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Interaction of straw amendment and soil NO_3^- content controls fungal denitrification and denitrification product stoichiometry in a sandy soil

Mehmet Senbayram[a,b], Reinhard Well[a], Roland Bol[c], David R. Chadwick[d], David L. Jones[d,e], Di Wu[d,*]

[a] *Thünen Institute of Climate-Smart Agriculture, Federal Research Institute for Rural Areas, Forestry and Fisheries, Bundesallee 65, 38116 Braunschweig, Germany*

[b] *Institute of Plant Nutrition and Soil Science, University of Harran, Osmanbey, 63000, Sanliurfa, Turkey*

[c] *Institute of Bio- and Geosciences, Agrosphere (IBG-3), Forschungszentrum Jülich GmbH, 52425 Jülich, Germany*

[d] *School of Natural Sciences, Bangor University, Gwynedd, LL57 2UW, UK*

[e] *UWA School of Agriculture and Environment, University of Western Australia, Crawley, WA 6009, Australia*

Corresponding author: Di Wu

Corresponding author address: School of Natural Sciences, Bangor University, Bangor, Gwynedd, LL57 2UW, UK

Corresponding author Tel: +44 1248 383062

Corresponding author E-mail: w.di@fz-juelich.de

Abstract

The return of agricultural crop residues are vital to maintain or even enhance soil fertility. However, the influence of application rate of crop residues on denitrification and its related gaseous N emissions is not fully understood. We conducted a fully robotized continuous flow incubation experiment using a Helium/Oxygen atmosphere over 30 days to examine the effect of maize straw application rate on: i) the rate of denitrification, ii) denitrification product stoichiometry (N_2O/N_2O+N_2 ratio), and iii) the contribution of fungal denitrification to N_2O fluxes. Five treatments were established using sieved, repacked sandy textured soil; i) non-amended control, ii) nitrate only, iii) low rate of straw + nitrate, iv) medium rate of straw + nitrate, and iv) high rate of straw + nitrate (n=3). We simultaneously measured NO, N_2O as well as direct N_2 emissions and used the N_2O ^{15}N site preference signatures of soil-emitted N_2O to distinguish N_2O production from fungal and bacterial denitrification. Uniquely, soil NO_3^- measurements were also made throughout the incubation. Emissions of N_2O during the initial phase of the experiment (0-13 days) increased almost linearly with increasing rate of straw incorporation and with (almost) no N_2 production. However, the rate of straw amendment was negatively correlated with N_2O, but positively correlated with N_2 fluxes later in the experimental period (13-30 days). Soil NO_3^- content, in all treatments, was identified as the main factor responsible for the shift from N_2O production to N_2O reduction. Straw amendment immediately lowered the proportion of N_2O from bacterial denitrification, thus implying that more of the N_2O emitted was derived from fungi (18±0.7% in control and up to 40±3.0% in high straw treatments during the first 13 days). However, after day 15 when soil NO_3^- content decreased to <40 mg NO_3^--N kg^{-1} soil, the N_2O ^{15}N site preference values of the N_2O produced in the medium straw rate treatment showed a sharp declining trend 15 days after onset of experiment thereby indicating a clear shift towards a more dominant bacterial source of N_2O. Our study singularly highlights the complex interrelationship between soil NO_3^-

kinetics, crop residue incorporation, fungal denitrification and $N_2O/(N_2O+N_2)$ ratio. Overall we found that the effect of crop residue applications on soil N_2O and N_2 emissions depends mainly on soil NO_3^- content, as NO_3^- was the primary regulator of the $N_2O/(N_2O+N_2)$ product ratio of denitrification. Furthermore, the application of straw residue enhanced fungal denitrification, but only when the soil NO_3^- content was sufficient to supply enough electron acceptors to the denitrifiers.

Keywords: Organic carbon; Denitrification product ratio; Greenhouse gas; Nitrogen cycling; Site preference

1. Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas with ca. 300 fold higher global warming potential than carbon dioxide (CO_2) and is also involved in the destruction of the stratospheric ozone layer (Ravishankara et al., 2009). Globally, soils are the largest anthropogenic source of N_2O, which is produced by several microbial and chemical processes (Butterbach-Bahl et al., 2013). Increasing evidence suggests that biological denitrification (fungal and bacterial) is the dominant process responsible for the soil-driven increase in atmospheric N_2O (Baggs, 2011). Microbial denitrification includes all or parts of the sequential reduction of NO_3^- to NO_2^-, NO, N_2O and N_2, which occurs under oxygen limited situations in soil (e.g., high water-filled pore space) (Weier et al., 1993). Due to the large background N_2 concentration in air and the large spatial and temporal heterogeneity of N_2 production, fluctuations in soil-borne N_2 fluxes are hard to determine. Therefore, a comprehensive and quantitative understanding of the controlling factors of denitrification in soil is still missing (Davidson and Seitzinger, 2006; Butterbach-Bahl et al., 2013).

