N₂O fluxes and related processes of denitrification in acidified soil

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Abstract:

In North China, high levels of N fertilizer and irrigation water are used in fields, which cause considerable N₂O fluxes via several pathways, especially anaerobic denitrification. Anaerobic denitrification is regarded as an important microbial process for N₂O production in soils with a low O₂ level and high N and labile C availability (the typical soil conditions caused by high levels of N fertilizer and irrigation water in the field). We conducted an anaerobic incubation experiment to determine the impact of soil acidification (with a series of soil pH levels, pH 6.2, pH 7.1, and pH
8.7) on N₂O source partitioning with the addition of KNO₃ and glucose. Natural abundance isotope
techniques and gas inhibitor technique were applied to analyze the N₂O flux derived from fungal
denitrification and bacterial denitrification and its isotopocule characteristics emitted from soils
after the addition of NO₃⁻ and glucose. A mapping approach was used to obtain further insight into
the N₂O production processes. Our findings confirmed that soil pH strongly controlled the N₂O
production and reduction rates of denitrification. Soil acidification significantly increased N₂O
emissions varied from 0.76 mg N kg⁻¹ for natural soil (pH 8.7), to 1.88 mg N kg⁻¹ for pH 7.1, and to
2.35 mg N kg⁻¹ for pH 6.2, and had a blockage effect on the reduction of N₂O to N₂. The addition
of carbon sources promoted complete denitrification. We assumed a higher contribution of fungal
denitrification to N₂O production compared to total N₂O emission associated with acidified soil. A
promotion of the contribution of fungal denitrification-derived N₂O was indeed observed with
decreasing pH, increasing from 0.28 mg N kg⁻¹ for pH 8.7 to 0.94 mg N kg⁻¹ for pH 6.2. The addition
of glucose further increased the contribution of fungal denitrification to N₂O production from 0.99
mg N kg⁻¹ for pH 8.7 to 3.66 mg N kg⁻¹ for pH 6.2. The mapping approach provided rational results
for correcting N₂O reduction compared with the acetylene inhibition method. The results calculated
by both methods indicated a reasonably large contribution of fungal denitrification to total N₂O
production in acidified soils.

**Keywords:** N₂O isotopocules; pH; fungal denitrification; glucose; nitrate fertilization

1. Introduction

As a trace gas in the atmosphere, N₂O is the third most important anthropogenic greenhouse
gas in terms of global climate change (Ciais et al., 2013; IPCC, 2014) and has a long atmospheric
lifetime (Ravishankara et al., 2009). Globally, agricultural soils account for approximately 60% of
atmospheric N₂O emissions, of which denitrification is the major source (Senbayram et al., 2015).

Denitrification is stepwise anaerobic reduction of NO₃⁻ to N₂, with NO₂⁻, NO and N₂O as
intermediates during the microbial-mediated process (Devol, 2015). Denitrification-derived N₂O
and N₂ fluxes can result in a considerable loss of N, while the reduction process of N₂O decreases N₂O fluxes (Butterbach-Bahl et al., 2013). Therefore, the net N₂O flux during the denitrification processes is the equilibrium of N₂O production and consumption in soil. The production ratio of N₂O/(N₂O+N₂) can be used as an adequate measure to understand information about both N₂O production and consumption. As an electron-consuming and heterotrophic process, denitrification is not only affected by nitrate concentration but also changes with the addition of carbon sources, which provides electrons and substrates for the growth and activity of denitrifiers (Heinen, 2006).

Soil pH is also an important factor that influences denitrification (Zhou et al., 2018) by acting on many physical, chemical and biological properties and processes of soil (Šimek and Cooper, 2002). To date, the relationships between soil pH and denitrification rate vary in different studies using different soil types, with each soil having particular physical, chemical and biological properties (Šimek and Cooper, 2002). The optimal pH for denitrification of 7-8 is commonly accepted (Sahrawat and Keeney, 1986; Peterjohn, 1991). During denitrification, N₂O emissions into the atmosphere could be an intermediate product caused by denitrifying bacteria or a terminal product by denitrifiers lacking nitrous oxide reductase in neutral and alkaline soils (Firestone et al., 1980; Richardson et al., 2009) depending on enzyme kinetics, physical and chemical factors. Fungi generally lack the nosZ gene used in the reduction of N₂O to N₂, and may play a more important role in N₂O production than bacteria under acidic conditions; therefore, N₂O is more likely to be emitted as the terminal product in acidic soil (Firestone and Davidson, 1989; Laughlin and Stevens, 2002; Hu et al., 2015), leading higher N₂O/(N₂O+N₂) ratios in acidic soils than in neutral and alkaline soils (Šimek and Cooper, 2002; Saggar et al., 2013).

Studies on the respective contribution of fungal and bacterial denitrification under a series of
pH levels may differ as conditions differed. Several methods have been used to quantify their relative importance to N₂O production, with each having advantages and challenges (Zou et al., 2014; Lewicka-Szczech et al., 2017). Inhibition of N₂O reduction with 10 vol% C₂H₂ (the acetylene inhibition method) based on the comparison of N₂O fluxes with and without acetylene application is used to quantify the reduction of N₂O to N₂ since N₂ quantification is challenging (Yanai et al., 2008; Bouwman et al., 2013; Saggar et al., 2013). Depletion of NO both in the presence of C₂H₂ and O₂ is most likely to occur under low water contents (Bollmann and Conrad, 1997; Nadeem et al., 2013a, b). While such NO depletion can be neglected in anaerobic environment (Murray and Knowles, 2003).

