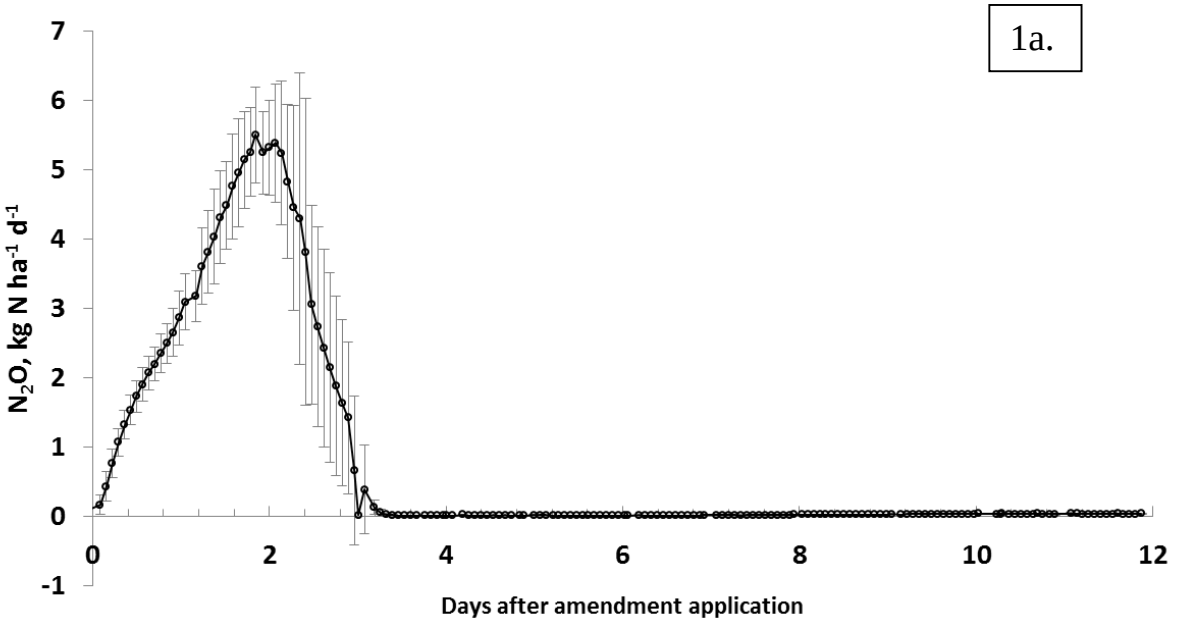
	$\delta^{15}N_{bulkAIR}$ (‰)			
Day	SAT/sat	HALFSAT/~~Sat~~sat	UNSAT/~~Sat~~sat	UNSAT/halfsat
-1	-3.8 (2.1)	-6.2 (1.5)	-14.2 (10.9)	-23.6 (1.1)
1	-18.9 (1.6)	-25.5 (4.6)	-20.3 (2.6)	-20.8 (2.3)
2	-7.7 (4.2)	-12.7 (2.7)	-12.2 (2.0)	-13.9 (5.7)
3	-2.4 (1.8)	14.0 (2.2)	-1.1 (7.6)	-4.4 (3.0)
4	-0.9 (2.2)	-0.3 (3.6)	-7.8 (4.6)	-9.3 (3.7)
5	-6.9 (0.9)	-4.3 (6.1)	-11.3 (3.7)	-8.9 (7.7)
7	-9.6 (1.5)	-10.0 (1.6)	-14.3 (4.7)	-13.4 (13.5)
12	-7.5 (1.2)	-8.6 (0.9)	-11.8 (2.6)	-21.3 (6.9)
	$\delta^{18}O_{SMOW}$ (‰)			
	SAT/sat	HALFSAT/~~Sat~~sat	UNSAT/~~Sat~~sat	UNSAT/halfsat
-1	33.3 (2.6)	32.7 (3.0)	31.4 (9.8)	25.2 (4.9)
1	42.9 (2.4)	37.1 (3.8)	32.3 (3.6)	33.3 (2.1)
2	54.0 (5.7)	48.7 (4.5)	42.7 (5.3)	40.5 (5.0)
3	45.7 (1.5)	59.7 (3.2)	53.4 (5.7)	41.2 (1.0)
4	42.5 (1.4)	42.0 (3.7)	38.1 (4.5)	39.9 (7.7)
5	36.0 (2.9)	34.6 (3.7)	30.4 (2.6)	36.5 (6.9)
7	32.2 (5.5)	31.6 (5.5)	28.4 (4.4)	32.7 (5.4)
12	34.9 (5.6)	34.1 (2.7)	32.4 (2.9)	28.5 (5.0)
	$\delta^{15}N\alpha_{AIR}$ (‰)			
	SAT/sat	HALFSAT/~~Sat~~sat	UNSAT/~~Sat~~sat	UNSAT/halfsat
-1	-0.3 (3.4)	-2.6 (1.8)	-9.5 (12.0)	-19.7 (2.1)
1	-17.4 (1.8)	-24.0 (5.8)	-20.2 (2.0)	-21.1 (2.6)
2	-4.6 (4.2)	-9.5 (3.6)	-11.1 (1.1)	-13.8 (5.9)
3	-0.8 (1.3)	17.2 (4.0)	7.6 (4.7)	-2.7 (3.2)
4	1.0 (2.5)	0.7 (2.2)	-3.5 (3.7)	-2.8 (7.7)
5	-5.9 (0.7)	-2.9 (5.4)	-9.4 (3.9)	-5.2 (7.9)
7	-7.8 (2.3)	-5.3 (4.2)	-12.3 (5.6)	-7.7 (11.5)
12	-3.3 (2.1)	-4.6 (0.6)	-8.1 (4.2)	-15.3 (5.5)
	SP_{AIR}			
	SAT/sat	HALFSAT/~~Sat~~sat	UNSAT/~~Sat~~sat	UNSAT/halfsat
-1	7.0 (3.9)	7.1 (4.2)	9.4 (2.1)	7.7 (1.9)
1	2.9 (0.6)	3.0 (2.3)	0.1 (1.8)	-0.7 (1.4)
2	6.3 (0.64)	6.4 (1.9)	2.2 (2.0)	0.2 (1.9)
3	3.3 (1.0)	6.4 (6.9)	11.9 (12.4)	5.9 (0.8)
4	3.7 (0.6)	2.0 (6.2)	8.7 (5.9)	5.4 (3.0)
5	2.0 (0.4)	3.0 (2.1)	3.9 (0.5)	7.4 (2.3)
7	5.0 (2.1)	9.2 (5.2)	3.9 (1.8)	11.2 (4.1)
12	8.4 (3.3)	7.9 (0.8)	7.3 (3.7)	11.8 (5.3)
	Estimated range of $\%B_{DEN}$			
	SAT/sat	HALFSAT/sat	UNSAT/sat	UNSAT/halfsat
-1	63-100	60-100	53-85	56-84
1-2	68-100	67-100	73-100	77-100
3-12	78-100	79-100	60-100	54-86

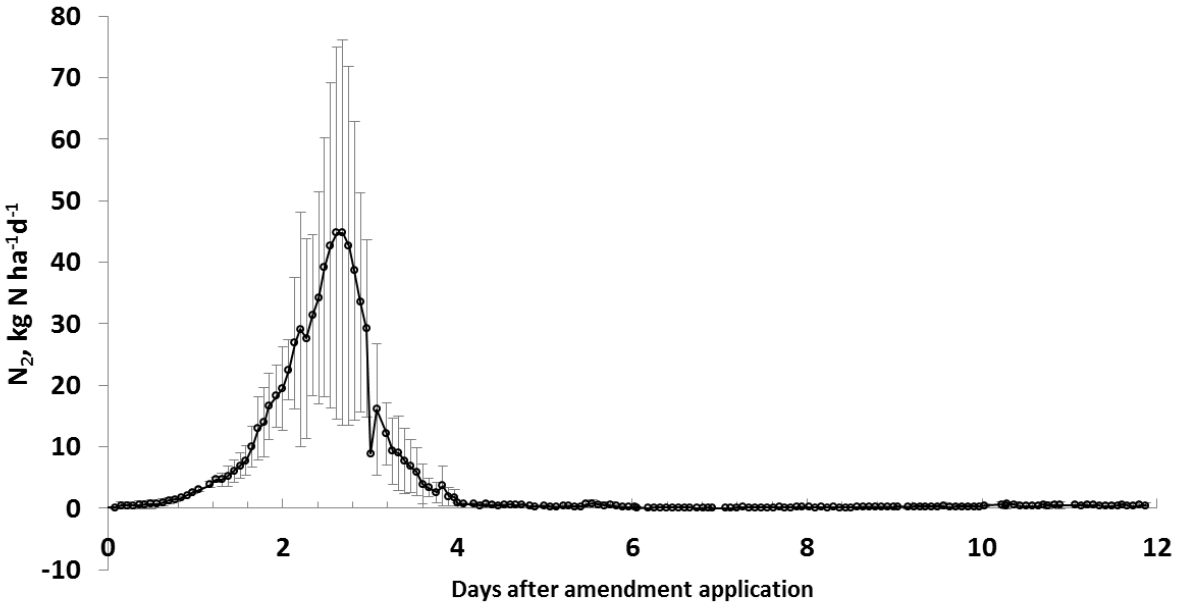
990 Table 7. Equations of fitted functions and correlation coefficients corresponding to Figure 5 for Site
991 Preference (SP) (Y axis) vs $\delta^{18}O$ (X axis) in all treatments for three periods. Correlations are
992 unadjusted, the P value tests if the slope is different from zero.
993