Soil carbon (C) availability is one of the most critical factors regulating denitrification rate, as labile C is the electron donor for all of the reduction steps from NO_3^- to N_2 (Burford and Bremner, 1975). Most laboratory studies have tested the effect of readily available C substrates (e.g. glucose) on denitrification pathways and its product stoichiometry (Weier et al., 1993; Meijide et al., 2010; Giles et al., 2017; Wu et al., 2017), however, only a few studies have used complex plant/animal residues (Miller et al., 2008; Köster et al., 2015). Straw incorporation in agricultural soils can improve soil quality (e.g. porosity, water-holding capacity, cation exchange capacity), increase land productivity and helps to sequester more C. However, concerns have also been raised about the effect of straw addition on soil N_2O emissions, as both positive and negative influences have been reported (Pan et al., 2017; Koebke et al., 2018; Xiao et al., 2018). This discrepancy may be

partly because, in addition to many other factors (e.g. moisture, oxygen, pH, temperature), labile soil C content alters the relative availability of reductant vs. oxidant compounds, which in turn also affects the final end products of denitrification, i.e. NO, N_2O or N_2. The higher ratio of electron donors (available organic C)/acceptors (N oxides) as a result of organic matter application to soil may favor N_2O reduction (Smith and Arah, 1990) due to electron donor abundance (Hutchinson and Davidson, 1993). The common hypothesis is that additional labile C amendment could promote denitrification rates in moist soils (Zhong et al., 2018) and also may enhance elemental N_2 losses via promoting sequential reduction of NO_3^-, NO_2, NO and N_2O to N_2 (Smith and Arah, 1990; Hutchinson and Davidson, 1993; Mathieu et al., 2006). Although a number of studies have indicated that N_2O emissions from soils can be lowered under conditions favoring N_2O reduction to N_2 (Firestone, 1982; Weier et al., 1993), it is still not clear how straw application in conjunction with mineral fertilizer would affect both production and reduction rate of N_2O. Furthermore, the $N_2O/(N_2O+N_2)$ product ratio of denitrification is regulated by the complex interrelationship between a number of soil parameters, e.g. NO_3^- concentration, available C content and O_2 availability (Blackmer and Bremner, 1978; Senbayram et al., 2012). For example, several studies have shown that higher soil NO_3^- concentration in soil can inhibit N_2O reductase activity, since NO_3^- is preferred over N_2O as a terminal electron acceptor (Firestone, 1982; Weier et al., 1993; Qin et al., 2017b). In this context, it is still not yet clear whether the amendment of soil with labile C would directly promote N_2O reduction to N_2 or whether its effect on the $N_2O/(N_2O+N_2)$ product ratio depends on other soil parameters, e.g. NO_3^- content.

In addition to bacteria, fungi are also capable of denitrification and N_2O production. Denitrifying fungi generally lack N_2O reductase, thus the gaseous emission from fungi is in the form of N_2O rather than N_2 (Laughlin et al., 2002). The possibility of significant contributions of fungi to soil N_2O production has been demonstrated in several studies, which reported fungal contributions of

between 40% and 89% of the emitted N_2O in different terrestrial ecosystems (Laughlin et al., 2002; Chen et al., 2014; Zhong et al., 2018). Since several studies have shown that organic C supply in moist soils could increase both fungal/bacterial biomass ratio and fungal N_2O production (Laughlin et al., 2002; Hayden et al., 2012; Zhong et al., 2018), we hypothesize that fungal denitrification may be a dominant source for N_2O emission in NO_3^- rich, crop residue amended, moist soil.

The different enzyme types of bacteria and fungi are known to produce a different intramolecular ^{15}N distribution in the linear asymmetric N_2O molecule, so-called ^{15}N site preference (SP). It has been found that the SP value of N_2O produced by bacterial denitrification ranges from -9‰ to +9‰, whereas nitrification and fungal denitrification produce N_2O with a SP range from +34‰ to +40‰ (Toyoda et al., 2017). This non-destructive, low cost gas sampling approach has been used previously to distinguish the different sources of N_2O production pathways in both lab and field scale studies (Decock and Six, 2013; Rohe et al., 2017).

Direct measurements of small amounts of N_2 produced from denitrification in soils are challenging due to the high atmospheric N_2 background and a lack of sufficiently sensitive equipment. Various approaches have been used to indirectly measure N_2 production from soil, e.g. the commonly used acetylene inhibition technique (Weier et al., 1993; Miller et al., 2008) and ^{15}N isotope labeling (Cai et al., 2001). However, neither are ideal, introducing their own artifacts (Terry and Duxbury, 1985; Groffman et al., 2006; Nadeem et al., 2013). In recent years, several automated soil incubation systems have been established for continuous direct N_2 measurement, based on the replacement of the soil atmosphere by He (Bol et al., 2003; Cardenas et al., 2003; Molstad et al., 2007; Liu et al., 2010; Köster et al., 2013; Qin et al., 2017b). In this study, we conducted our incubation experiment with a newly-designed fully robotic continuous flow incubation system (ROFLOW) that enables us to determine directly very low ($\geq$10 g N_2-N ha^{-1}) soil N_2 fluxes using sealed vessels and steel

components (<10 ppm N_2 background concentration). Furthermore, the system is uniquely equipped with a filter membrane at the base for soil water sampling and moisture adjustment (Fig. 1), which allows simultaneous monitoring of soil NO_3^- dynamics during experiments.