In this study, we combined the acetylene inhibition method and the natural abundance isotope technique to investigate N₂O and N₂ fluxes and their production processes in laboratory experiments. N₂O isotopic characteristics include ¹⁸O, ¹⁵Nμbulk, and ¹⁵N site preference (SP) within the linear N₂O molecule. SP, the difference between ¹⁵Nα and ¹⁵Nβ, which represent the central N position (α) and the peripheral N position (β), respectively (Toyoda and Yoshida, 1999), is a promising alternative to assess N₂O production pathways (Well et al., 2008; Opdyke et al., 2009) to differentiate various microbial processes (Sutka et al., 2008; Rohe et al., 2014a). However, SP alone is not concrete enough to quantify the respective contribution of fungal and bacterial denitrification to N₂O emitted since N₂O reduction leads to an enrichment in ¹⁵N, particularly at the α position, and ¹⁸O, furthermore, causes an increase in SP. This increase would result in a shift away from SP values associated with bacterial denitrification towards those associated with fungal denitrification by using the two end-member mixing model and thus move values closer to those of fungal denitrification (Ostrom et al., 2007). Furthermore, the abundance of ¹⁸O also varies among N₂O
emission routes. In summary, $\delta^{18}O$ together with SP may help to further distinguish N$_2$O pathways (Ostrom et al., 2007; Snider et al., 2013; Köster et al., 2015) and provide insight into the dynamics of N$_2$O reduction (Well and Flessa, 2009).

In our previous field study, the application of KNO$_3$ followed by lower soil pH, and led to a higher cabbage yield and lower N$_2$O fluxes compared to other inorganic fertilizers (Lin, 2019), causing less nitrogen loss and environmental pollution. We assumed there existed appreciable contribution of fungal denitrification-derived N$_2$O because of the higher SP values of produced N$_2$O.

For the above, we report an anaerobic incubation experiment to determine the impact of soil acidification on N$_2$O fluxes derived from fungal denitrification and bacterial denitrification with the addition of KNO$_3$ and carbon sources. Besides, a method used for correcting N$_2$O reduction will be tested by comparing with the acetylene inhibition method. Moreover, this study will give insight into the dynamics of N$_2$O production and reduction processes and provide a theoretical basis for alleviating N$_2$O emissions by modulating agricultural management activities.

2. Materials and methods

2.1. Soil collection and characterization

Soil samples used for the incubation experiment were collected from the upper 20 cm of three locations at a temperate arable field site at the Environmental Research Station of the Chinese Academy of Agricultural Sciences, located in the Shunyi District, Beijing, China (40°15′N, 116°55′E) in September 2016. The soils – classified as calcareous Fluvo-aquic were manually sieved to 4 mm and air dried, and sub-samples were shipped to Environmental Stable Isotope Lab., CAAS for analysis. The basic soil properties before the beginning of the experiment were: pH, 8.7; organic C,
15.40 g·kg⁻¹; total N, 1.10 g·kg⁻¹; bulk density, 1.48 g·cm⁻³.

2.2. Treatments and experimental set-up

7 kg air dried soil (oven dry basis equivalent) was adjusted by adding different volume of 1 M H₂SO₄ (two low pH groups) or water (control group) at 50% WFPS at 25 °C for 14 days to initiate and stabilize the microbial activity and soil pH prior to imposing treatments. To obtain a homogenous distribution and equal content of substrates and soil moisture tension in all soil samples, soil was mixed evenly with KNO₃ (100 mg N kg⁻¹) and glucose (0 and 300 mg C kg⁻¹) which were added in particles and then was homogenized with distilled water to their target WFPS (70% WFPS) using a shovel prior to packed into culture flasks.

On each sampling day, equivalent of 40 g dry soil was packed into each 0.28 L culture flask with a bulk density of 1.2 g cm⁻³ and a number of 48 flasks were covered with airtight rubber seals and air in flasks was evaluated after which He was injected into the evaluated flasks to create anaerobic conditions. 30 mL C₂H₂ was injected into selected flasks by replacing corresponding volume of He, resulted in 10 kPa C₂H₂ in the headspace. The flasks were incubated at 25 ± 1 °C for 2 h in the dark. Gas samples were collected each incubation day (day 1, 2, 3, 5, 7, 9, 12, 15) in pre-evacuated 20-ml vials after 2 hours incubation after which destructive sampling was done to collect 20 g soils from the flask for soil properties analysis. Soil samples were incubated without seals and were covered with plastic film to keep soil moisture on non-sampling day. Soil moisture was kept constant at 70% WFPS by weighing the flasks and adding distilled water at alternative days. Each treatment contained four replicates. A total of 384 flasks were used.