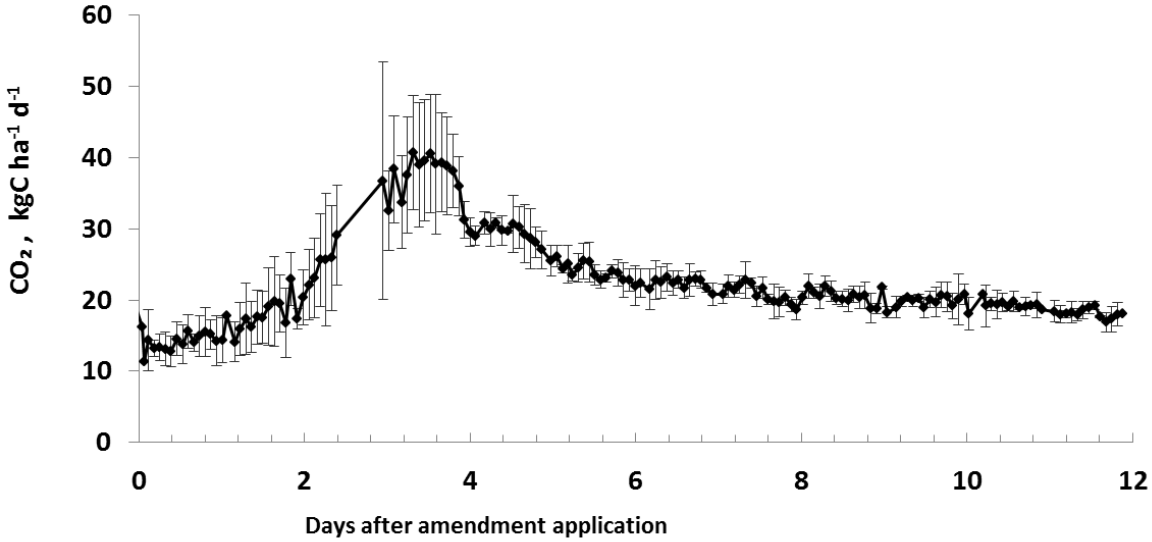
Treatment	Days 1-2	Days 3-5	Days 7-12
SAT/sat	$y = 0.2151x - 5.8386$, $R^2 = 0.6529$ P=0.05	$y = 0.1204x - 1.848$, $R^2 = 0.397$ P=0.129	$y = 0.5872x - 12.223$, $R^2 = 0.985$ P<0.001
HALFSAT/sat	$y = 0.3447x - 10.129$, $R^2 = 0.9048$ P=0.004	$y = 0.18x - 4.5966$, $R^2 = 0.1728$ P=0.266	$y = 0.4063x - 6.2632$, $R^2 = 0.6876$ P=0.171
UNSAT/sat	$y = 0.2709x - 8.9968$, $R^2 = 0.8664$ P=0.007	$y = 0.7248x - 18.874$, $R^2 = 0.507$ P=0.031	$y = 0.6848x - 15.236$, $R^2 = 0.7156$ P=0.034
UNSAT/halfsat	$y = -0.0146x + 0.2506$, $R^2 = 0.0024$ P=0.927	$y = 0.3589x - 7.2194$, $R^2 = 0.4839$ P=0.037	$y = -0.318x + 21.261$, $R^2 = 0.1491$ P=0.450

994
995

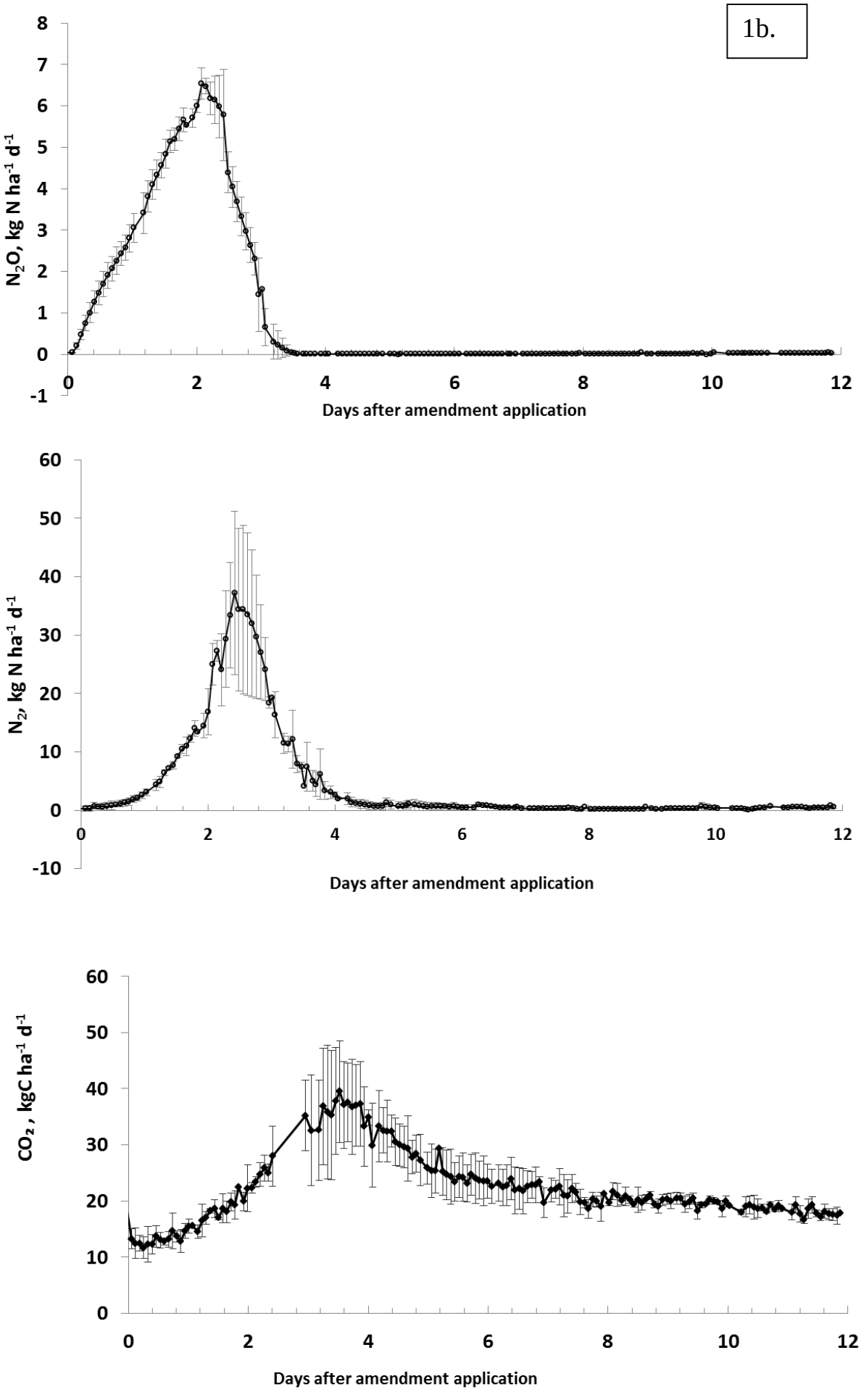
1a.


7
6
5
4
3
2
1
0
-1
0
2
4
6
8
10
12
N_2O, kg N ha^{-1} d^{-1}
Days after amendment application


80
70
60
50
40
30
20
10
0
-10
0
2
4
6
8
10
12
N_2, kg N $ha^{-1}d^{-1}$
Days after amendment application

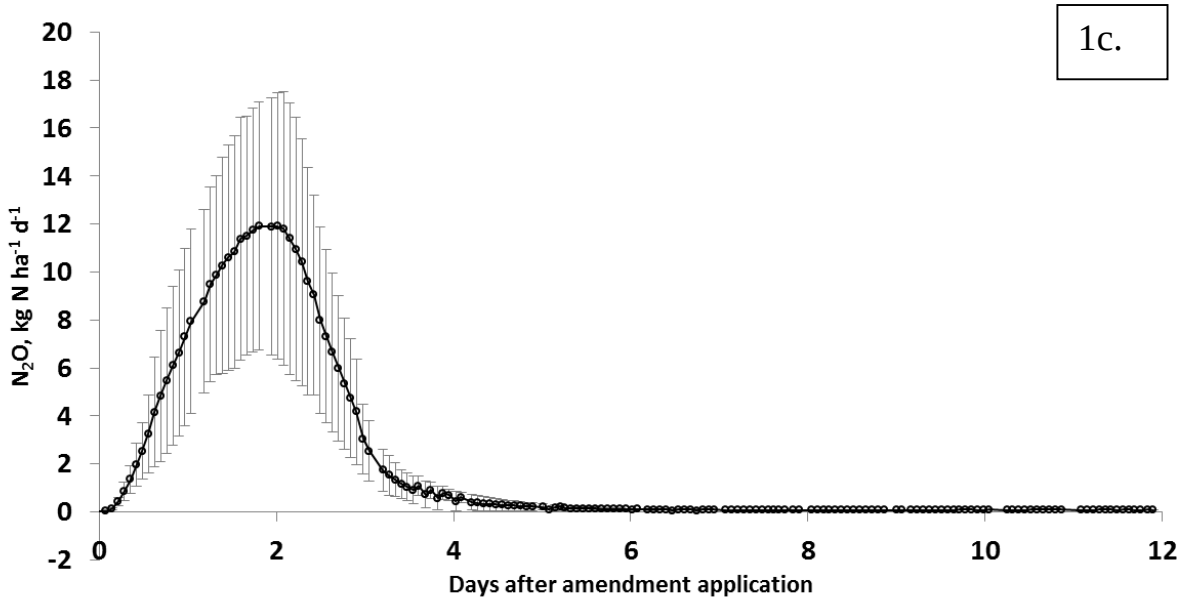

60
50
40
30
20
10
0
0
2
4
6
8
10
12
CO_2, kgC ha^{-1} d^{-1}
Days after amendment application

