N2O Fluxes and Related Processes of Denitrification in Acidified Soil

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N_2O 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 N_2O fluxes via several pathways, especially anaerobic denitrification. Anaerobic denitrification is regarded as an important microbial process for N_2O production in soils with a low O_2 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 N_2O source partitioning with the addition of KNO_3 and glucose. Natural abundance isotope techniques and gas inhibitor technique were applied to analyze the N_2O flux derived from fungal denitrification and bacterial denitrification and its isotopocule characteristics emitted from soils after the addition of NO_3^- and glucose. A mapping approach was used to obtain further insight into the N_2O production processes. Our findings confirmed that soil pH strongly controlled the N_2O production and reduction rates of denitrification. Soil acidification significantly increased N_2O 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 N_2O to N_2. The addition of carbon sources promoted complete denitrification. We assumed a higher contribution of fungal denitrification to N_2O production compared to total N_2O emission associated with acidified soil. A promotion of the contribution of fungal denitrification-derived N_2O 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 N_2O 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 N_2O reduction compared with the acetylene inhibition method. The results calculated by both methods indicated a reasonably large contribution of fungal denitrification to total N_2O production in acidified soils.

Keywords: N_2O isotopocules; pH; fungal denitrification; glucose; nitrate fertilization

1. Introduction

As a trace gas in the atmosphere, N_2O 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 N_2O emissions, of which denitrification is the major source (Senbayram et al., 2015).

Denitrification is stepwise anaerobic reduction of NO_3^- to N_2, with NO_2^-, NO and N_2O as intermediates during the microbial-mediated process (Devol, 2015). Denitrification-derived N_2O

and N_2 fluxes can result in a considerable loss of N, while the reduction process of N_2O decreases N_2O fluxes (Butterbach-Bahl et al., 2013). Therefore, the net N_2O flux during the denitrification processes is the equilibrium of N_2O production and consumption in soil. The production ratio of $N_2O/(N_2O+N_2)$ can be used as an adequate measure to understand information about both N_2O 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, N_2O 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 N_2O to N_2, and may play a more important role in N_2O production than bacteria under acidic conditions; therefore, N_2O 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 $N_2O/(N_2O+N_2)$ 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 N_2O production, with each having advantages and challenges (Zou et al., 2014; Lewicka-Szczebak et al., 2017). Inhibition of N_2O reduction with 10 vol% C_2H_2 (the acetylene inhibition method) based on the comparison of N_2O fluxes with and without acetylene application is used to quantify the reduction of N_2O to N_2 since N_2 quantification is challenging (Yanai et al., 2008; Bouwman et al., 2013; Saggar et al., 2013). Depletion of NO both in the presence of C_2H_2 and O_2 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 N_2O and N_2 fluxes and their production processes in laboratory experiments. N_2O isotopic characteristics include ^{18}O, $^{15}N^{bulk}$, and ^{15}N site preference (SP) within the linear N_2O molecule. SP, the difference between $^{15}N^{\alpha}$ and $^{15}N^{\beta}$, which represent the central N position (α) and the peripheral N position (β), respectively (Toyoda and Yoshida, 1999), is a promising alternative to assess N_2O 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 N_2O emitted since N_2O reduction leads to an enrichment in ^{15}N, particularly at the α position, and ^{18}O, 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 ^{18}O also varies among N_2O

95 emission routes. In summary, $\delta^{18}O$ together with SP may help to further distinguish N_2O pathways
96 (Ostrom et al., 2007; Snider et al., 2013; Köster et al., 2015) and provide insight into the dynamics
97 of N_2O reduction (Well and Flessa, 2009).

98 In our previous field study, the application of KNO_3 followed by lower soil pH, and led to a
99 higher cabbage yield and lower N_2O fluxes compared to other inorganic fertilizers (Lin, 2019),
100 causing less nitrogen loss and environmental pollution. We assumed there existed appreciable
101 contribution of fungal denitrification-derived N_2O because of the higher SP values of produced N_2O.
102 For the above, we report an anaerobic incubation experiment to determine the impact of soil
103 acidification on N_2O fluxes derived from fungal denitrification and bacterial denitrification with the
104 addition of KNO_3 and carbon sources. Besides, a method used for correcting N_2O reduction will be
105 tested by comparing with the acetylene inhibition method. Moreover, this study will give insight
106 into the dynamics of N_2O production and reduction processes and provide a theoretical basis for
107 alleviating N_2O emissions by modulating agricultural management activities.