2.3. Gas analysis

Gas samples and air were measured by the isotope ratio mass spectrometer with a concentrator
system (IRMS; Delta V Plus-Precon, Thermo Fisher Scientific, Bremen, Germany). Since the peak area and the molar concentration of N$_2$O have a linear correlation, the N$_2$O concentration could be determined basing on the peak area m/z 44 with known isotopic ratios and concentration of standard N$_2$O gas samples (Air Liquide America, Specialty Gases LLC). N$_2$O emission rates were calculated as follows:

$$f_{N_2O} = \frac{\rho \times V \times \Delta C}{m \times \Delta t \times 1000} \times \frac{273}{273+T} \times 24$$

(1)

where $f$ is the emission rate of N$_2$O, $\mu$g kg$^{-1}$ h$^{-1}$; $\rho$ is the N$_2$O density (1.25 kg m$^{-3}$), at 273 K and 101 kPa; $V$ is the effective headspace volume of a jar, 0.25 L; $\Delta C/\Delta t$ is the variation of N$_2$O concentration per unit time (nL L$^{-1}$ h$^{-1}$); $m$ is the mass of dry soil, 40$\times$10$^{-3}$ kg; $T$ is the air temperature during incubation, 25$^\circ$C; 24 is the 24 hours of one day, and used for the translation gaseous emission rate of per hour to per day.

The dual isotope and isotopocules of N$_2$O ($\delta^{15}$N$^{\text{bulk}}$, $\delta^{15}$N$^{\alpha}$ and $\delta^{18}$O) were measured by the IRMS with Delta V Plus-Precon, with the precision of 0.5‰ for $\delta^{15}$N$^{\text{bulk}}$, 0.9‰ for $\delta^{15}$N$^{\alpha}$, and 0.6‰ for $\delta^{18}$O.

$$\delta X = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1\right) \times 1000$$

(2)

where X is $^{15}$N$^{\text{bulk}}$, $^{15}$N$^{\alpha}$ or $^{18}$O. The standard reference gases were used for calibration.

$$\delta^{15}N^{\text{bulk}} = (\delta^{15}N^{\alpha} + \delta^{15}N^{\beta})/2$$

(3)

$$\text{SP} = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta}$$

(4)

where $\alpha$ and $\beta$ is the position of central and terminal N atoms, respectively, SP is the difference of $\delta^{15}$N between the central and terminal N position.

2.4. Soil analysis

Soil pH was measured in suspension of soil (1:2.5, soil: 0.01 M CaCl$_2$) using a Thermo Orion
pH meter (Mettler Toledo, China pH). Soil water content was determined after weight loss for 24 h drying in 105°C. The NO$_3^-$-N and NH$_4^+$-N content were determined by extracting soil with 2 M KCl solution (1:5, soil: KCl). The extract was analyzed for the concentration of NO$_3^-$-N and NH$_4^+$-N using a Lachat Flow-Injection Auto-analyzer (Lachat Instruments, USA).

2.5. Identification of N$_2$O production processes

In this study, the contribution of fungal denitrification ($f_{BD}$) and bacterial denitrification ($f_{FD}$; $f_{BD} = 1 - f_{FD}$) to total N$_2$O production were calculated by the two end-member mixing model (equation (5) and (6); Sutka et al., 2003) using two different approaches. In this model, SP values are used to investigate the respective contribution of the two pathways, one from lower SP group and one from higher SP group, which are selected as the end members. Four cases should be considered depending on this model: (i) bacterial nitrification and bacterial denitrification, (ii) bacterial nitrification and nitrifier denitrification, (iii) fungal denitrification and bacterial denitrification, (iv) fungal denitrification and nitrifier denitrification. This experiment was conducted under anaerobic environment, for which denitrification was the only process in soil. Therefore, the two end-members are fungal denitrification ($f_{FD}$) and bacterial denitrification ($f_{BD}$). Isotopic endmembers for SP values of produced N$_2$O were assumed to be 35.5‰ ($SP_{FD}$) for fungal denitrification and -5‰ ($SP_{BD}$) for bacterial denitrification (Sutka et al., 2008; Rohe et al., 2014a).

$$f_{FD} = \frac{SP - SP_{BD}}{SP_{FD} - SP_{BD}}$$ (5)

$$f_{BD} = 1 - f_{FD}$$ (6)

The reduction of N$_2$O to N$_2$ can cause an enrichment in SP values, thus moving the values closer to those of fungal denitrification. If N$_2$O reduction process is not blocked, SP values for fungal and bacterial denitrification could be confounded.
In this study, two methods (i.e. acetylene inhibition approach and mixing approach) were chosen to eliminate this confusion.

2.5.1. Acetylene inhibition method

In this method, the reduction of N\textsubscript{2}O to N\textsubscript{2} can be inhibited by the addition of 10 kPa (10 vol%) C\textsubscript{2}H\textsubscript{2}. Therefore, N\textsubscript{2}O emissions are the total (N\textsubscript{2}O+N\textsubscript{2}) emissions for treatments with C\textsubscript{2}H\textsubscript{2} and the net N\textsubscript{2}O production after the reduction of N\textsubscript{2}O for treatments free of C\textsubscript{2}H\textsubscript{2}. The SP values obtained from groups with 10 kPa C\textsubscript{2}H\textsubscript{2} which are not influenced by N\textsubscript{2}O reduction are able to distinguish fungal and bacterial denitrification calculated by the two end-member mixing model (equation (5) and (6); Sutka et al., 2003). The SP values derived from bacterial denitrification as the lower end-member and from fungal denitrification as the higher one. Results derived from this method are the measured results in Figure 4.