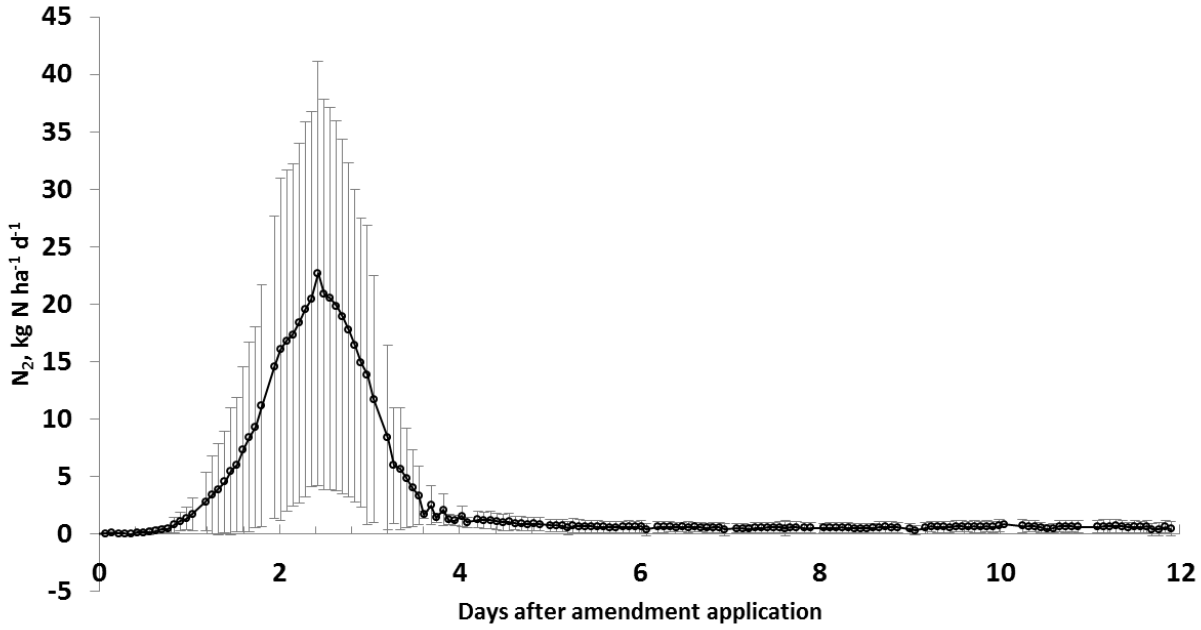
996

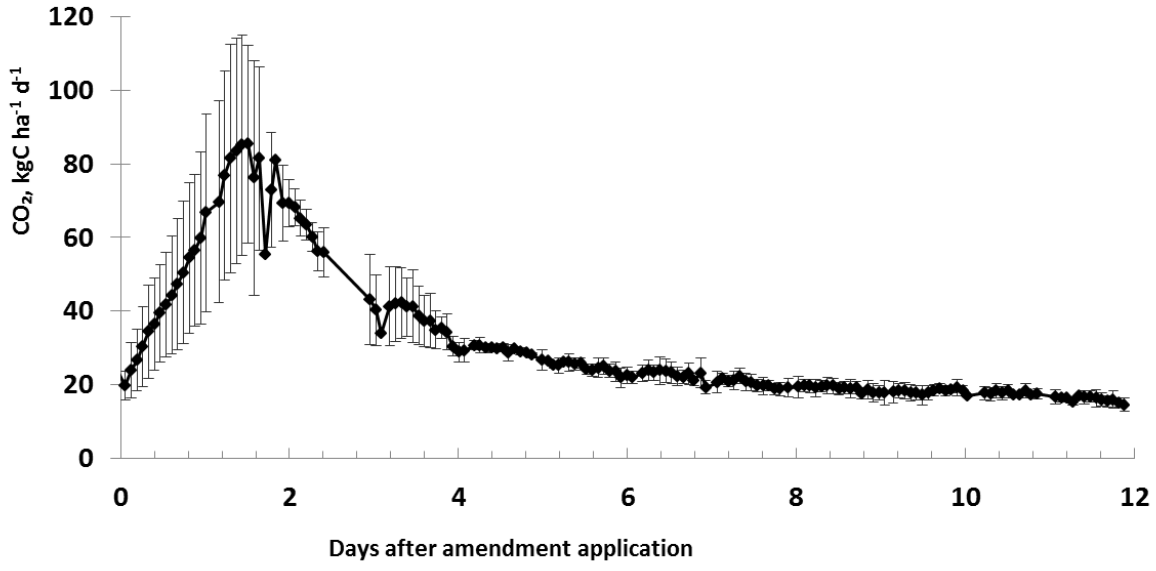

1b.
N_2O, kg N ha^{-1} d^{-1}
Days after amendment application
N_2, kg N ha^{-1} d^{-1}
Days after amendment application
CO_2 , kgC ha^{-1} d^{-1}
Days after amendment application

997

1c.

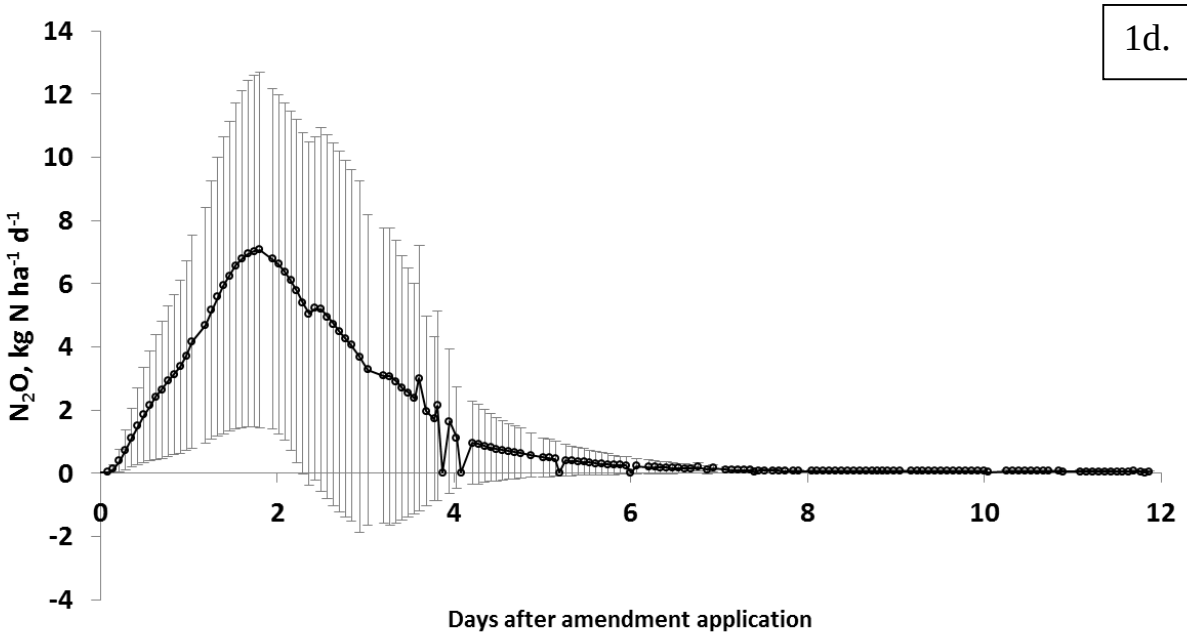

20
18
16
14
12
10
8
6
4
2
0
-2
N_2O, kg N ha^{-1} d^{-1}
0
2
4
6
8
10
12
Days after amendment application

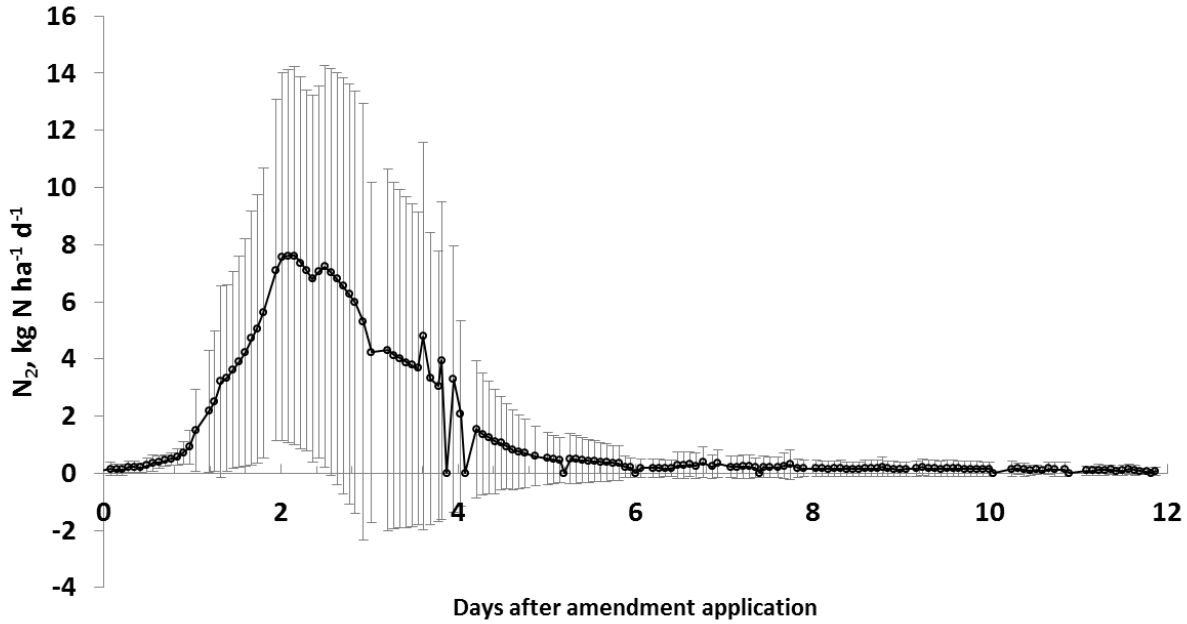

45
40
35
30
25
20
15
10
5
0
-5
N_2, kg N ha^{-1} d^{-1}
0
2
4
6
8
10
12
Days after amendment application

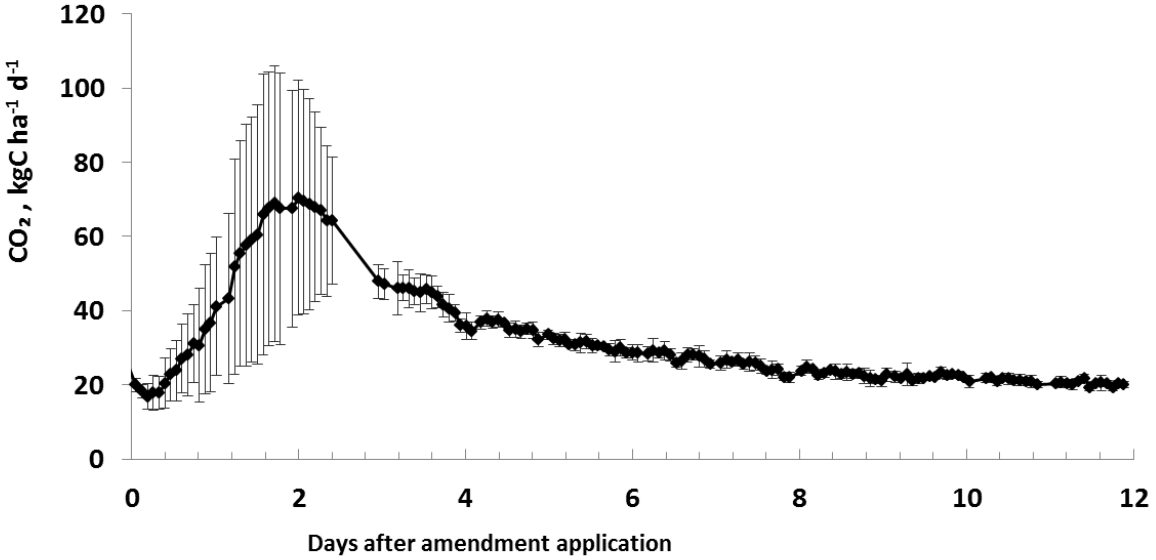

120
100
80
60
40
20
0
CO_2, kgC ha^{-1} d^{-1}
0
2
4
6
8
10
12
Days after amendment application

998

1d.