108

109 **2. Materials and methods**

110 *2.1. Soil collection and characterization*

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

117 15.40 g·kg^{-1}; total N, 1.10 g·kg^{-1}; bulk density, 1.48 g·cm^{-3}.

118 *2.2. Treatments and experimental set-up*

119 7 kg air dried soil (oven dry basis equivalent) was adjusted by adding different volume of 1 M
120 H_2SO_4 (two low pH groups) or water (control group) at 50% WFPS at 25 ℃ for 14 days to initiate
121 and stabilize the microbial activity and soil pH prior to imposing treatments. To obtain a
122 homogenous distribution and equal content of substrates and soil moisture tension in all soil samples,
123 soil was mixed evenly with KNO_3 (100 mg N kg^{-1}) and glucose (0 and 300 mg C kg^{-1}) which were
124 added in particles and then was homogenized with distilled water to their target WFPS (70% WFPS)
125 using a shovel prior to packed into culture flasks.

126 On each sampling day, equivalent of 40 g dry soil was packed into each 0.28 L culture flask
127 with a bulk density of 1.2 g cm^{-3} and a number of 48 flasks were covered with airtight rubber seals
128 and air in flasks was evaluated after which He was injected into the evaluated flasks to create
129 anaerobic conditions. 30 mL C_2H_2 was injected into selected flasks by replacing corresponding
130 volume of He, resulted in 10 kPa C_2H_2 in the headspace. The flasks were incubated at 25±1 ℃ for
131 2 h in the dark. Gas samples were collected each incubation day (day 1, 2, 3, 5, 7, 9, 12, 15) in pre-
132 evacuated 20-ml vials after 2 hours incubation after which destructive sampling was done to collect
133 20 g soils from the flask for soil properties analysis. Soil samples were incubated without seals and
134 were covered with plastic film to keep soil moisture on non-sampling day. Soil moisture was kept
135 constant at 70% WFPS by weighing the flasks and adding distilled water at alternative days. Each
136 treatment contained four replicates. A total of 384 flasks were used.

137 *2.3. Gas analysis*

138 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_2O have a linear correlation, the N_2O concentration could be determined basing on the peak area m/z 44 with known isotopic ratios and concentration of standard N_2O gas samples (Air Liquide America, Specialty Gases LLC). N_2O emission rates were calculated as follows:

$$f_{N_2O} = \frac{\rho \times V \times \Delta C}{m \times \Delta t \times 1000} \times \frac{273}{273+T} \times 24 \quad (1)$$

where f is the emission rate of N_2O, μg kg^{-1} h^{-1}; ρ is the N_2O 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_2O concentration per unit time (nL L^{-1} h^{-1}); m is the mass of dry soil, 40×10^{-3} kg; T is the air temperature during incubation, 25°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_2O ($\delta^{15}N^{bulk}$, $\delta^{15}N^{\alpha}$ and $\delta^{18}O$) were measured by the IRMS with Delta V Plus-Precon, with the precision of 0.5‰ for $\delta^{15}N^{bulk}$, 0.9‰ for $\delta^{15}N^{\alpha}$, and 0.6‰ for $\delta^{18}O$.

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

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

$$\delta^{15}N^{bulk} = (\delta^{15}N^{\alpha} + \delta^{15}N^{\beta})/2 \quad (3)$$

$$SP = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta} \quad (4)$$

where α and β 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

161 pH meter (Mettler Toledo, China pH). Soil water content was determined after weight loss for 24 h
162 drying in 105°C. The NO_3^--N and NH_4^+-N content were determined by extracting soil with 2 M KCl
163 solution (1:5, soil: KCl). The extract was analyzed for the concentration of NO_3^--N and NH_4^+-N using
164 a Lachat Flow-Injection Auto-analyzer (Lachat Instruments, USA).