2.5.2. Isotopocule mixing approach

To distinguish the fraction of N\textsubscript{2}O derived from fungal denitrification (f\textsubscript{FD}) or bacterial denitrification (f\textsubscript{BD}), we used an isotopocule mixing approach adopted by others (Zou et al., 2014; Deppe et al., 2017; Lewicka-Szczebak et al., 2017; Congreves et al., 2019) in treatments free of C\textsubscript{2}H\textsubscript{2}, that showed the calculation of f\textsubscript{FD} and f\textsubscript{BD} with this approach. The isotopocule map consists of SP and δ\textsuperscript{18}O values of gas samples (Fig. 1) whose end-member areas are given for fungal denitrification and bacterial denitrification. We used soil-specific endmembers derived from average literature in previous work to perform the isotopocule map. The values are characteristic for soil incubation and not influenced by N\textsubscript{2}O reduction (bacterial denitrification: SP\textsubscript{BD} -10‰ to 0‰ (Toyoda et al., 2005; Sutka et al., 2006); δ\textsuperscript{18}O\textsubscript{BD} : +10‰ to +20‰ (Snider et al., 2013; Lewicka-Szczebak et al., 2014); fungal denitrification: SP\textsubscript{FD} : +34‰ to +37‰, δ\textsuperscript{18}O\textsubscript{FD} : +30‰ to +40‰ (Sutka et al., 2003)).
et al., 2008; Rohe et al., 2014a)). The mean mixing line connects average values for both endmembers.

The influence of N\textsubscript{2}O reduction on SP could not be neglected for source partitioning. The isotope effect caused by this reduction was calculated by the reduction and mixing line intercept approach (Deppe et al., 2017; Lewicka-Szczebak et al., 2017; Congreves et al., 2019). The reduction line used the average of the reported reduction slope (0.35; Ostrom et al., 2007; Jinuntuya-Nortman et al., 2008; Well and Flessa, 2009; Lewicka-Szczebak et al., 2015) and SP and $\delta^{18}\text{O}$ values of each gas sample as origin of the reduction line. The point of the intersection between the mean mixing line and the reduction line gave the estimated initial isotope values (SP\textsubscript{0} and $\delta^{18}\text{O}_{0}$) of produced N\textsubscript{2}O before reducing to N\textsubscript{2}. If the SP\textsubscript{0} value was lower than the measured SP value of N\textsubscript{2}O, the calculated value (SP\textsubscript{0}) was used, since N\textsubscript{2}O reduction could not be negligible.

From the calculated SP\textsubscript{0} values (or SP), the fraction of N\textsubscript{2}O derived from fungal denitrification ($f_{FD}$) or bacterial denitrification ($f_{BD}$) can be estimated using the two end-member mixing model.

Results derived from this method are the calculated results (calculated $f_{FD}$; Figure 4).

2.6. Statistical analysis

Statistical analysis was done using SPSS version 19.0 and figures were drawn using SigmaPlot (version 14.0) and the software R (version 3.5.1). Tukey’s HSD post-hoc test was used to reveal significant pairwise differences among different treatments. Pearson's correlation was used to analysis the correlation between gas flux (N\textsubscript{2}O, N\textsubscript{2}) and isotopocule characteristics of N\textsubscript{2}O ($\delta^{15}\text{N}_{\text{bulk}}$, $\delta^{18}\text{O}$, SP) with $p < 0.05$ used as the criterion for statistical significance.

3. Results
3.1. Effect of soil acidification on inorganic nitrogen

To obtain different pH levels, soils at natural pH (pH 8.7) were preincubated for a period of two weeks after acidification. Data for all the pH groups are presented in Table 1. With H₂SO₄ added, significant differences among pH groups were observed and varied from 0.9 to 2.5 units (p < 0.0001). Moreover, there were also obvious changes in both NH₄⁺ and NO₃⁻ concentrations. The average NH₄⁺ content at pH 6.2 was significantly different from that in natural soil (pH 8.7; p < 0.01) and increased 2.1 times by the end of preincubation (Table 1) in conjunction with a slight decrease of the NO₃⁻N content (5.9 mg N kg⁻¹).

3.2. N₂O and N₂ fluxes

Compared to the groups free of C₂H₂, the addition of C₂H₂ significantly increased N₂O production in all treatments (p < 0.05; Fig. 2). With substrate and carbon source consumption, both the N₂O production and reduction rates generally deceased with incubation progress in all groups (Fig. 2). Soil pH strongly controls the N₂O production and reduction rates of denitrification. Both soil N₂O and (N₂O+N₂) cumulative emissions in acidic soils increased significantly compared to soils of natural pH (p < 0.0001). No significant difference was observed between soils of pH 7.1 and pH 8.7, but higher values occurred in soils of pH 7.1.