14
12
10
8
6
4
2
0
-2
-4
0
2
4
6
8
10
12
N_2O, kg N ha^{-1} d^{-1}
Days after amendment application


16
14
12
10
8
6
4
2
0
-2
-4
0
2
4
6
8
10
12
N_2, kg N ha^{-1} d^{-1}
Days after amendment application


120
100
80
60
40
20
0
0
2
4
6
8
10
12
CO_2, kgC ha^{-1} d^{-1}
Days after amendment application

999

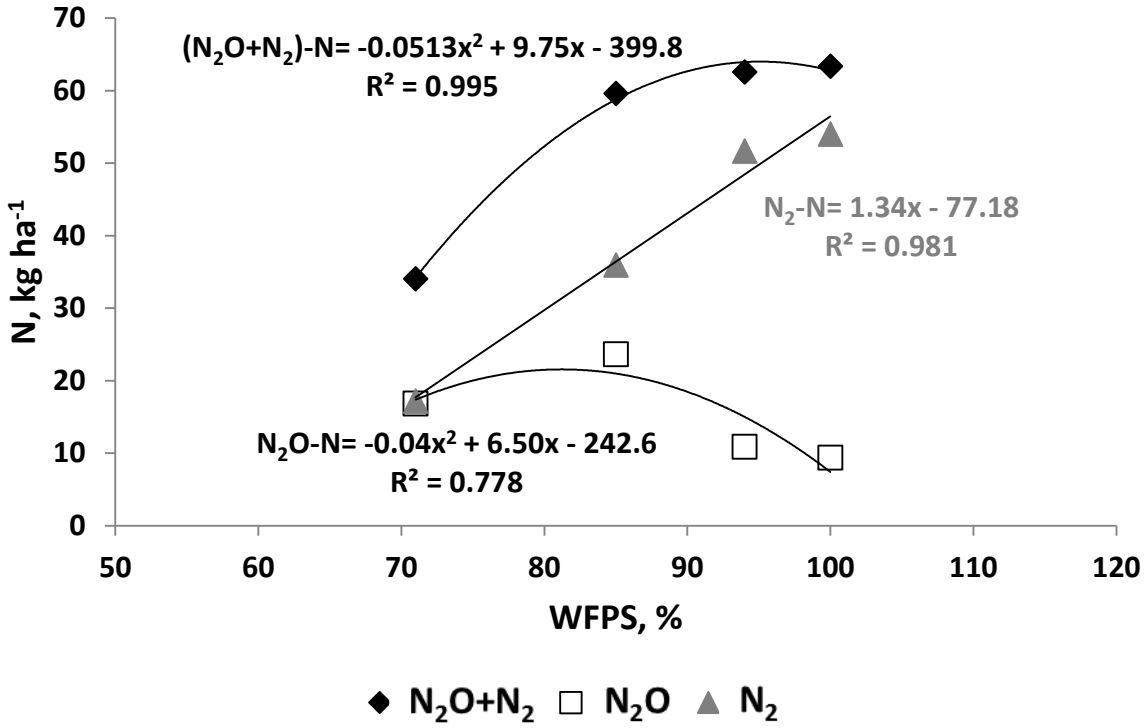

(N2O+N2)-N= -0.0513x2 + 9.75x - 399.8
R² = 0.995
N2-N= 1.34x - 77.18
R² = 0.981
N2O-N= -0.04x2 + 6.50x - 242.6
R² = 0.778
N, kg ha-1
WFPS, %
N2O+N2
N2O
N2

Figure 2

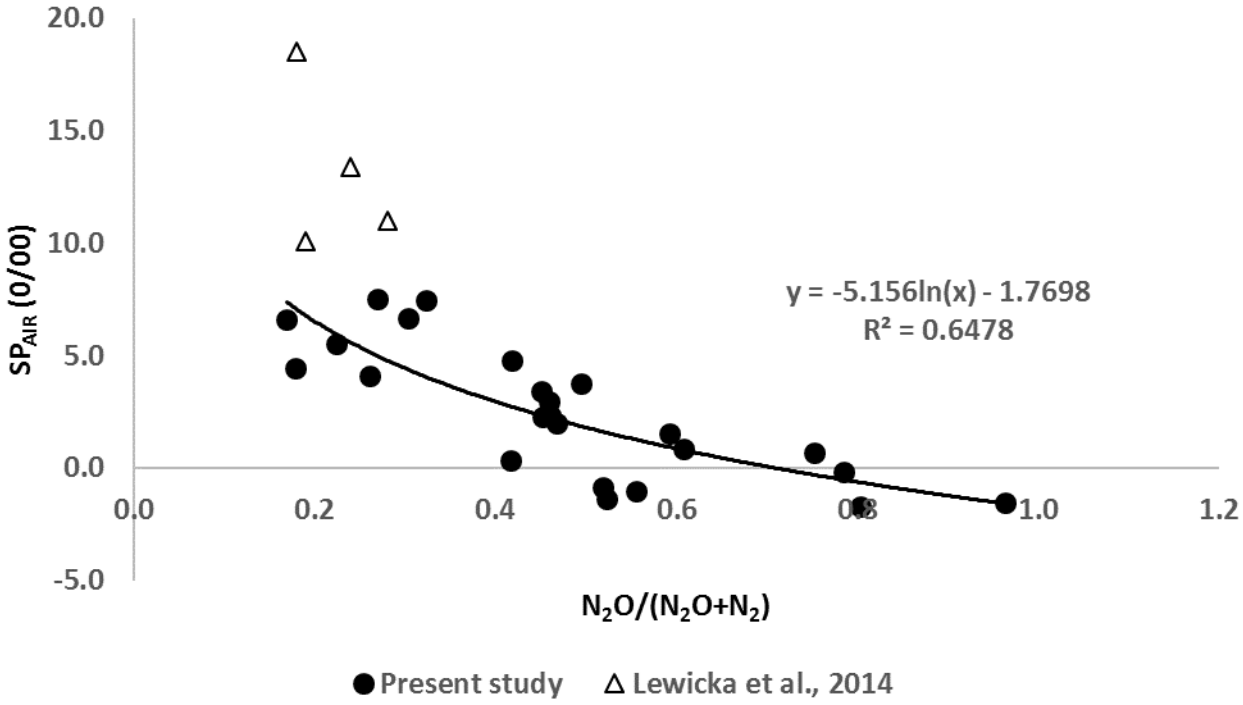
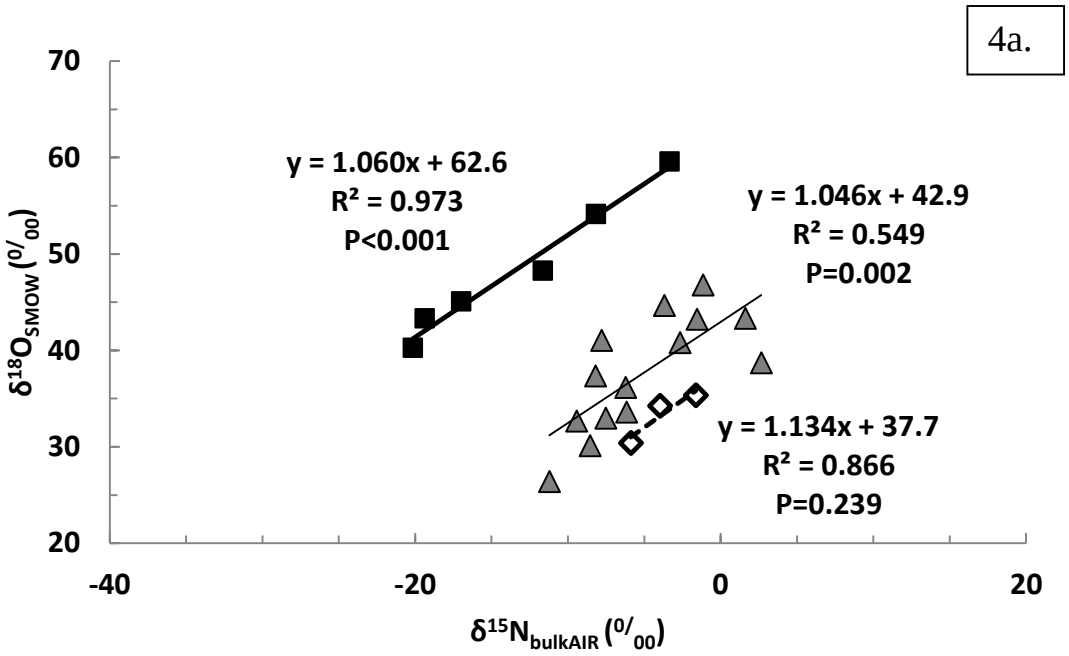
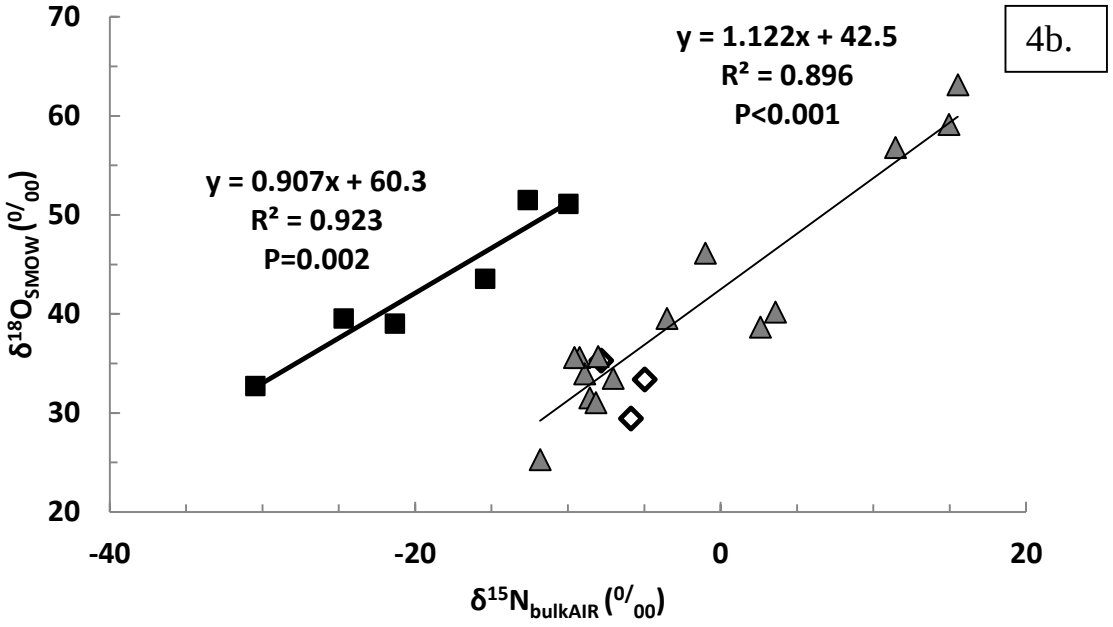
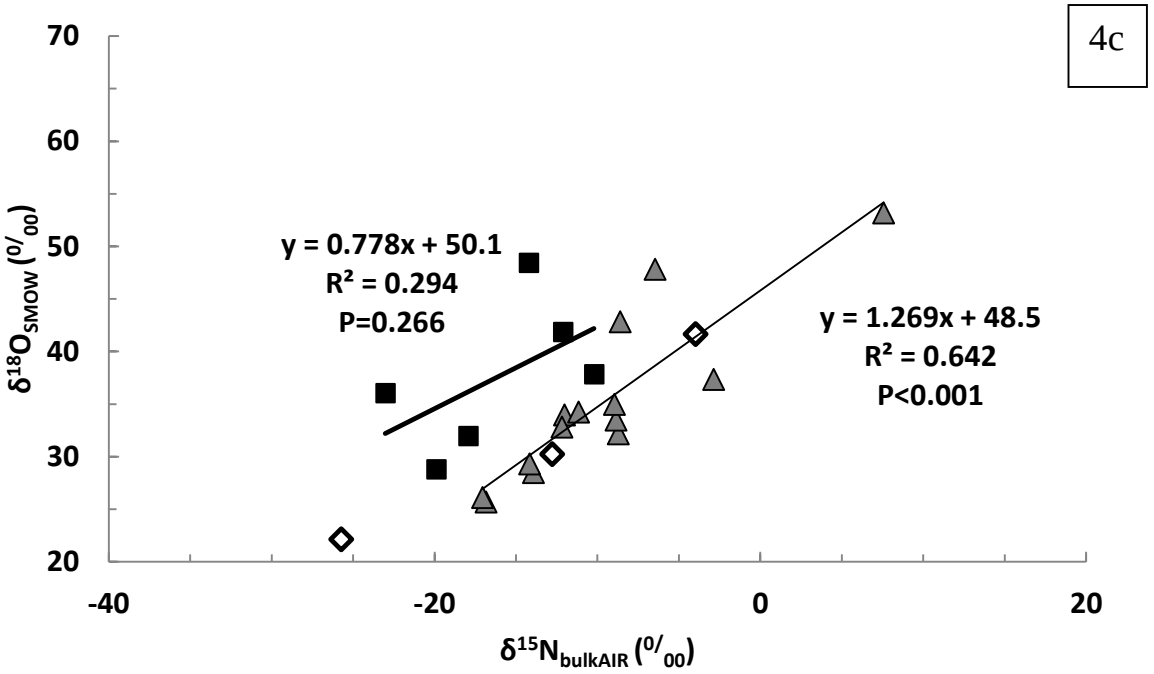