We studied a sandy textured arable soil with low ammonium (NH_4^+) content and examined i) whether or not there is a potential for higher N_2O emission when straw in conjunction with nitrate (NO_3^-) based fertilizer is incorporated into soil, ii) does the straw amendment directly regulate the N_2O/N_2O+N_2 product ratio of denitrification, and iii) will the straw amendment increase the contribution of fungal denitrification to N_2O fluxes? This was achieved through the use of a unique experimental platform that allowed online simultaneous measurements of NO, N_2O and N_2 fluxes, and soil water sampling for NO_3^-. Furthermore, we coupled this with N_2O isotopomer measurements to distinguish N_2O production between fungal and bacterial denitrification.

2. Materials and methods

2.1. Soil

The soil was collected from farmland in Fuhrberg, Lower Saxony, Germany (52° 33' 6" N, 9° 50' 49" E). Winter wheat had been grown prior to soil sampling. The sandy textured soil was classified as a Gleyic Podzol (sand 90.1%, silt 3.1%, clay 5.9%) and contained 0.1% total N, 0.5 mg NH_4^+-N kg^{-1} soil, 43.7 mg NO_3^--N kg^{-1} soil and 1.8% organic carbon with a pH of 5.6 (H_2O). The upper 5 cm of soil and roots were removed and soil was collected from the first 10 cm below the removed layer. The soil was sieved to <10 mm, air-dried and stored at 4 °C before packing into cores. Prior to the experiment, soil was wetted to ca. 40% water holding capacity (WHC) for a week and stored at room temperature to minimize the drying-wetting effect.

150 *2.2. Robotized soil incubation experiment and trace gas measurements*

151 The incubation experiment was carried out at Thünen Institute of Climate-Smart Agriculture
152 Braunschweig, Germany in the ROFLOW system using a make-up atmosphere containing 80% He
153 and 20% O_2 (Köster et al., 2013). The cylindrical incubation vessels consisted of acrylic glass with
154 an inner diameter of 140 mm and 150 mm height. Each incubation vessel was equipped with a
155 polyamide filter membrane (EcoTech, Bonn, Germany - hydrophilic; pore size 0.45 μm) at the
156 bottom, which allowed adjustment of the soil moisture and the removal of the soil water samples.
157 The experiment consisted of five treatments (n=3); i) non-amended control treatment (CK) with no
158 addition, ii) treated with 20 mmol KNO_3 (KNO_3), iii) low rate of straw + 20 mmol KNO_3 (LS+N),
159 iv) medium rate of straw + KNO_3 (MS+N) and iv) high rate of straw + KNO_3 (HS+N). The pre-
160 incubated soils were mixed by hand with 1, 2.5 or 5 g kg^{-1} dry soil maize straw (0.78% total N and
161 44.05% total C) in the LS+N, MS+N, and HS+N treatments, respectively prior to the experiment
162 and 1 kg dry soil was packed into each vessel (with a density of 1.25 g cm^{-3}). Oven-dried maize
163 straw was ground through a 2 mm mesh sieve for homogeneity. By applying a vacuum from the
164 top of each vessel, the repacked soil cores were flooded from the bottom of the vessels with either
165 20 mmol KNO_3 solution (in KNO_3, LS+N, MS+N, and HS+N) or distilled water (in CK) and then
166 drained to 28.3% gravimetric water content (67% WFPS) by applying a vacuum to the ceramic
167 plate. The incubation vessels were then sealed and the atmospheric air in the vessels was replaced
168 by a pure He/O_2 mixture (to remove any CO_2, NO, N_2O or N_2 in the soil pores or headspace) by
169 applying a vacuum from the top and filling with He/O_2 mixture in three cycles that were completed
170 within 6 h. Subsequently, the headspace of each vessel was flushed continuously with a gas mixture
171 of He (80%) and O_2 (20%) at a flow rate of ca. 25 mL min^{-1}. The temperature of the incubation
172 room was set at 20°C during the 30 days of incubation.

The airflow from each vessel was directed sequentially to a gas chromatograph by two multi-positional valves (VICI, Houston, USA), where the gas sample was analyzed a thermal conductivity detector (TCD) for N_2, O_2, and CO_2, and an electron capture detector (ECD) for N_2O quantification. The sample outlet of GC was connected to the inlet of the online NO analyzer (Eco-Physics, Dürnten, Switzerland). A microcontroller unit (Arduino Mega 2560 REV3) was programmed to control the system via giving/receiving signals i) to/from the multi-positional VICI valves for setting the target position, ii) to/from the GC for ready signal or start/stop method and iii) to the computer to start/stop data acquisition (for a schematic overview of the system see Fig. 1).

2.3. Mineral N analysis

Soil samples were collected at the end of the incubation period from each vessel. The soil samples were extracted with 2 M KCl solution (1:5 w/v) by shaking for 1 hour. Additionally, ca. 15 ml of soil solution was collected on two occasions from each vessel during the incubation period (during moisture adjustment at the beginning of the incubation and 13 days after onset of treatments) by opening the valve at the bottom of the membrane filter and applying slight overpressure from the top. The KCl extracts and soil solution were then filtered through Whatman 602 filter paper and stored at −20°C until analysis. The concentrations of NH_4^+ and NO_3^- in soil extracts and soil solution were measured using a continuous flow analyzer (Smartchem 200S/N1104238, WESTCO, France).