The total N₂O production determined in soils with C₂H₂ was significantly larger (p < 0.001) in the presence of glucose, varying among 5.85 mg N kg⁻¹ d⁻¹ for pH 8.7, 8.89 mg N kg⁻¹ d⁻¹ for pH 7.1 and 12.67 mg N kg⁻¹ d⁻¹ for pH 6.2, compared to groups in the absence of glucose, varying among 0.89 mg N kg⁻¹ d⁻¹ for pH 8.7, 2.14 mg N kg⁻¹ d⁻¹ for pH 7.1 and 2.93 mg N kg⁻¹ d⁻¹ for pH 6.2 (Fig. 2; Table 2). The differences in N₂O production among soils with different pH values were all statistically significant under the same glucose conditions (p < 0.001).
Net N$_2$O production in the absence of C$_2$H$_2$ varied between 0.76 and 2.35 mg N kg$^{-1}$ d$^{-1}$ (Fig. 2; Table 2), and was significantly increased after adding glucose ($p < 0.001$) with a variation between 2.76 and 8.44 mg N kg$^{-1}$ d$^{-1}$. The N$_2$O reduction rate, calculated based on the comparison between the groups with and without C$_2$H$_2$, varied from 0.12 to 4.23 mg N kg$^{-1}$ d$^{-1}$ and was significantly higher in the presence of glucose, showing a higher time-weighted mean value for a lower pH, although higher values among all the pH groups were not observed in the first 3 days. Significant differences of N$_2$O reduction rate between pH 6.2 and 7.1 and between pH 6.2 and pH 8.7 were observed in the absence of glucose ($p < 0.05$), but the addition of glucose decreased the differences ($p = 0.240$ between pH 6.2 and 7.1; $p = 0.054$ between pH 6.2 and pH 8.7). This C-effect was also reflected in the production ratio (N$_2$O/(N$_2$+N$_2$O)), which was lower for all pH levels in the presence of glucose varying from 0.47 for pH 8.7 to 0.67 for pH 6.2 compared to the groups free of glucose varying from 0.80 for pH 6.2 to 0.88 for pH 8.7.

3.3. Isotopocules of N$_2$O

Similar to N$_2$O production, the $\delta^{15}$N$^{\text{bulk}}$ values generally decreased with incubation time in all treatments (Fig. 3) and were depleted compared to $\delta^{15}$N-NO$_3^-$ at the beginning of the incubation (the initial values of $\delta^{15}$N-NO$_3^-$ of pH 8.7, pH 7.1 and pH 6.7 were -2.39‰, -2.18‰ and -3.63‰, respectively; $\delta^{15}$N-NO$_3^-$ and $\delta^{18}$O-NO$_3^-$ values were measured by bacterial denitrification method), ranging from -55.49‰ to -27.67‰ (Table 2). Significant differences were found among different soil pH values ($P < 0.001$). In the absence of C$_2$H$_2$, the $\delta^{15}$N$^{\text{bulk}}$ values of natural soils were significantly higher than that in the presence of C$_2$H$_2$ ($P < 0.05$), and this statistical significance was not observed in soils after acidification. The $\delta^{15}$N$^{\text{bulk}}$ of produced N$_2$O in soils with glucose varied between -43.72‰ and -27.67‰ and was significantly higher in more acidic soils ($p < 0.001$).
The $\delta^{18}O$ values showed no evident changes for pH 6.2 with incubation time, decreased in the first three days and then increased for the other two soils (Fig. 3). The $\delta^{18}O$ values of produced N$_2$O in soils with C$_2$H$_2$ varied from 16.83‰ to 20.64‰ (Table 2) and were significantly decreased ($p < 0.001$) than that free of C$_2$H$_2$ and were considerably depleted compared to $\delta^{18}O$-NO$_3$ at the beginning of the incubation (the initial $\delta^{18}O$-NO$_3$ values of pH 8.7, pH 7.1 and pH 6.7 were 49.43‰, 50.16‰ and 51.57‰, respectively); the $\delta^{18}O$ value was approximately 1‰ higher for pH 6.2 than for natural soils ($p = 0.051$). These differences clearly increased after the addition of glucose ($p < 0.001$). Contrary to the groups with C$_2$H$_2$, $\delta^{18}O$ values in soil with higher pH values were larger than that lower ones in the absence of C$_2$H$_2$. In general, the addition of glucose significantly increased $\delta^{18}O$ values in the absence of C$_2$H$_2$, and this difference was weakened in the presence of C$_2$H$_2$.

The SP values in groups with C$_2$H$_2$ varied between 4.90‰ and 21.73‰, with a wider variation for a lower pH (Table 2; Fig. 3), and were higher for groups free of glucose compared to groups with glucose in the early stage of the incubation. The highest SP values in groups free of glucose were observed before day 5, with a general decline after day 5. Conversely, the lowest SP values in the presence of glucose were observed before day 5 except for pH 7.1, which was observed on day 9. The addition of glucose increased the SP values on and after day 5 compared to the groups free of glucose, and the differences between groups with and without glucose were higher for a lower pH, varying among 2.89‰ for pH 8.7, 3.66‰ for pH 7.1 and 7.33‰ for pH 6.2. The higher average SP values were followed by lower pH conditions.

### 3.4. The relationship between isotopocules and gaseous nitrogen fluxes

Pearson correlation analysis were used to identify isotopocules of N$_2$O linked to N$_2$O and N$_2$ flux (Table 3). For all groups, the $\delta^{15}$N$_{\text{bulk}}$, $\delta^{18}$O and SP values of N$_2$O were all significantly
correlated with N₂O flux, with positive correlations between $\delta^{15}\text{N}_{\text{bulk}}$ and N₂O and between SP and N₂O ($p < 0.01$) and negative correlations between $\delta^{18}\text{O}$ and N₂O ($p < 0.01$) in the absence of glucose. Additionally, a higher correlation coefficient was found between $\delta^{15}\text{N}_{\text{bulk}}$ and N₂O at pH 8.7, a negative correlation was found between SP and N₂O in all soils, and a positive correlation was found between $\delta^{18}\text{O}$ and N₂O at pH 6.2 after adding glucose. In the absence of glucose, no significant correlation was observed between N₂ flux and isotopocules of N₂O at pH 8.7 and pH 7.1 except for $\delta^{18}\text{O}$ and N₂ at pH 7.1. Positive correlations were found between $\delta^{15}\text{N}_{\text{bulk}}$ and N₂ and between SP and N₂ ($p < 0.01$), and a negative correlation was found between $\delta^{18}\text{O}$ and N₂ at pH 6.2 that was consistent with the groups after the addition of glucose. For soils at pH 8.7 and pH 7.1, positive correlations were observed between $\delta^{15}\text{N}_{\text{bulk}}$ and N₂, followed by negative correlations between $\delta^{18}\text{O}$ and N₂ and between SP and N₂.