165 *2.5. Identification of N_2O production processes*

166 In this study, the contribution of fungal denitrification (f_{BD}) and bacterial denitrification (f_{FD};
167 f_{BD} =1 - f_{FD}) to total N_2O production were calculated by the two end-member mixing model
168 (equation (5) and (6); Sutka et al., 2003) using two different approaches. In this model, SP values
169 are used to investigate the respective contribution of the two pathways, one from lower SP group
170 and one from higher SP group, which are selected as the end members. Four cases should be
171 considered depending on this model: (i) bacterial nitrification and bacterial denitrification, (ii)
172 bacterial nitrification and nitrifier denitrification, (iii) fungal denitrification and bacterial
173 denitrification, (iv) fungal denitrification and nitrifier denitrification. This experiment was
174 conducted under anaerobic environment, for which denitrification was the only process in soil.
175 Therefore, the two end-members are fungal denitrification (f_{FD}) and bacterial denitrification (f_{BD}).
176 Isotopic endmembers for SP values of produced N_2O were assumed to be 35.5‰ (SP_{FD}) for fungal
177 denitrification and -5‰ (SP_{BD}) for bacterial denitrification (Sutka et al., 2008; Rohe et al., 2014a).

178 $$f_{FD}=\frac{SP-SP_{BD}}{SP_{FD}-SP_{BD}} \quad (5)$$

179 $$f_{BD}=1-f_{FD} \quad (6)$$

180 The reduction of N_2O to N_2 can cause an enrichment in SP values, thus moving the values
181 closer to those of fungal denitrification. If N_2O reduction process is not blocked, SP values for fungal
182 and bacterial denitrification could be confounded.

183 In this study, two methods (i.e. acetylene inhibition approach and mixing approach) were
184 chosen to eliminate this confusion.

185 *2.5.1. Acetylene inhibition method*

186 In this method, the reduction of N_2O to N_2 can be inhibited by the addition of 10 kPa (10 vol%)
187 C_2H_2. Therefore, N_2O emissions are the total (N_2O+N_2) emissions for treatments with C_2H_2 and the
188 net N_2O production after the reduction of N_2O for treatments free of C_2H_2. The SP values obtained
189 from groups with 10 kPa C_2H_2 which are not influenced by N_2O reduction are able to distinguish
190 fungal and bacterial denitrification calculated by the two end-member mixing model (equation (5)
191 and (6); Sutka et al., 2003). The SP values derived from bacterial denitrification as the lower end-
192 member and from fungal denitrification as the higher one. Results derived from this method are the
193 measured results in Figure. 4.

194 *2.5.2. Isotopocule mixing approach*

195 To distinguish the fraction of N_2O derived from fungal denitrification (f_{FD}) or bacterial
196 denitrification (f_{BD}), we used an isotopocule mixing approach adopted by others (Zou et al., 2014;
197 Deppe et al., 2017; Lewicka-Szczebak et al., 2017; Congreves et al., 2019) in treatments free of
198 C_2H_2, that showed the calculation of f_{FD} and f_{BD} with this approach. The isotopocule map consists
199 of SP and $\delta^{18}O$ values of gas samples (Fig. 1) whose end-member areas are given for fungal
200 denitrification and bacterial denitrification. We used soil-specific endmembers derived from average
201 literature in previous work to perform the isotopocule map. The values are characteristic for soil
202 incubation and not influenced by N_2O reduction (bacterial denitrification: SP_{BD} -10‰ to 0‰
203 (Toyoda et al., 2005; Sutka et al., 2006); $\delta^{18}O_{BD}$: +10‰ to +20‰ (Snider et al., 2013; Lewicka-
204 Szczebak et al., 2014); fungal denitrification: SP_{FD}: + 34‰ to +37‰, $\delta^{18}O_{FD}$: +30‰ to +40‰ (Sutka

et al., 2008; Rohe et al., 2014a)). The mean mixing line connects average values for both endmembers.

The influence of N_2O 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 $\delta^{18}O$ 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_0 and $\delta^{18}O_0$) of produced N_2O before reducing to N_2. If the SP_0 value was lower than the measured SP value of N_2O, the calculated value (SP_0) was used, since N_2O reduction could not be negligible.

From the calculated SP_0 values (or SP), the fraction of N_2O 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_2O, N_2) and isotopocule characteristics of N_2O ($\delta^{15}N^{bulk}$, $\delta^{18}O$, SP) with $p < 0.05$ used as the criterion for statistical significance.