2.4. Isotope analysis and N_2O source partitioning

Additional gas samples for isotopic analysis were taken from each incubation vessel by attaching 120-mL serum bottles to the outlets in flow-through mode (Well et al., 2008) for around 2 h. The

196 N_2O $\delta^{15}N^{bulk}$, $\delta^{15}N^{\alpha}$, and $\delta^{18}O$ isotope signatures were then determined by analyzing *m/z* 44, 45,
197 and 46 of intact N_2O^+ molecular ions, and *m/z* 30 and 31 of NO^+ fragment ions (Toyoda and
198 Yoshida, 1999) on an isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen,
199 Germany) at Thünen Institute Braunschweig, Germany. The SP value of the produced N_2O (SP_0),
200 i.e. prior to its partial reduction to N_2, was calculated using a Rayleigh-type model, assuming that
201 isotope dynamics followed closed-system behavior (Lewicka-Szczebak et al., 2017). The model
202 can be described as follows:

203 $$SP_{N2O-r} = SP_0 + \eta_r \ln\left(\frac{C}{C_0}\right) \quad (1)$$

204

205 In this equation, SP_{N2O-r} is the SP value of the remaining substrate (i.e. residual N_2O), SP_0 is the
206 SP value of the initial substrate (i.e. produced N_2O before reduction occurred), η_r is the net isotope
207 effect associated with N_2O reduction, and C and C_0 are the residual and the initial substrate
208 concentration (i.e. C/C_0 expresses the $N_2O/(N_2O+N_2)$ product ratio). In this study an η_r of -5‰ was
209 used based on previously reported average values (Lewicka-Szczebak et al., 2014). For source
210 partitioning, the end-member values (SP_{fD}) were defined as 37‰ for nitrification and fungal
211 denitrification, and -5‰ (SP_D) for bacterial denitrification (Toyoda et al., 2017). The source
212 partitioning of N_2O production was based on the two end-member isotopic mass balance equation:

213

214 $$SP_0 = SP_D \times f_{D\text{-}SP} + SP_{fD} \times f_{fD\text{-}SP} \quad (2)$$

215

216 It should be noted that distinguishing the N_2O produced between nitrification and fungal
217 denitrification based on SP values is impossible because of the overlapping SP signature from those
218 pathways (Frame and Casciotti, 2010; Lewicka-Szczebak et al., 2014; Toyoda et al., 2017). In this
219 equation, $f_{D\text{-}SP}$ and $f_{fD\text{-}SP}$ represent the contribution of bacterial denitrification and

nitrification+fungal denitrification to total N_2O release calculated on the basis of SP_0 values, respectively. In the present study, however, considering that the specific experimental conditions were set up to favor denitrification, i.e. i) N was applied in the form of NO_3^-; ii) initial soil NH_4^+ content was under detection limits (<0.5 mg NH_4^+-N kg^{-1} soil) with constantly low NH_4^+ content during incubation; and iii) high soil moisture (67% WFPS), the contribution of nitrification and nitrifier denitrification were assumed to be negligible (See Discussion). Thus, only the most plausible scenario (bacterial denitrification vs fungal denitrification) was discussed for the SP_0 source partitioning calculation.

2.5. Calculations and statistical analysis

The cumulative gas emissions were calculated by linear interpolation between measured fluxes. Statistically significant differences were tested using Tukey's honest significant difference post-hoc tests at a 5% significance level by SPSS 21.

3. Results

3.1. Soil mineral N

Soil NH_4^+ concentrations in all treatments were very low (1-3 mg kg^{-1} soil) at the end of the experiment (Table 1). Soil NO_3^- concentrations decreased over time in all treatments and the observed rate of decrease was more rapid with an increasing rate of straw application (Fig. 2A). Soil NO_3^- contents at the end of the 30-day incubation period followed the trend: KNO_3 > LS+N = CK > MS+N > HS+N (Table 1). Soil NO_3^- was completely depleted in the HS+N treatment after 13 days, whereas 84%, 59% and 12% of the soil NO_3^- were depleted in MS+N, LS+N and KNO_3 at the end of the incubation, respectively.

3.2. Emission of NO, N_2O, N_2 and CO_2

Significant NO emission peaks were observed in straw-amended treatments (HS+N, MS+N and LS+N) immediately after onset of the experiment, whereas the NO emissions from the CK and KNO_3 treatments remained low throughout the experiment. Here the maximum NO emission rates were 7 (±2), 38 (±18) and 22 (±6) g NO-N ha^{-1} day^{-1} in the LS+N, MS+N and HS+N treatments, respectively. Total emissions of NO over the 30 day incubation were significantly greater in the HS+N and MS+N treatments than in the LS+N, with the lowest seen in KNO_3 and CK, indicating the importance of labile C on NO formation and losses (Table 2).