3.5. Source partitioning

The mapping approach based on $\delta^{18}\text{O}/\text{SP}$ plots for soil-derived N₂O was used for source partitioning (Fig. 1). $\delta^{18}\text{O}$ values were highest in natural soils with glucose, coupled with the SP values. These individuals were outside the vector that is interpretable by mixing N₂O from fungal denitrification and bacterial denitrification as source processes. This shift to higher values could be interpreted by the incorporation of mixed production from fungal and bacterial denitrification and the partial reduction of N₂O to N₂ during bacterial denitrification. Such a shift was only observed in individuals from natural soil, where no acid was added. Individuals in the absence of glucose were more concentrated, with a narrow range of 16.53‰ to 28.11‰ for $\delta^{18}\text{O}$ and of 7.61‰ to 22.93‰ for SP, compared to groups after adding glucose (18.48‰ to 34.28‰ for $\delta^{18}\text{O}$ and 6.80‰ to 25.73‰ for SP).
Based on the endmember signatures, $f_{FD}$ calculated from Equation (5) ranged from 23% to 76% (Table S1). The highest SP values were measured in individuals at pH 6.2 with glucose, and closer SP values were observed from samples in groups without carbon sources, especially natural soils. Using the mean values for the SP and δ$^{18}$O values of fungal denitrification and bacterial denitrification (i.e., center values of endmember areas in Fig. 1), the contribution of fungal denitrification to N$_2$O production was 45% for pH 8.7, 48% for pH 7.1 and 49% for pH 6.2 and was lower for pH 8.7 (37%) and pH 7.1 (43%) and higher for pH 6.2 (57%) after adding carbon sources. From the calculated SP (or SP$_0$) values estimated by the isotopocule map (calculated SP values) and the measured SP values directly measured from the groups with C$_2$H$_2$ (measured SP values), the contribution of fungal denitrification-derived N$_2$O based on the above two approaches was calculated by using the end-members mixing model (the calculated and measured $f_{FD}$ were estimated by the above calculated and measured SP values, respectively). Hence, we compared the calculated and measured $f_{FD}$ and found good agreement between them with a significant fit to the 1:1 line (Fig. 4), especially for the group in the absence of glucose ($R^2 = 0.81$). The mean absolute difference between the measured and calculated $f_{FD}$ was 0.11 in the absence of glucose and 0.04 in the presence of glucose. The mean relative error in the determination of the contribution of fungal denitrification to N$_2$O production was 28% in the absence of glucose and 9% in the presence of glucose. A better fit was not obtained when the calculated and measured $f_{FD}$ for individual pH values were determined and applied separately. From the correlation tested among different pH levels, we found that only for acidified soils free of glucose ($R^2 = 0.77$ for pH 6.2; $R^2 = 0.43$ for pH 7.1) were the measured and calculated $f_{FD}$ correlated. In the presence of glucose, although no correlation was observed for individual pH values, a slightly significant fit to the 1:1 line was obtained when soils of different
pH values were determined and applied together ($R^2 = 0.40$), which indicated that these $f_{iD}$ values were associated with the incubation conditions.

4. Discussion

It is well established that soil pH is a main controlling factor for N$_2$O production/reduction through regulating N mineralization, nitrification and denitrification processes (Xiao et al., 2013; Qu et al., 2014), especially in anaerobic environments. Modifying soil pH on site is difficult due to the high buffering capacity of most soils. Nevertheless, soils differing in natural pH also have differences in many other properties. Hence, the influence of soil pH is often conducted by modulating the pH by liming, which requires repeated lime applications and is a gradual and typical method that can take years (Adams and Adams, 1983; Nicol et al., 2008). In this study, natural soil (pH 8.7) was significantly modified by the application of H$_2$SO$_4$ with a short-term preincubation. This process was carried out under highly controlled conditions of pH and carbon sources (glucose), adopted homogenized and sieved soil free of plants and animals, imposed conditions optimal for denitrification in the anaerobic incubation, and examined N$_2$O and (N$_2$O+N$_2$) emissions in response to pH amendments, and distinguished the fraction of N$_2$O derived from fungal or bacterial denitrification by using an isotopocule mixing approach.