20.0
15.0
10.0
5.0
0.0
-5.0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
SP$_{AIR}$ (0/00)
N$_2$O/(N$_2$O+N$_2$)
y = -5.156ln(x) - 1.7698
R² = 0.6478
Present study
Lewicka et al., 2014

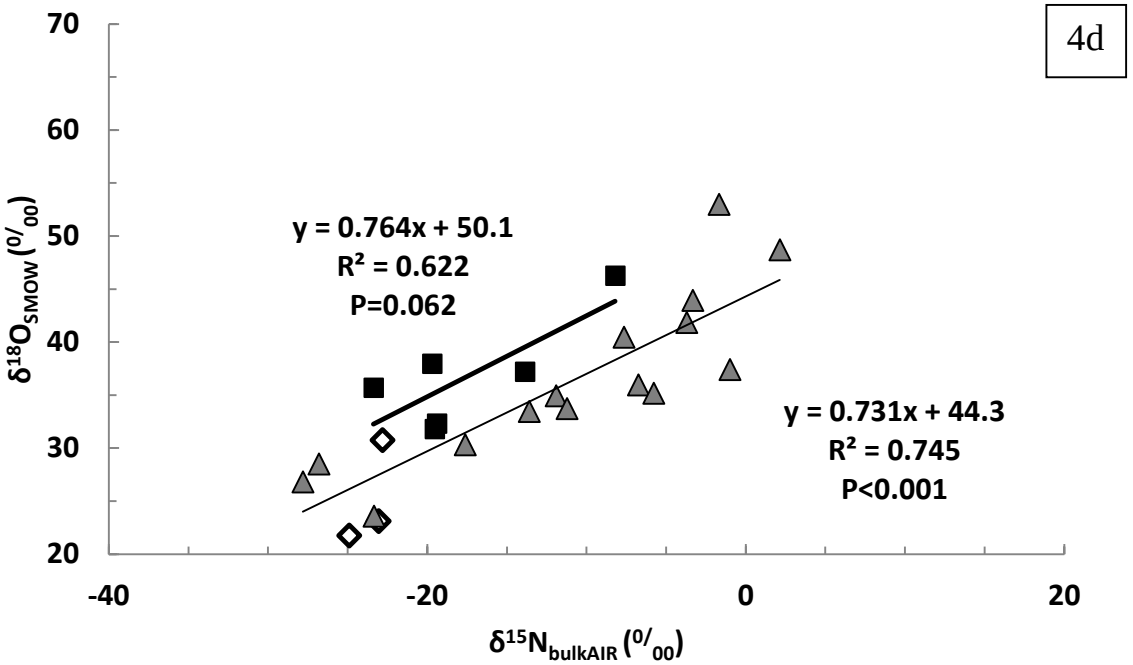
Figure 3


4a.
y = 1.060x + 62.6
R² = 0.973
P<0.001
y = 1.046x + 42.9
R² = 0.549
P=0.002
y = 1.134x + 37.7
R² = 0.866
P=0.239
70
60
50
40
30
20
-40
-20
0
20
δ18OSMOW (0/00)
δ15NbulkAIR (0/00)


4b.
y = 1.122x + 42.5
R² = 0.896
P<0.001
y = 0.907x + 60.3
R² = 0.923
P=0.002
70
60
50
40
30
20
-40
-20
0
20
δ18OSMOW (0/00)
δ15NbulkAIR (0/00)