The daily N_2O flux rate increased over time in all treatments, reaching a maximum at around day 7 and then decreased afterwards with different declining rates between the treatments (Fig. 2B-F). Maximum daily N_2O emission rates were 269 (±13), 414 (±27), 631 (±24), 734 (±64), and 899 (±36) g N_2O-N ha^{-1} day^{-1} in the CK, KNO_3, LS+N, MS+N and HS+N treatments, respectively. In the HS+N treatment, fluxes of N_2O decreased sharply after day 10, and remained low throughout the experimental period, whereas the N_2O flux rates decreased gradually in all the other treatments, but were less pronounced for decreasing rates of added straw. At the end of the incubation period, N_2O fluxes were below the detection limit in the HS+N and MS+N treatments, but significant N_2O fluxes were still detected in all the other treatments.

The decrease in N_2O fluxes followed almost the same trend as the decrease in NO_3^- concentrations in different treatments. From our measurements, when soil NO_3^- concentrations decreased below 40 mg NO_3^--N kg^{-1} soil, the emission of N_2O also decreased. Thus, we can separate the experiment into two Phases; Phase I (0-13 days – no limitation of NO_3^- in any treatments) and Phase II (13-30 days – NO_3^- limited, specifically in high straw rate treatments). As shown in Table 2, emission of N_2O in Phase I increased almost linearly with higher rates of straw incorporation in N fertilized soils. However, application of KNO_3 only slightly increased N_2O fluxes during this period

compared to CK. In Phase II, almost no N_2O emissions were detected in the HS+N treatment, and the cumulative emissions during this phase were now negatively correlated with the rate of straw amendment. Here, the highest cumulative N_2O fluxes were measured in the LS+N and the KNO_3 treatments and the lowest from the HS+N treatment. Overall, application of N fertilizer alone significantly increased the cumulative N_2O emissions by 80% compared with the CK, while this increase was 125%, 85% and 49% in the LS+N, MS+N and HS+N treatments, respectively (Table 2).

Fluxes of N_2 in the CK and the KNO_3 treatments were consistently low throughout the experimental period and increased only slightly during the last 10 days of incubation, being more pronounced in the CK than in the KNO_3 treatment. In straw amended treatments, N_2 emissions were very low during the first 10 days of incubation, but peaked over a relatively short period in the HS+N treatment at 13 day (Fig. 2B-F). Subsequently, the N_2 emissions increased gradually over time in all straw treatments and the rate of increase was larger at higher rates of straw application. Here, the increase in N_2 emission rates was closely associated with the decrease in N_2O emissions and soil NO_3^- concentrations (Fig. 2). Emissions of N_2 became dominant in the HS+N and the MS+N treatments in Phase II. Total N_2 fluxes were more than 10-fold higher in Phase II than in Phase I in all treatments. Between the treatments, the highest cumulative N_2 emissions were observed in HS+N and MS+N, while the lowest were from the CK and KNO_3 (Table 2). The $N_2O/(N_2O+N_2)$ ratio decreased significantly in all treatments in Phase II compared to Phase I. However, this decrease in $N_2O/(N_2O+N_2)$ ratio was lowest in both KNO_3 and LS+N treatments and highest in the HS+N. In the MS+N treatment, the emission of N_2O (48%) was very similar to the emission of N_2 (52%) in Phase II, while in contrast it had been 99% N_2O and only 1% N_2 in Phase I.

Daily fluxes of CO_2 increased significantly over time in Phase I and remained relatively constant in Phase II (Fig. 3). Cumulative CO_2 fluxes were almost doubled in the HS+N treatment compared

to CK, whereas an increase of about 70% was observed in MS+N compared to CK and KNO_3 treatments.

3.3. N_2O SP values and source partitioning

The SP_0 values ranged from -4‰ to 4‰ on day 1 in all treatments, being lowest in KNO_3 treatment (-4‰ ±0.3) and highest in straw amended treatments (4‰ ±4.6 in HS+N) (Fig. 2). Addition of straw in combination with KNO_3 increased SP_0 values from the first day ($P < 0.05$) up to 8‰. The SP_0 values increased gradually over time in all treatments until day 13 and the rate of increase was higher with higher levels of straw amendment. After day 13, different SP_0 value dynamics were observed in different treatments, indicating multiple N_2O sources. The SP_0 values continued to increase in the CK, KNO_3 and LS+N treatments until the end of the incubation, reaching maximum value of 30.5 ‰, whereas the SP_0 values sharply decreased in the MS+N treatment, reaching -2.6 ‰ at day 29. It was not possible to detect SP_0 values in the HS+N treatment after day 13 due to extremely low N_2O concentrations (less than 100 ppb).

To calculate the proportion of each N_2O emitting process, source partitioning based on the two-end-member model was used. During the initial period of the experiment, very low SP_0 values suggest that almost all emitted N_2O originated from bacterial denitrification, however, the share of fungal denitrification derived N_2O increased almost linearly over time in all treatments. In later periods, specifically in Phase II, the SP_0 values showed a decreasing trend in the MS+N treatment (no N_2O was emitted in HS+N), which paralleled the decreasing trend in N_2O emission and soil NO_3^- content. This clearly indicates that when soil NO_3^- content decreases, bacterial denitrification recovers and even then may dominate again in parallel to the increase in N_2O reduction rates. The contribution of fungal denitrification to the cumulative N_2O emitted during the incubation period varied between 29% and 40% between the treatments, being significantly greater in the straw

315 amended soils (Fig. 4A). Note, we acknowledge that the SP_0 source partitioning approach provides
316 only an estimation about the source of emitted N_2O due to the i) overlapping SP signals of different
317 processes, ii) variability of isotopologue enrichment factors of N_2O reduction, and iii) variation in
318 SP signals between different microbial strains (see Discussion). Nevertheless, the technique
319 provides useful insights of the effects of straw addition on the underlying soil microbial processes.