4.1. Effect of soil acidification on inorganic nitrogen

Acidification of soil was accompanied by changes in other soil properties (Table 1) resulting from changes in soil function. Therefore, the significantly higher NH$_4^+$ content ($p < 0.01$) coupled with the decrease in NO$_3^-$ content in acidic soil during soil preincubation was partially associated with a process of producing NH$_4^+$, i.e., DNRA. DNRA is the process by which NO$_3^-$ can be directly
and rapidly converted into NH$_4^+$ in soils under similar conditions (i.e., low redox potential, available NO$_3^-$ and labile C) for denitrification (Zumft, 1997; Silver et al., 2001). In soils of pH 6.2, the addition of glucose made a gradual promotion of NH$_4^+$ contents and changed the reduction of NO$_3^-$, moving this process closer to denitrification and farther from DNRA compared to the groups free of glucose. This influence caused by the addition of glucose may be due to the promotion of the N$_2$O reduction process (Weier et al., 1993; Azam et al., 2002). Soil acidification resulted in this effect, which did not occur in neutral and alkaline soils.

4.2. N$_2$O and N$_2$ fluxes

The results showed that soil acidification was associated with both increasing denitrification rates and the N$_2$O/(N$_2$O+N$_2$) product ratios in the presence of glucose, in good agreement with previous findings (Scholefield et al., 1997; Hanaki et al., 1992). The influence of pH may be indirect – the availability of organic carbon and nitrogen mineralization and the microbial community are regulated by the shift in soil pH, further leading to changes in the denitrifying component. A previous study reported that the optimum pH for bacterial denitrification was from 7.0 to 8.0 (Sahrawat and Keeney, 1986). Therefore, slightly acidic soil may have a partial blockage effect on bacterial denitrifiers, whose significantly higher N$_2$O production may be attributed to fungal denitrification since acidic soil conditions favor the existence of fungal denitrifiers (Laughlin and Stevens, 2002; Chen et al., 2015). Moreover, the activity of NO reductase for fungal denitrifiers (P450nor) has been shown to be 5-fold higher than that of bacterial denitrifiers (Morozkina and Kurakov, 2007); hence, it can be assumed that fungal denitrifiers are capable of higher N$_2$O production with more substrates due to the partial blockage of bacterial denitrifiers by acidic environments. Furthermore, a lower soil pH increased the N$_2$O/(N$_2$O+N$_2$) product ratio due to the lower tolerance of N$_2$O reductase to
acidic soil than the other denitrification reductases (Knowles, 1982), also leading to the promotion of N₂O release (Šimek and Cooper, 2002; Saggar et al., 2013).

4.3. Isotopocules of N₂O

The isotopocule characteristics of produced N₂O reflect multiple sources that are influenced by microbial mixing processes, the extent of the N₂O reduction process and the variation of the relevant isotope effects (Lewicka-Szczebak et al., 2015). The trends of the isotopocule characteristics of produced N₂O were associated with those of the N₂O and (N₂O+N₂) product rates. The results from the isotopocule data (Table 2, 3 and Fig. 3) showed that the addition of glucose promoted the reduction of N₂O to N₂ and the enrichment of ¹⁵N and ¹⁸O of residual N₂O (Weier et al., 1993; Azam et al., 2002). The complicated oxygen isotope fractionation during microbial N₂O production by denitrification has led δ¹⁸O to be a poor tracer of microbial origins in numerous previous studies (Kool et al., 2007; Ostrom et al., 2007; Kool et al., 2009). Nonetheless, a combination of δ¹⁸O and SP has been used to correct N₂O reduction in this study, indicating a viable approach to resolve the confusion for N₂O source partitioning (Deppe et al., 2017; Lewicka-Szczebak et al., 2017).

Lower δ¹⁸O values in the absence of C₂H₂ were found in acidified soils, which were caused by the reduced N₂O reduction resulting from the inhibition of the N₂O reduction by lower pH (Ostrom et al., 2007), consistent with the product ratio of N₂O/(N₂O+N₂). In addition, the lower δ¹⁸O values could also be attributed to the greater O-exchange for lower pH since soil acidification may have a partial blockage effect on bacterial denitrification but favors fungal denitrification (Sahrawat and Keeney, 1986; Laughlin and Stevens, 2002; Chen et al., 2015), during which increased O exchange may occur than in bacterial denitrification (Rohe et al., 2017). Higher δ¹⁸O values in the presence
of C$_2$H$_2$ were also found after soil acidification, which could be interpreted as increased N$_2$O reduction leading to a greater enrichment of heavy isotopes of $^{15}$N and $^{18}$O. Thus, the inhibition of N$_2$O reduction by C$_2$H$_2$ has a larger negative effect on the $\delta^{15}$N$_{bulk}$ and $\delta^{18}$O values of the produced N$_2$O in higher pH soils with higher N$_2$O reduction. In addition, other co-occurring microbial processes, i.e., DNRA and anammox, may also be involved in the above process.

For each pH level, generally, the mean SP values in the absence of glucose decreased compared to the groups with glucose. The results in this study visually showed that there was a transition between fungal denitrification-derived and bacterial denitrification-derived N$_2$O release during the whole incubation. Relative to the whole incubation, higher SP values were generally observed in the first 3 days in the absence of glucose (18.91‰ to 21.55‰ for pH 6.2, 19.94‰ to 22.93‰ for pH 7.1, 12.37‰ to 17.04‰ for pH 8.7; Fig. 3). These values come closer to the expected SP values derived from fungal denitrification (Sutka et al., 2008; Rohe et al., 2014a), and a higher contribution to N$_2$O production was also observed (45% to 66% for pH 6.2, 45% to 54% for pH 7.1, 36% to 48% for pH 8.7, derived from the measured $f_{FD}$; Fig. 4). SP continued to decrease for the first 5 days at all pH levels, which is in line with the N$_2$O product rates, suggesting the transition of moving N$_2$O production processes closer to bacterial denitrification and farther from fungal denitrification and that the higher N$_2$O release in the first 5 days was associated with fungal denitrification, especially for the two lower pH values. The higher correlation coefficient between the N$_2$O product rates and SP values at the two lower pH values ($r = 0.82$ for pH 7.1, $r = 0.79$ for pH 6.2) also demonstrated the above since soil acidification may favor the existence of fungal denitrifiers but partially inhibits bacterial denitrifiers (Sahrawat and Keeney, 1986; Laughlin and Stevens, 2002; Chen et al., 2015).