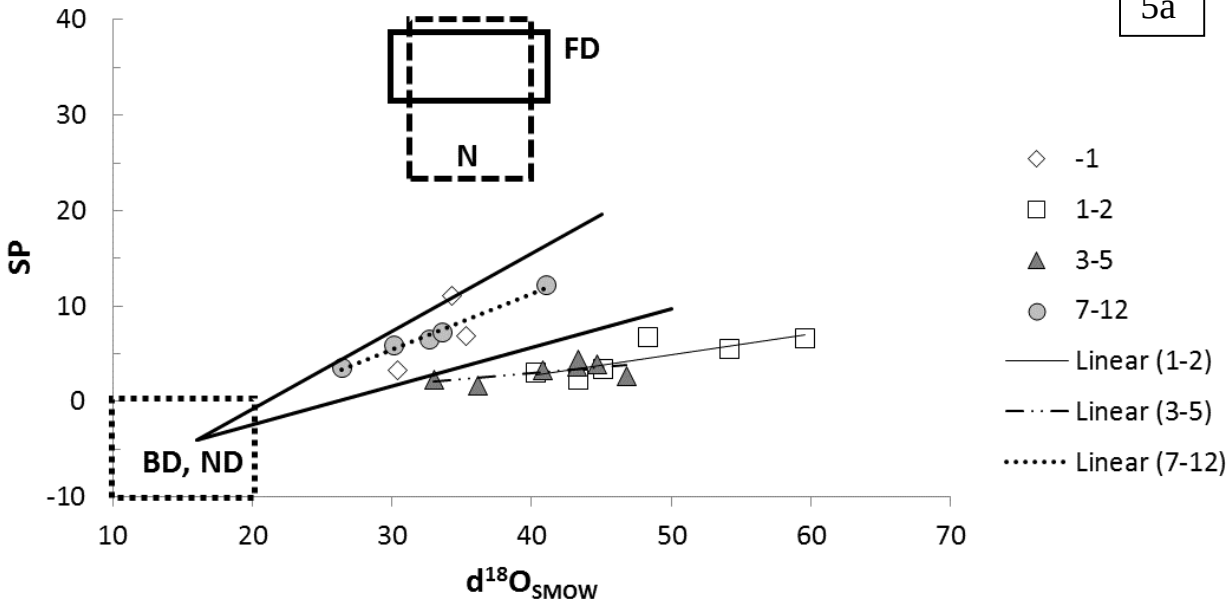


1014
1015
1016

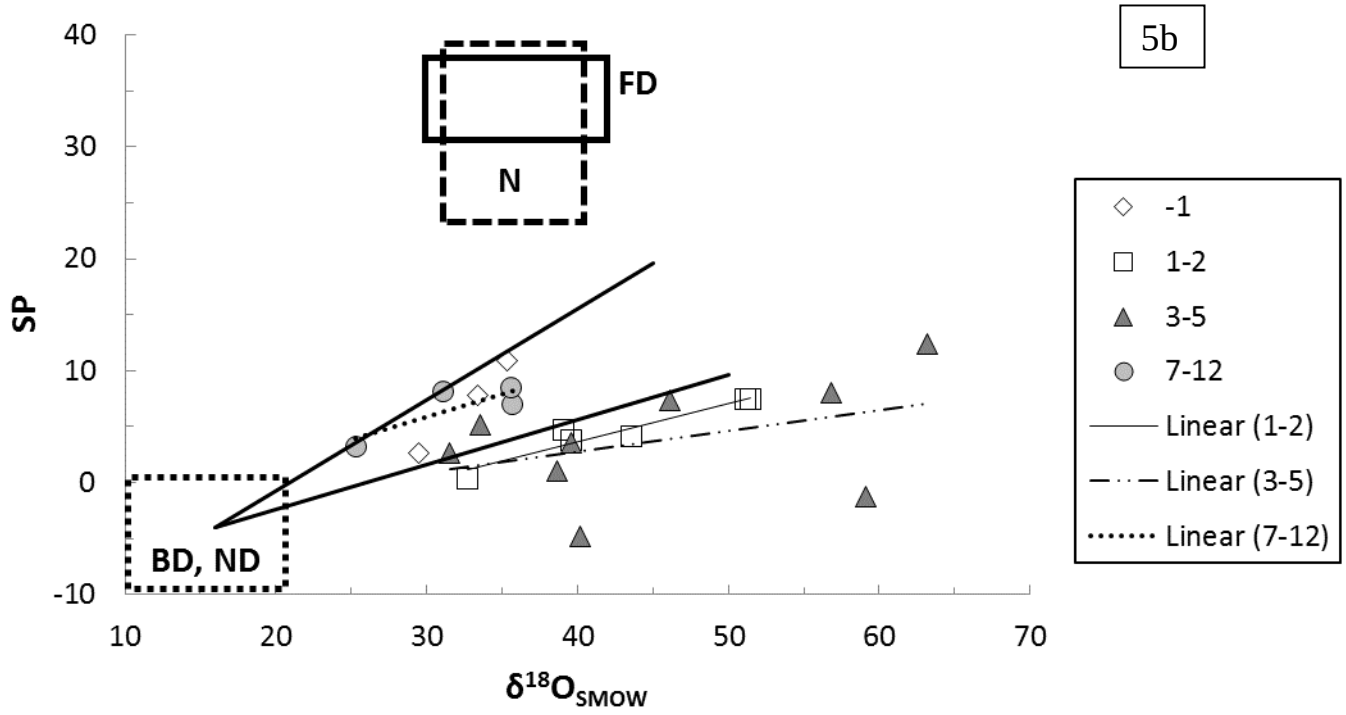


1017
1018
1019

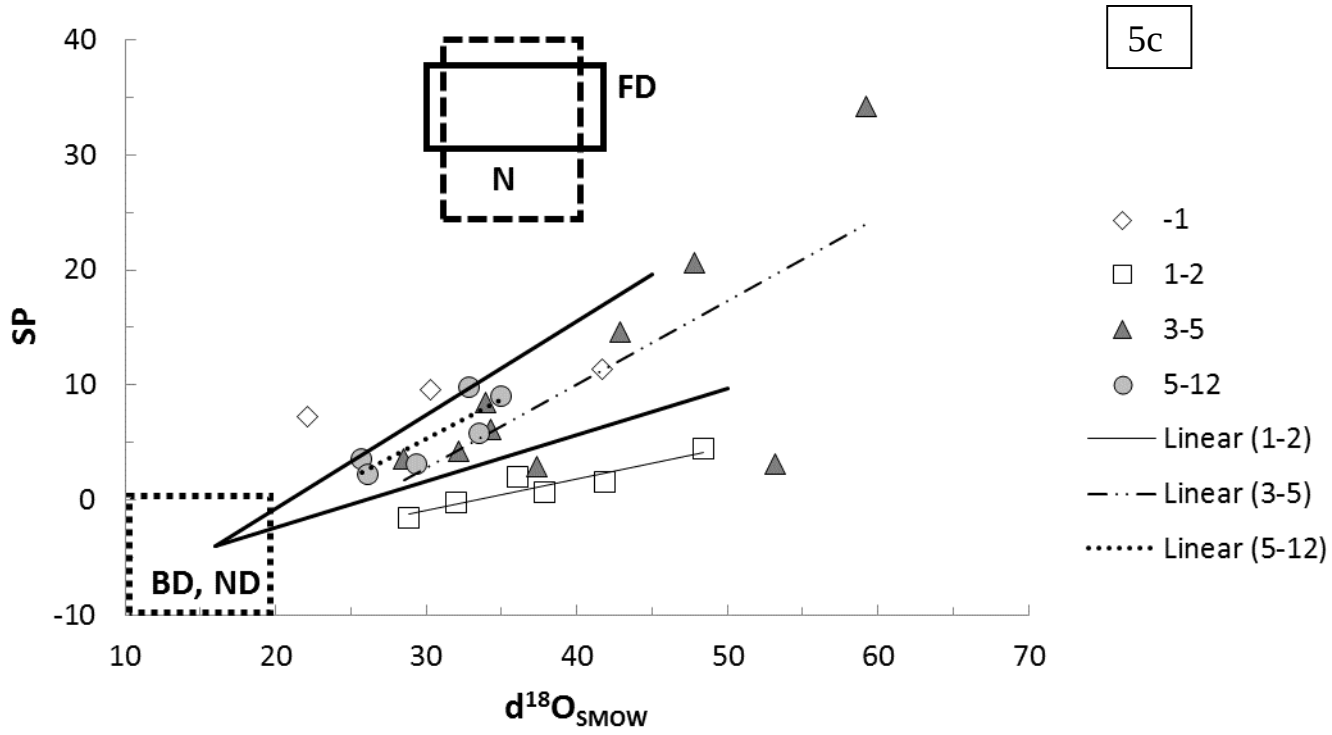
5a


FD
N
BD, ND
SP
d18OSMOW
-1
1-2
3-5
7-12
Linear (1-2)
Linear (3-5)
Linear (7-12)

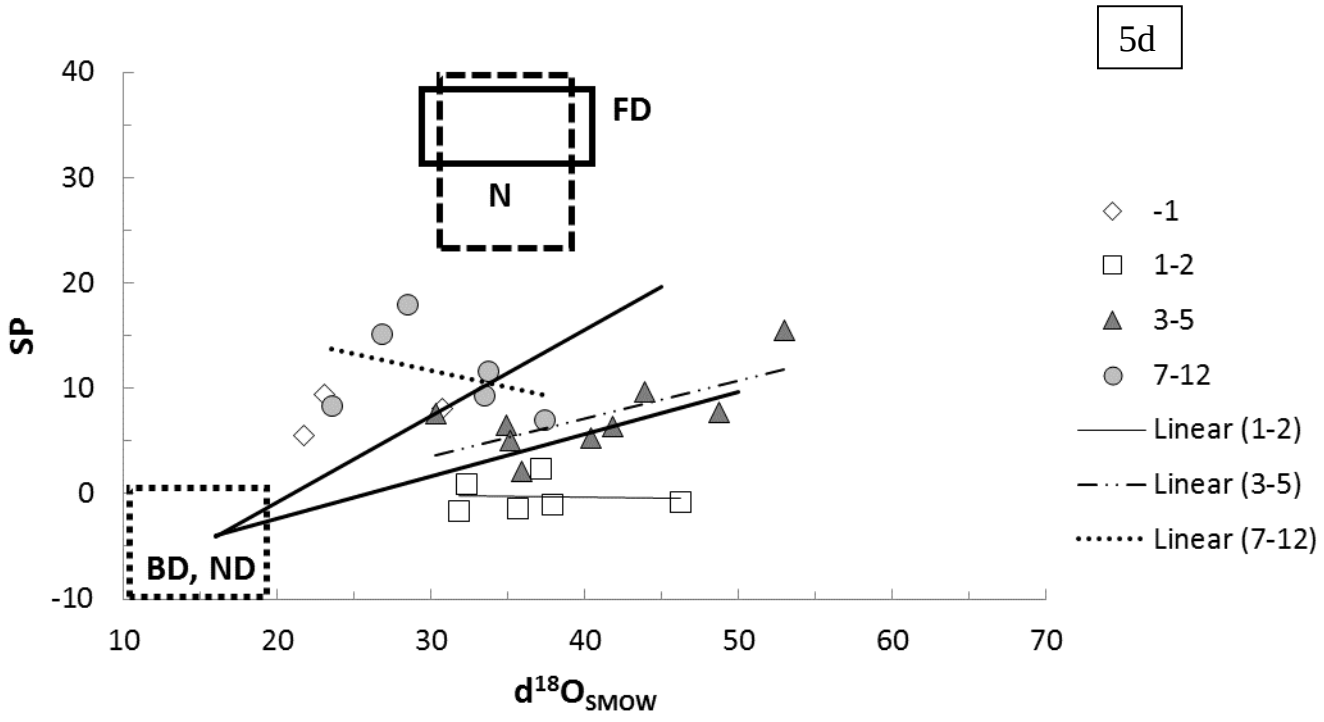
5b


FD
N
BD, ND
SP
δ18OSMOW
-1
1-2
3-5
7-12
Linear (1-2)
Linear (3-5)
Linear (7-12)

1032



1033
1034
1035



1036
1037

REFEREE 1

The paper aims to quantify N2O and N2 production process in grassland soils and its dependence on compaction. N2O and N2 emissions and their isotopic signature have been monitored over a period of 12 days after amendment of KNO3. The presented laboratory studies simplify the complex soil pore system into macro and micropores and uses four stages in a rather narrow range of 70 to 95% "mean" WFPS.

The experimental setup is described in detail. The results agree with the expected values, i.e. domination of bacterial denitrification processes for the higher water content and an increasing share of other contribution for when part of the pores is dry. The measurement of the isotopic signature allows to distinguish different production processes and their dependence on the water status of the macro and micropores.

I had difficulties to follow the argumentation and get quickly lost in too many in details. I also miss a discussion of the significance of the presented findings for the characterization of the emissions of N-species for real grassland systems, although in the introduction (e.g. lines 62 and 63) the study is set in this context.

The used soil stem from a long-term permanent grassland. But the preparation of the samples (a necessary step for the laboratory study) destroys the specific characterization of a grassland soil. Roots and the organization of the aggregates are removed and there is no plant growth that greatly influence the distribution and availability of N substrate as well as the oxygen supply. It should also be mentioned that a large share of N-input in agricultural system occurs in reduced N-form (excrement's, urea or ammonium nitrate). In grazed system, spatial heterogeneity is related to the urine patches with a very high N-input on a very limited area. Also, compaction (trampling by animal, tractor tracks) is spatially very heterogeneous and likely uncoupled to N-substrate input.

R: the authors agree that soil structure is destroyed, but as the referee says himself, this is a laboratory study, so we are not trying to reproduce the field conditions but to understand soil processes. In fact, we are assessing the potential for this soil to emit N_2O and for this reason we have optimised the conditions for denitrification. The plant is not included for the same reason, as we aim to understand the processes in the soil, although we agree that the plant plays a major role in modifying these processes. The soil used in this study is not sourced from a grazed grassland, but a grassland that is cut, so the effect of the animal, via grazing, soil compaction and excreta deposition is not relevant.

The results from the present study shows for N2O as well as (N2O and N2) emission a remarkably low variability among the four treatment, much lower as typically experienced in field measurements.

Below are given specific comments as a guideline to improve the manuscript

Abstract:

Lines 16 and 17: The soil emitted N2O is predominantly derived from denitrification and to a smaller extent, nitrification in soils,

This is a too crude generalization. There are many ways to produce N2O and the share between them depends in a complex manner from the main driver, such as oxygen content, substrate availability, etc.

R: the authors agree with the referee point and in fact the sentence goes on to say: 'both processes controlled by environmental factors and their interactions, and are influenced by agricultural management'. We have however made it clear that it is a generalisation.

Lines 20 and 21: Soil water content expressed as water filled pore space (WFPS) is a major controlling factor of emissions and its interaction with compaction, has not been studied at the micropore scale.

This is slightly misleading as the experimental setup can only measure net fluxes across the surface of the entire soil samples and naturally does not allow to determine N2O production/consumption in and out of the micropores.

R: yes, the referee is right in that we are not looking at production and consumption separately; but we only claim the control is on emissions (not production and/or consumption) and we are controlling moisture at the micropore scale.

Introduction

Lines 210 and 211: concentration) for 24 h, or until the system and the soils atmosphere were emitting low background levels of both N2 and N2O (N2 can get down to levels of 280 ppm much smaller than atmospheric values).

Please indicate these „background" values.

R: the flushing goes on until there is no further decrease in the background signal. This normally occurs within 24 hours. Values can reach a few gN/ha/d (much lower than atmospheric values of 70%).

Lines 222 and 223: Flushing was carried out with He for half an hour before the solution was required for application to the soil cores and continued during the application process to avoid atmospheric N2 contamination (a total of one and a half hours).

How this affects the oxygen availability?

R: the flushing is done to the amendment outside the incubation vessel, so we remove N_2 from the liquid before application. The incubation vessel on the other hand continues to receive He/O_2 so it should not affect O_2 availability, in fact the increase in CO_2 in later experiments supports this assumption.

Lines 304 and 305: We accepted these as unavoidable features of the experimental set-up, but we suggest that the main response of the gaseous emissions occurred under the initial conditions, prior to the loss of water over subsequent days.