320

321 **4. Discussion**

322 *4.1. Sources of N_2O as affected by straw amendment and soil NO_3^- kinetics*

323 Using SP values and the two end-member approach enables an estimation of the relative
324 contributions of fungal and bacterial denitrification to N_2O emission, which are occurring
325 simultaneously in amended soils. However, this approach is only valid if i) the N_2O reduction
326 fractionation effect on SP values can be corrected, and ii) the N_2O derived from nitrification and
327 nitrifier denitrification were negligible. In the present study, the following conditions were set to
328 fit this specific case. Firstly, the direct measurement of N_2 production enabled us to calculate the
329 initial SP values (SP_0) by considering the N_2O reduction fractionation effect (Lewicka-Szczebak
330 et al., 2017), which minimizes the possibility of overestimation of fungal
331 denitrification/nitrification (Wu et al., 2016). Secondly, a sandy soil with very low NH_4^+ content
332 and high soil moisture (WFPS=67%) was chosen, and N was applied in the form of NO_3^- to
333 suppress N_2O formation from nitrification during the incubation period. Nevertheless, in the
334 present experiment fungal denitrification may still be overestimated due to the possible
335 contribution of nitrification derived N_2O related to the mineralization of the organic matter during
336 the experiment. However, in our recent study, the contribution of mineralization related N_2O
337 formation from various straw treatments was found to be < 5% of the emitted N_2O in a fertilized

sandy soil over 40 days of incubation (Koebke et al., 2018). Therefore, we believe that the present experimental set up enabled a reliable estimation of fungal and bacterial denitrification derived N_2O using the N_2O SP source partitioning approach.

During the initial period of the experiment, the very low SP_0 values (-4 to 4‰) suggested that almost all emitted N_2O originated from bacterial denitrification. However, the linear increase in SP_0 values until day 13 in all treatments indicated that the share of fungal denitrification derived N_2O increased over time. Dominancy of bacterial N_2O during the early phase of the experiment with a subsequent shift (almost linear increase over time) towards fungal activity is in agreement with previous studies (Laughlin and Stevens, 2002; Zhong et al., 2018). This indicated that bacterial activity started almost immediately after the start of the experiment, whereas the fungal colonization and activity increased somewhat slower, but became dominant in the latter phase. Similarly, Henriksen and Breland (2002) found that bacterial activity dominated immediately after residue incorporation in soils, whereas biological activity gradually shifted towards a dominance of fungal activity in later phases. The observed higher proportion of fungal N_2O production in straw amended treatments is consistent with previous studies in which the fungal N_2O production was increased under an enhanced organic C supply in moist soil (Laughlin et al., 2002; Zhong et al., 2018).

The sharp decrease in SP_0 values after day 15 in the MS+N treatment indicated a clear shift of N_2O source from fungal denitrification to bacterial denitrification, which was in parallel with the decreasing trend in N_2O emission and soil NO_3^- content. Unlike bacterial denitrifiers, fungi generally lack nitrous oxide reductase (*nos*), which means fungal denitrification mainly relies on the availability of NO_3^- and NO_2^- as electron acceptors (Baggs, 2011). We therefore presume the shift from fungal to bacterial N_2O in high straw amended treatments is attributed to the depletion of electron acceptors in soil (NO_3^-, and NO_2), causing a decrease in denitrifying fungal community.

As most denitrifying bacteria have *nos* and thus can use N_2O as an electron acceptor, bacterial denitrification recovered and dominated again when soil NO_3^- concentrations became limited.

In the present study, the contribution of fungal denitrification to N_2O emission was similar to the 18% fungal contribution in control soil measured by Herold et al. (2012) (where the acetylene inhibition technique was used), 40-51% in residue added soils reported by Zhong et al. (2018) (acetylene inhibition technique was used), and 36%-70% in NO_3^- treated coastal sediments reported by Wankel et al. (2017) (isotopomer and stable isotope labelling was used). On the other hand, Laughlin and Stevens (2002) reported a much greater contribution of fungi to N_2O production (89%) in grassland soils where soil organic C content was expected to be high. In this context, we conclude that the application of crop residues could enhance N_2O emission through fungal denitrification, however, only when soil NO_3^- content is sufficiently high for supplying enough electron acceptors to denitrifying organisms. However, in straw amended soils, a depletion of NO_3^- in soil may cause a shift from fungal to bacterial denitrification derived N_2O. Nevertheless, we should note that in view of the uncertainties of the SP approach, and that there are limited comparisons of studies using the same approach to estimate fungal N_2O production there is still a need to confirm these results in future studies.