The SP values in natural soils were generally lower than 15‰, indicating a smaller contribution of
fungal denitrification (Zou et al., 2014), and Fig. 4 shows the lower contribution derived from fungal
denitrifiers for soils of pH 8.7 (36% for pH 8.7) than the two other soils (42% for pH 7.1, 43% for
pH 6.2).

4.4. Source partitioning

The combination of δ¹⁸O and SP has only recently been applied for correcting N₂O reduction
(Deppe et al., 2017; Lewicka-Szczebak et al., 2017), indicating a viable approach to resolve the
confusion regarding N₂O source partitioning.

This study was conducted at a relatively higher soil moisture favoring denitrification; therefore,
it is undisputable to consider oxygen isotope exchange with soil water for the analysis of δ¹⁸O of
N₂O (Lewicka-Szczebak et al., 2016). Rohe et al. (2017) reported that the extent of oxygen exchange
varied between 83% and 94% for fungal denitrification and between 17% and 73% for bacterial
denitrification under the conditions tested. The δ¹⁸O values used for the endmember values of fungal
denitrification (Fig. 1) were derived from pure culture studies (Sutka et al., 2008; Rohe et al., 2014a),
which excluded extreme values from pure cultures that were not considered representative of soil-
produced N₂O since they showed more variable oxygen exchange than that of soil incubations. The
lower δ¹⁸O values on the left side of the mixing line might be interpreted as the δ¹⁸O (-11.52‰)
values of H₂O used in this study being lower than in studies applied to determine endmember values
(-8.9‰; (Rohe et al., 2014b)). Additionally, the pure culture used by Rohe et al. (2014) was
conducted at pH 7.4, which is slightly higher than the two soils with lower pH values (pH 6.2 and
pH 7.1) used in this study. Although fungi can contribute to N₂O release at low pH (Yanai et al.,
2007), some fungi prefer neutral to weakly alkaline environments for N₂O release (Burth and Ottow,
1983; Shoun et al., 1992). It was assumed that the slight pH difference between the two studies may
also be one of the reasons that some points with lower $\delta^{18}$O and higher SP values were outside the vector. Soil acidification resulted in increasing N$_2$O release associated with fungi-mediated denitrification, leading to higher SP values derived from fungal denitrification (Maeda et al., 2015). The isotopocule characteristics (SP, $\delta^{15}$N$_{\text{bulk}}$, $\delta^{18}$O) for the analysis of the produced N$_2$O are informative. The isotopocule map used for the correction of N$_2$O reduction based on SP/$\delta^{18}$O somehow made a difference for N$_2$O source partitioning, especially in the absence of glucose. The validation shows a certain agreement between the calculated and measured $f_{FD}$, indicating that the SP and $\delta^{18}$O values were sufficient within soil incubation experiments for calculating $f_{FD}$ using the two end-member mixing model. Although a slightly weaker fit was observed in the presence of glucose ($R^2 = 0.40$), there was also some significance of the calculated $f_{FD}$ relative to the measured $f_{FD}$. A weaker fit to the 1:1 line in the presence of glucose had a lower absolute error than the group free of glucose. Summarizing the results of the validation between the calculated and measured $f_{FD}$, we can conclude that this research can provide certain references for the correction of N$_2$O reduction and N$_2$O source partitioning, although there is some bias between the calculated and measured $f_{FD}$.

5. Conclusion

This study observed an accordant variation in soil pH with the addition of KNO$_3$ comparable to field conditions and confirmed the hypothesis in a field study that fungal denitrification greatly contributes to N$_2$O production.

The facilitation of soil acidification by fungal denitrification-derived N$_2$O and N$_2$O release from soil has been confirmed. In addition, acidified soils have a negative effect on the reduction of N$_2$O to N$_2$. Although the addition of glucose weakened this negative effect, a higher N$_2$O/(N$_2$O+N$_2$)
ratio occurred in more acidic soils. Therefore, the addition of glucose promoted both N₂O reduction and production. The mapping approach provided rational results for correcting N₂O reduction in comparison with the acetylene inhibition method and indicated a reasonably large contribution of fungal denitrification to total N₂O production in acidified soils. However, there is a gap between the mapping approach and the C₂H₂ inhibition method for correcting N₂O reduction among soils of different pH groups. Therefore, the bias caused and the extent to which such an approach may be suitable for various incubation conditions and field environments require further confirmation. Despite all this, there is also some significance for the mapping approach in correcting N₂O reduction since it offsets the deficiency of the acetylene inhibition method in field research, as well as in quantifying and qualifying N₂O source partitioning derived from fungal and bacterial denitrification.

Declaration of competing interest

The authors declare that they have no conflicts of interest to influence this work reported in this paper.

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