"We suggest" is a strange formulation, either the time coarse of the emissions clearly shows this, or it is an assumption.

R: this statement came after a comment from a previous reviewer. We have changed the text now to say 'we assume'.

Results

Lines 311 UNSAT/halfsat (50-100 N kg- dry soil)

Unit of NO3- seems incorrect. Also, the header of Table 2 is wrong (twice UNSAT/SAT)

R: the referee is correct, units and heading have been amended.

Lines 349 to 351: The results showed that the total N emission (N2O+N2) (Table 3) had a consistent decreasing trend, with decreasing soil moisture i.e. from 63.4 for SAT/sat (100% WFPS) to 34.1 kg N ha-1 (71% WFPS) for UNSAT/halfsat.

I don't see a consistent decreasing trend. Only the driest treatment shows a lower emission.

R: we have modified the text to reflect this properly: 'The results showed that the total N emission (N_2O+N_2) (Table 3) decreased between the highest and the lowest soil moistures i.e. from 63.4 for SAT/sat (100% WFPS) to 34.1 kg N ha^{-1} (71% WFPS) for UNSAT/halfsat'

It also would make more sense to use the same reference for the mineral N content as well as the cumulative gaseous emissions (e.g. per g soil).

R: we agree this is a good suggestion. So we have included this extra information in table 3.

Lines 351 and 352: The maximum cumulative N2O occurred at around 80% WFPS as Fig. 2 shows. This is an overinterpretation. There are four values and a fit with three unknown is applied.

R: we agree that there are no many points, but the value of this analysis is that for a narrow soil moisture range (70-100%) there seems to be a linear response for the N_2 but not for the N_2O and the total flux. Those shown were the best fits.

Noticeable emissions of N2O and N2 occur in all four treatment only up to day four. Bacterial denitrification is identified as the main production pathway. This is due to the experimental setup

with a combined amendment of KNO3 and glucose, a setup that produce good conditions for denitrification irrespective of the specific treatment.
R: as mentioned earlier, we optimised conditions for denitrification, except for soil moisture that is the factor we are studying.

REFEREE 2

General remarks

This paper presents results from a sophisticated laboratory experiment in which an agricultural soil was compacted and adjusted to 4 different moisture conditions. Glucose and nitrate was added and the formation, isotopic and isoptomeric composition of gasesous N was measured over a period of 12 days. Using those data the authors try to determine the contribution of different processes to N gas formation. The paper is a good example how much information you can get from experimental data if you spend a lot of energy in calculations and data analysis. However, in my eyes the paper has three critical weaknesses:

1.) The results are not really new. It is known for a long time that addition of nitrate and glucose stimulates denitrification in soils and that denitrification is favored under wetterconditions. All the points in the conclusions are not new. If there is new knowledge obtained from the study, it has to be elaborated more clearly.
R: we agree that some of the general points are known, for example the effect of soil moisture on emissions, but this is normally considered in relation to ranges of <60%, 60-75% and >75%. We have looked at a more detailed moisture adjustment, four levels at a relatively high moisture range, between 70 to 100% WFPS. We have also studied the isotopocules of N_2O and found isotopic similarities at similar moisture levels. Moreover, for the first time we have conducted N_2 +N_2O flux measurements at defined saturation of pores size fractions as a prerequisite to model denitrification as a function of water status.

2.) The paper is lacking a clear story. It is not really clear to me what was the final purpose of all those detailed analysis. There are some hypothesis mentioned at the end of the introduction but the rest of the manuscript is not tailored to address those hypotheses. The hypothesis that wetter conditions reduce heterogeneity could be answered from just looking at the error bars in figure 1 – you do not need sophisticated analysis to prove this point. Aiming to understand what is going on in one0s own experiment (as stated in the last sentence of the introduction) is not a sufficient aim of a paper.
R: We have done a detailed control of soil moisture in the soil and in order to do this we had to do the detailed analysis the reviewer refers to in terms of the moisture adjustment. In this way we ensured that the four moisture levels above 70% WFPS were as accurate as possible. We also used tools such as the isotopomers to confirm source processes, and this is the result of our research in the last 15 years, when we have built up a large database of isotopomers of N_2O to improve the uncertainty in the determination of the sources. In this particular experiment we have been able to elucidate the effect of saturation on processes at relatively high moisture levels when combined with the measurements of N_2O and N_2 emissions.

3.) There are some problems with the experimental approach which limit interpretation of the data. First, moisture conditions were not constant but changed a lot during the experiment. The second treatment, for example at the end of the experiment had the same water content as the third treatment in the beginning. They had changing substrate concentrations in parallel to changing moisture conditions. Thus, the interpretation

of moisture effects during the course of the experiment is difficult. A way to minimize that effect would have been to moisten the supplied He/O2 gas. I would also expect that water loss was highest in the beginning, when the surface layer was drying. A way to get some information about temporal changes of water content would have been to weigh the incubation vessels during the incubation. Second, they measured gas emission – not gas production. They mention this problem in the paper but somehow ignore its consequences. The emitted gas probably originates from those sites which are physically linked to the atmosphere, while gas production, e.g. in the center of aggregates did probably contribute less to the emitted gas. So, the conclusions drawn from the analysis could be valid only for a part of the soil volume.
R: we are aware there are limitations to the experimental approach. In order to moist the gas we would have to have an extra vessel where we flush the gas through. Measuring N_2 is very difficult due to background atmospheric levels and any additions to the experimental system poses a risk of leaks. In addition, adding moist gas will likely block the tubing as these are very narrow (1/8"o.d.). The flow of the gas is very slow (10 ml/min) simulating a low wind speed so normally this would dry the soil in field conditions too. It would represent a rainfall event where the initial moisture differs between treatments but some drying occurs due to the wind flow. We believe the effect of drying will be more relevant (and significant relative to the initial moisture) later in the incubation. We also know that if drying is significantly affecting the microbes, we would see an increase in CO_2 emissions which did not happen later in the incubation. We have introduced changes in the text to make the reader aware of this and have reflected this as 'the effect of initial soil moisture'.

Detailed comments

l.17: remove "soils"
R: removed

l.40: What do you mean with "benign" for the environment. Do you mean the process is important because it closes the global N cycle because it reverses N-fixation?
R: no, it is benign because it does not cause harm to the environment.

l.64-73: I would move this paragraph to an earlier point, before talking about compactation.
R: we have placed this paragraph after the compaction, as it follows from the previous paragraph where we discuss the effect of livestock on compaction. It also leads to the following text on effect of compaction on soil water: 'reducing the soil air volume and therefore increasing the WFPS'.

l.72: I would replace "powerful tool" by "basis".
R: changed

l.81: If there are several references for one statement, present them in chronological order.
R: changed

l.81-82: Remove sentence
R: removed

l.83: ": : :under the conditions: : :"
R: changed

l.92: Be more specific. What do you mean by “other steps of denitrification”?
R: we agree that this sentence was not clear enough so we rewrote to: “Simultaneous occurrence production and reduction of N_2O as in natural conditions presents a challenge for isotopic factors determination due to uncertainty on N_2 reduction and the co-existence of different microbial communities producing N2O (Lewicka-Szczebak *et al.*, 2014).
l.93: “reported here”.
R: changed

l.100: Does that mean that those results are only relevant at elevated C and N?
R: We have modified the text as follows: ‘The results generally confirmed the range of values of η (net isotope effects) and $\eta^{18}O/\eta^{15}N$ ratios reported by previous studies for N_2O reduction for that part of the soil volume were denitrification was enhanced by the N+C amendment. This did not apply for the other part of the soil volume not reached by the N+C amendment, showing that the validity of published net isotope effects for soil conditions with low denitrification activity still needs to be evaluated’.

l.108: Why CO2?
R: we have changed the text: ‘soil to assess the impact of different levels of soil saturation on N_2O and N_2 emissions after compaction. CO_2 emissions were measured in addition as an estimate of respiration and thus of O2 consumption’**.**
l.112: “controlled laboratory conditions”
R: changed but this text is now in section 2.4 as recommended by another referee.

l.119: What do you mean by “heterogeneity in N emissions”?
R: spatial distribution of emissions, text changed to clarify

l.120: I am not a soil scientist, but is that really new?
R: prediction of N_2O emissions is very difficult in part due to their spatial variability. We are trying to understand how this effect occurs in a relatively narrow range of moisture (70-100%). As far as we know there no other studies going to this level of detail. This has been included in the text (end of introduction section).

l.121: Aiming to understand what is going on in one0s own experiment is not a sufficient aim of a paper.
R: we have changed the text: ‘We aimed to understand changes in the ratio $N_2O/(N_2O+N_2)$ at the different moisture levels studied in a controlled manner on soil micro and macropores. Moreover, we used isotopocule values of N2O to evaluate if the contribution of bacterial denitrification to the total N2O flux was affected by moisture status’

l.137: Verb missing. “was applied”?
R: the verb is early on in the paragraph. The paragraph is now split to make it clear.

l.228: “CO2 was measured: : :”
R: changed

l.230: replace “pulled together in one sample” by “pooled”
R: changed

l.232: Remove sentence. There is a similar sentence in the results section.
R: removed

l.268: Were the data normal distributed?

R: yes, all datasets were tested by fitting a Gaussian model resulting in Fprob<0.001. this was added in the results section.

l.275: “mixing model was then used” (use past tense)
R: changed

l.283: When did this occur and what is a possible explanation?
Wrong fractionation factors?
We clarified the variability of endmember values and fractionation factors in the introduction: “The analysis comprised measurements of the N_2O and N_2 fluxes combined with isotopocule data. Net isotope effects (η values) are variable to a certain extent as they result from a combination of several processes causing isotopic fractionation (Well et al., 2012). The results generally confirmed the range of of η values and $η^{18}O/η^{15}N$ ratios reported by previous studies for N_2O reduction for the soil volume reached by the N+C amendment. This did not apply for the soil volume not reached by the N+C amendment.”

l.290: A TCD is an detector – not an analyzer.
R: changed analysed for determined

l.303 Why was the gas stream not bubbled through water to saturate it with water?
R: see our explanation above in point 3.

l.305: I would expect the highest water loss right in the beginning.
R: the flowrate is very low so drying will take a while, we are assuming that the significant water loss will affect later in the incubation, later than the peaks appear. However, as explained earlier, we have now referred to the effect of the initial soil moisture in the treatments.

l.306. But they were similar between treatments in the end although different starting conditions.
R: yes

l.314-316: There was a high variability in the data.
R: but only for NH_4^+ it was not significant. A sentence was added

l.318: Remove “The results showed that”
R: removed

l.329: I do not see that in Figure 1. In Unsat/sat the N2O maximum was at 12 kg N/ha d, not
around 7.
R: the referee is correct, we have now amended the text to reflect this: ‘The N_2O maximum in the SAT/sat and HALFSAT/sat treatments was of similar magnitude (means of 5.5 and 6.5 kg N ha^{-1} d^{-1}, respectively) and but not those of UNSAT/sat and UNSAT/halfsat (means of 7.1 and 11.9 kg N ha^{-1} d^{-1}, respectively).