4.2. N_2O production and reduction as affected by straw amendment and soil NO_3^- kinetics

Straw application can increase the rate of the denitrification (microbial or fungal) (Baggs, 2011; Qin et al., 2017a; Xiao et al., 2018), mainly due to the extra substrate supply (electron donors as energy source) (Giles et al., 2017). During the initial period of our experiment (in Phase I), total gaseous N ($NO+N_2O+N_2$) and CO_2 fluxes increased almost linearly with the higher straw application rate, thereby showing a significant relationship between respiration and denitrification rates (Burford and Bremner, 1975; Miller et al., 2008; Xiao et al., 2018).

Contradictory observations have been reported on the impact of crop straw incorporation on N_2O emissions (Chen et al., 2014; Shan and Yan, 2013). This discrepancy may be partly because of the effect of labile C on the end product of bacterial or fungal denitrification (N_2O or N_2), which may vary under different conditions (Qin et al., 2017b). In our study, gaseous N fluxes during Phase I were dominated by N_2O, with minor NO fluxes and almost no N_2 emissions even in the straw treatments. In Phase I, application of KNO_3 alone slightly increased N_2O fluxes compared to CK, whereas N_2O fluxes increased more than 3-fold in HS+N indicating that labile organic C was likely limiting and controlling the rate of the N_2O production (Fig. 2). It has been suggested that addition of crop residues would decrease N_2O emissions by lowering N_2O/N_2 ratio and stimulating microbial immobilization in soil (Mathieu et al., 2006; Frimpong and Baggs, 2010). It is striking that in contrast to the expected outcome, even with excess organic C input (5 g straw kg^{-1} dry soil in HS+N), high NO_3^- content in soil would still inhibit N_2O reduction, causing very high N_2O emission and also relatively high NO fluxes. Compared to N_2O fluxes, the NO fluxes in straw amended soils were very low. However, compared to CK and KNO_3, straw amendment did induce significant NO losses during the initial phase of the experiment. Because straw amendment also enhanced fungal denitrification during this phase, the increase in NO fluxes may be attributed to the leakage from fungal denitrification. We may speculate that NO_3^- and NO_2^- reducing fungal strains developed faster than the NO reducers shortly after amendments causing such leakage, however, further research at the molecular level is needed to prove this hypothesis.

In the present study, the increase in N_2 fluxes became greater when soil NO_3^- contents decreased below 40 mg NO_3^--N kg^{-1} soil (in Phase II), and N_2 fluxes dominated when concentrations decreased below 30 mg NO_3^--N kg^{-1} soil in the HS+N and MS+N treatments (Fig. 4B). This is likely because the supply of NO_3^- at the denitrifying microsites became lower than the demand for terminal electron acceptors, which is in agreement with earlier reports (Weier et al., 1993;

Senbayram et al., 2012; Qin et al., 2017a). It should be noted that measured total soil NO_3^- concentration was likely much higher than the concentrations in the soil microsites where denitrification occurs (Myrold and Tiedje, 1985). In this context, further research is needed perhaps with new measurement approaches to better quantify the direct relationship between NO_3^- concentration and the product stoichiometry of denitrification in soil hotspots.

In contrast to a number of studies (Cookson et al., 1998; Mathieu et al., 2006), our results showed that N_2O reduction was found not to be directly affected by C supply. Higher labile C seems to favor N_2O reduction only when soil NO_3^- content decreases to a threshold concentration, which seemed to occur when the bulk NO_3^- concentration ranged between 20 and 50 mg N kg^{-1} soil in our study. This is possibly because, NO_3^- is usually preferred over N_2O as a terminal electron acceptor and N_2O can escape from the soil whenever NO_3^- supply is greater than the reducing demand of denitrifiers (Swerts et al., 1996). We believe that the present study explains the contradictory reports of straw addition on N_2O fluxes as i) firstly we show in Phase I, straw addition triggered N_2O fluxes (when NO_3^- is high) with no N_2O reduction effect, and ii) secondly in Phase II, almost all N_2O was reduced to N_2 when soil NO_3^- content decreased below a certain level. In support of our findings, Xiao et al. (2018) recently showed that crop residue application drastically stimulated N_2O fluxes when applied with KNO_3, compared to other nitrogen forms.

5. Conclusion

Based on the results in this experiment, there are four key take-home messages;

i) Straw amendment in moist sandy soil enhances soil denitrification rate and triggers gaseous N losses.

ii) When soil NO_3^- content is high, denitrification produces almost solely N_2O with little NO and N_2 emissions from straw amended soils. Thus, our data suggests that straw

application, even at very high rates, does not directly affect the product stoichiometry of denitrification ($N_2O/(N_2O+N_2)$ product ratio).

iii) The effect of crop residue application on soil N_2O emissions is related to the soil NO_3^- content, since NO_3^- appears to be the ultimate regulator of the $N_2O/(N_2O+N_2)$ product ratio of denitrification.

iv) Application of straw residue predominantly enhances fungal denitrification when soil NO_3^- content is sufficient, however, when soil NO_3^- is low, bacterial denitrification dominates.

Thus, the present study suggests that in agricultural systems where large amount of organic plant residues are incorporated into soil, risk of N_2O emissions can be minimized by keeping soil NO_3^- concentrations under site-specific threshold values (e.g. using NO_3^--free N fertilizers and/or fertilizers containing nitrification inhibitors). Another way of mitigating N_2O in these soils could be to develop management practices which slow down fungal growth after residue amendment as the present study suggests that fungal denitrification seems to be an important processes contributing to N_2O losses in residue-amended soils. Further field validations are needed to test the efficiency of these hypotheses. Overall, our study shows the importance of continuous direct measurement of N_2 fluxes alongside N_2O and NO fluxes and soil NO_3^- concentrations, and the use of the N_2O ^{15}N site preference approach in improving our understanding of the complex interrelation between crop straw incorporation and gaseous denitrification N losses.