l.348. Right. But what are the consequences of this for your experiment and its interpretation?
R: this belongs to the discussion (4.1) so have been moved in there to explain the potential underestimation of the production due to low diffusion.

l.354: You probably mean “CO2 fluxes”. Why was CO2 measured?
R: yes, added fluxes in the sentence. CO2 indicates aerobic respiration and as explained above (l.108) **is also affected by the soil moisture and level of compaction.**

l.360: The carbon budget is interesting but complicated. Could you calculate recovery rates for the added glucose? It looks as if there are recoveries higher than 100%. Can this be interpreted as a priming effect? A problem with using CO2 for carbon budgeting is, that depending on pH you also have other IC species in the soil solution. Do you know the pH in your soils?
R: pH is 5.63 as shown in Table 1. We did not do a C budget, but it is possible that soil C would have also contributed to the CO_2 emitted but to a lower extent compared to the added glucose.

l.370: Add article before “period”
R: added

l.375: The SP data have a high standard deviation. Are the differencers discussed in this paragraph real?
R: we think the larger variation (high SD) of SP around day 3 corresponds to the with highest variation of N_2 and N_2O fluxes (which is evident from Figs

l.391:You may consider adding these data to the plot.
R: data added to figure

l.394: Separate into two sentences.
Start second one with “In our data, maximum : : :.”
R: changed

l.404 So what is the message ofthis paragraph with respect to the first sentence of the paragraph?
R: we have rewritten: ‘the question arises to which extent the relationships between the d18O and d15Nbulk and between d18O and SP within the individual treatments denitrification dynamics. We checked this to evaluate the robustness of isotope effects during N_2O reduction as a prerequisite to calculate the percentage of bacterial denitrification in N_2O production.”

l.405: Why was this done?
R: we have found that the isotopologues seem to be potentially more powerful than initially thought. By looking at these relationships we have learnt how the responses relate to the sources of these gases.

l.428: Why was this plot done?
R: the same reason as above

l.441: I do not see data within those areas in the plots.
R: we have not been so clear, and we refer to the vectors more than the areas. Text has been changed to reflect this.

l.456: “sat” page 19: It is difficult to detect the storyline on this page.
R: we are explaining that from our results we are providing a refinement in the soil moisture (WFPS) thresholds previously established as borderline for nitrification-denitrification. We are also proposing that WFPS which was previously established

as a normalised parameter for these type of soil moisture thresholds, might actually change with soil type.

L513: Could it be that there was C limitation in the dryer treatments because glucose was metabolized aerobically?
R: if glucose was metabolised we would have expected C to have been less limiting

l.534-537: The message of the CO2 paragraph is not really clear. Are the CO2 data helpful in this manuscript?
R: we have deleted the paragraph as suggested.

l.539: How much is the unacounted N-loss in comparison to the accounted gasesous losses?
R: we added: " unaccounted-for N loss is two to three times the total measured gas loss (Table 3)".

l.541: NO: What are typical NO fluxes in the literature? Can the NO flux have a significant magnitude? The same applies to microbial biomass: Is the microbial biomass potentially formed from the unaccounted N-loss in a realistic order of magnitude?
R: we are now able to measure NO fluxes in the system. Loick et al reports a ratio N_2O/NO of 0.4 for example, so yes, it can be significant. We did not do microbial biomass in this instance.

l.567: How should nitrification contribute to BDEN? Do you mean nitrifier-denitrification?
R: thus large contributions to the total N_2O flux from nitrification were not probable

l.636: I do not understand the content and purpose of this paragraph.
R: text changed to: The question arises, if the poor coincidence of Pool 2 isotopologue fluxes with previous N_2O reduction studies reflects the variability of isotope effects of N_2O reduction or if the contribution of other processes like fungal denitrification could explain this (Lewicka-Szczabek et al, 2017). The latter explanation is evaluated in section 4.3.

l.719: Don0t you have 4 periods in the figure? Table 3: Unit missing for Total emitted N. Tables 5 and 6: I wonder whether these data could be presented better in figures.
R: no, only three. Units included. Yes, figures can illustrate better, but as we explained in the initial review, this data is very useful for models and we think providing the values will be more useful.

Figure 5: the four sub-graphs are quite similar. Isn0t a conclusion that the results were not much influenced by soil moisture?
Do you really need 4 graphs?
R: we concluded that there were similarities between the 2 high moisture and 2 low moisture treatments. We believe this is an important finding due to the relatively narrow range of soil moisture we have studied, above 70%, in which we still find differences in fluxes. Davidson stated that the threshold for nitrification-denitrification lies at about 60%, in our case we have managed to refine this.

REFEREE 3

This is an interesting study that addresses the roles of soil compaction and water saturation

levels on N2O production and the microbial origins of N2O. The results are not terribly profound but this is an important contribution to the literature as the precise causes of N2O hot spot production are still unresolved. Overall I found the writing to suffer from incorrect grammar and English writing style. Further, the manuscript is much longer than it needs to be. The manuscript would greatly benefit from a major rewrite and could be re-written as a short concise note rather than a full research paper. I've identified some issues with the writing below but there are numerous problems beyond what I have listed.

R: the majority of the authors consist of native English speakers and the English has been revised by them, so we believe the quality of the English is good. We think that providing the current level of detail in this manuscript as a full research paper is required to give further evidence for the need to use isotopic signatures and modelling approaches of N_2O in order to describe the driving source processes of this gas as emitted from soils.

Line 26 to 29: As this sentence contains both a colon and a semi-colon it needs to be broken into at least two sentences. I do not understand the meaning of the portion after the colon (28-29).

R: thanks for the suggestion, paragraph has been split.

Line 73 and 74: Please check with Coplen (2011) regarding the correct usage of "isotopologues"and " isotopomers".

R: we have now modified the text according to Coplen's definitions below and used isotopocule always if SP AND d^{18}O are addressed, isotopomer if ONLY SP is addressed.
According to Coplen: 'The molecular species can be an isotopologue, an isotopomer, or neither. For example, the three molecular species $^{15}N_2\,^{16}O$, $^{14}N^{15}N^{16}O$, and $^{15}N^{14}N^{16}O$ are isotopocules, but they are neither isotopologues (because the latter two do not differ in isotopic composition) nor isotopomers (only the latter two are isotopomers). Isotopolog: Molecular species that differ only in isotopic composition (number of isotopic substitutions) and relative molecular Mass. Isotopomers: Molecular species having the same number of each isotopic atom (thus, the same relative molecular mass) but differing in their positions.'
We defined these in the introduction as: 'Isotopologues of N_2O represent the isotopic substitution of the O and/or the two N atoms within the N_2O molecule. The isotopomers of N_2O, are those differing in the peripheral (β) and central N-positions (α) of the linear molecule' which we believe agree with the definition given by Coplen.

Line 97-98: Why is "soil volume" the key control on the net isotope effect? This seems more like an experimental condition rather than a governing soil process.

R: we changed the text for: "The results generally confirmed the range of values of η (net isotope effects) and $\eta^{18}O/\eta^{15}N$ ratios reported by previous studies for N_2O reduction for that part of the soil volume were denitrification was enhanced by the N+C amendment. This did not apply for the other part of the soil volume not reached by the N+C amendment."

Line 111-112: Generally avoid one-sentence paragraphs. This statement belongs more appropriately in the Methods section and could be deleted here.

R: text has been moved as suggested

Line 159: This paragraph is much longer and more detailed than it needs to be.

R: section has been moved to a supplementary material.

Line 323-324: Use past tense here.
R: all throughout this section (3.2) there is only past tense. I am not sure where the reviewer refers to.

Line 338: Delete “already”.
R: deleted as suggested.

Line 351: Incorrect word use. SP values don’t “show”; rather they are obtained. Use past tense to describe trends in the experimental data throughout this paragraph.
R: text has been amended.

Line 363: Don’t describe “the plot”; rather simply refer to the trends between the parameters.
R: text amended.

Line 365: Regressions don’t suggest but simply describe a (presumably significant) relationship between two parameters. You can state that the intercept of the regression equation relating SP and the N2O/(N2O+N2) was – 2 per mil.
R: changes have been introduced.

Line 367-369: The writing is confusing here; I cannot follow the meaning of this sentence.
R: These are the lines in the submitted pdf: “This is in juxtaposition with the situation when the N emissions are dominated by N_2 or N_2O is low, where the SP values of soil emitted N_2O were much higher (Fig. 3), pointing to an overall product ratio related to an ‘isotopic shift’ of 10 to 12.5o/oo.”
We modified to (including previous sentence):
“The plot of the N_2O / (N_2O + N_2) ratio vs SP for all treatments in the first two days (when N_2O was increasing and the N_2O / (N_2O + N_2) ratio decreasing) shows a significant negative response of the SP when the ratio increased (Fig. 3). The regression suggests that when the emitted gaseous N is dominated by N_2O (ratio close to 1) the SP values will be slightly negative with values around -2 (Fig. 3), i.e. within the range SP range of bacterial denitrification. With decreasing N_2O / (N_2O + N_2) ratio the SP values of soil emitted N_2O were increasing to values up to 8 per mil.”

Line 370: It is not helpful to refer to data in a figure of another paper. Describe the main significance to the similarity between these data sets.
R: I think the reviewer here refers to line 389. We are not referring to a figure necessarily but to the data from Lewicka-Szczebak et al. (2014). The significance was explained in the discussion: ‘These results confirm from 2 independent studies Lewicka-Szczebak et al., 2014) that there is a relationship between the product ratios and isotopic signatures of the N_2O emitted.’

Line 374: Again, don’t state what is plotted in Figure 4, describe the relationships between the variables and refer to the figure.
R: This is in line 406. We have edited the text as suggested.

Line 383: The r2 values by themselves are not very relevant. What is relevant is if the relationships are significant and their associated p values.
R: R^2 are reported in lines 412 onwards. We have analysed the regressions and introduced the P values as suggested.

Line 389: See comment for line 374.
R: I think reviewer refers to line 428. We have stated the new figure was done similarly to the previous one, so we have left the text as it was.

Tables 1, 4, 5 and 6: These tables could readily be placed in the Supplementary Documents.
R: yes, it would be possible, but we would like to have the editor's view before moving them.

Figure 5: These figures are not well organized. Put a box around the legends so that we know they are legends. Within the legend, the line should be placed through the data points rather than defining each line as "Linear". The y-axis title should display delta not "d".
R: Legends have now been enclosed by a box. The 'Linear' word in the legend clarifies that a linear function was fitted so we have left this as it was. The reviewer refers to the X axis, delta has been changed.