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Table 1 Soil nitrate (NO_3^-) and ammonium (NH_4^+) concentrations at the end of the experiment in non-amended control (CK), KNO_3 (KNO_3), low rate of straw + KNO_3 (LS+N), medium rate of straw + KNO_3 (MS+N) and high rate of straw + KNO_3 (HS+N) treatments. Means denoted by a different letter in the same column differ significantly according to the Tukey's HSD post-hoc tests at α=0.05.

Parameter	NO_3^- (mg N kg^{-1} dry soil)	NH_4^+ (mg N kg^{-1} dry soil)
CK	33±8.3 [b]	2±1.1 [a]
KNO_3	81±5.6 [a]	1±0.3 [a]
LS+N	37±4.8 [b]	3±0.8 [a]
MS+N	15±8.6 [c]	2±1.2 [a]
HS+N	0±0.0 [d]	3±0.1 [a]

Table 2 Cumulative emissions of N_2O, N_2, NO and CO_2 at Phase I (0-13 days) and during the whole incubation period (0-30 days) in non-amended control (CK), KNO_3 (KNO_3), low rate of straw + KNO_3 (LS+N), medium rate of straw + KNO_3 (MS+N) and high rate of straw + KNO_3 (HS+N) treatments. Means (n=3) denoted by a different letter in the same column differ significantly according to the Tukey's HSD post-hoc tests at α=0.05.

	N_2O (g N ha^{-1}) Day 0-13	N_2O (g N ha^{-1}) Total	N_2 (g N ha^{-1}) Day 0-13	N_2 (g N ha^{-1}) Total	NO (g N ha^{-1}) Day 0-13	NO (g N ha^{-1}) Total	CO_2 (kg C ha^{-1}) Day 0-13	CO_2 (Kg C ha^{-1}) Total
CK	2448±145 ^{d}	4555±606 ^{b}	38±1.0 ^{b}	697±93.0 ^{b}	1.4±0.1^{c}	1.7±0.1^{c}	77±19.3^{a}	156±35.4^{b}
KNO_3	4033±106 ^{c}	8115±792 ^{a}	45±7.8 ^{b}	564±78.7 ^{b}	1.6±0.0^{c}	1.9±0.1^{c}	71±14.9^{a}	160±23.8^{b}
LS+N	5616±151 ^{b}	10192±771 ^{a}	103±18.4 ^{ab}	819±62.8 ^{ab}	25.0±5.7^{bc}	25.3±5.7^{bc}	74±18.6^{a}	176±41.8^{b}
MS+N	6907±567 ^{a}	8797±1378 ^{a}	81±3.0 ^{b}	1656±139.7 ^{ab}	71.2±11.6^{a}	71.6±11.6^{a}	120±19.3^{a}	252±17.8^{ab}
HS+N	7594±302 ^{a}	7604±295 ^{a}	197±45.3 ^{a}	2049±597.0 ^{a}	42.3±11.9^{ab}	42.7±12.0^{ab}	131±14.6^{a}	307±30.7^{a}

Figure captions:

Figure 1. Simplified diagram of the robotized continuous flow incubation system (ROFLOW) used in the experiment. The system is controlled by a Arduino-based microcontroller unit (Arduino Mega attached with 16 position relay). This control unit adjusts the position of VICI valves, gives signals to the GC (start/stop method) and the computer (start and stop data acquisition).

Figure 2. (A) NO_3^- dynamics, and (B-F) daily emissions of N_2O, N_2, NO and SP_0 values during the incubation period (30 days) in non-amended control (CK), KNO_3 (KNO_3), low rate of straw + KNO_3 (LS+N), medium rate of straw + KNO_3 (MS+N) and high rate of straw + KNO_3 (HS+N) treatments. Error bars shows the standard error of each treatments (n=3).

Figure 3. Soil daily cumulative CO_2 emissions during the incubation (30 days) in non-amended control (CK), KNO_3 (KNO_3), low rate of straw + KNO_3 (LS+N), medium rate of straw + KNO_3 (MS+N) and high rate of straw + KNO_3 (HS+N) treatments. Error bars shows the standard error of each treatment (n=3). Means denoted by a different letter differ significantly according to the Tukey's HSD post-hoc tests at α=0.05.

Figure 4. (A) Contribution of fungal and bacterial denitrification derived N_2O emissions to the cumulative N_2O fluxes, and (B) the ratio of $N_2O/(N_2O+N_2)$ during the Phase I (0-13 days), Phase II (13-30 days), and whole incubation period (0-30 days) in non-amended control (CK), KNO_3 (KNO_3), low rate of straw + KNO_3 (LS+N), medium rate of straw + KNO_3 (MS+N) and high rate of straw + KNO_3 (HS+N) treatments. Error bars shows the standard error of each treatment (n=3). DAO indicates days after onset of the treatments.