3. Results

227 *3.1. Effect of soil acidification on inorganic nitrogen*

228 To obtain different pH levels, soils at natural pH (pH 8.7) were preincubated for a period of
229 two weeks after acidification. Data for all the pH groups are presented in Table 1. With H_2SO_4 added,
230 significant differences among pH groups were observed and varied from 0.9 to 2.5 units ($p < 0.0001$).
231 Moreover, there were also obvious changes in both NH_4^+ and NO_3^- concentrations. The average NH_4^+-N
232 content at pH 6.2 was significantly different from that in natural soil (pH 8.7; $p < 0.01$) and
233 increased 2.1 times by the end of preincubation (Table 1) in conjunction with a slight decrease of
234 the NO_3^--N content (5.9 mg N kg^{-1}).

235 *3.2. N_2O and N_2 fluxes*

236 Compared to the groups free of C_2H_2, the addition of C_2H_2 significantly increased N_2O
237 production in all treatments ($p < 0.05$; Fig. 2). With substrate and carbon source consumption, both
238 the N_2O production and reduction rates generally deceased with incubation progress in all groups
239 (Fig. 2). Soil pH strongly controls the N_2O production and reduction rates of denitrification. Both
240 soil N_2O and (N_2O+N_2) cumulative emissions in acidic soils increased significantly compared to
241 soils of natural pH ($p < 0.0001$). No significant difference was observed between soils of pH 7.1
242 and pH 8.7, but higher values occurred in soils of pH 7.1.

243 The total N_2O production determined in soils with C_2H_2 was significantly larger ($p < 0.001$) in
244 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
245 7.1 and 12.67 mg N kg^{-1} d^{-1} for pH 6.2, compared to groups in the absence of glucose, varying
246 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
247 6.2 (Fig. 2; Table 2). The differences in N_2O production among soils with different pH values were
248 all statistically significant under the same glucose conditions ($p < 0.001$).

Net N_2O production in the absence of C_2H_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_2O reduction rate, calculated based on the comparison between the groups with and without C_2H_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_2O 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 ($N_2O/(N_2+N_2O)$), 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_2O

Similar to N_2O production, the $\delta^{15}N^{bulk}$ values generally decreased with incubation time in all treatments (Fig. 3) and were depleted compared to $\delta^{15}N\text{-}NO_3^-$ at the beginning of the incubation (the initial values of $\delta^{15}N\text{-}NO_3^-$ of pH 8.7, pH 7.1 and pH 6.7 were -2.39‰, -2.18‰ and -3.63‰, respectively; $\delta^{15}N\text{-}NO_3^-$ and $\delta^{18}O\text{-}NO_3^-$ 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_2H_2, the $\delta^{15}N^{bulk}$ values of natural soils were significantly higher than that in the presence of C_2H_2 ($P < 0.05$), and this statistical significance was not observed in soils after acidification. The $\delta^{15}N^{bulk}$ of produced N_2O in soils with glucose varied between -43.72‰ and -27.67‰ and was significantly higher in more acidic soils ($p < 0.001$).

271 The $\delta^{18}O$ values showed no evident changes for pH 6.2 with incubation time, decreased in the
272 first three days and then increased for the other two soils (Fig. 3). The $\delta^{18}O$ values of produced N_2O
273 in soils with C_2H_2 varied from 16.83‰ to 20.64‰ (Table 2) and were significantly decreased ($p <$
274 0.001) than that free of C_2H_2 and were considerably depleted compared to $\delta^{18}O\text{-}NO_3^-$ at the
275 beginning of the incubation (the initial $\delta^{18}O\text{-}NO_3^-$ values of pH 8.7, pH 7.1 and pH 6.7 were 49.43‰,
276 50.16‰ and 51.57‰, respectively); the $\delta^{18}O$ value was approximately 1‰ higher for pH 6.2 than
277 for natural soils ($p = 0.051$). These differences clearly increased after the addition of glucose ($p <$
278 0.001). Contrary to the groups with C_2H_2, $\delta^{18}O$ values in soil with higher pH values were larger than
279 that lower ones in the absence of C_2H_2. In general, the addition of glucose significantly increased
280 $\delta^{18}O$ values in the absence of C_2H_2, and this difference was weakened in the presence of C_2H_2.

281 The SP values in groups with C_2H_2 varied between 4.90‰ and 21.73‰, with a wider variation
282 for a lower pH (Table 2; Fig. 3), and were higher for groups free of glucose compared to groups
283 with glucose in the early stage of the incubation. The highest SP values in groups free of glucose
284 were observed before day 5, with a general decline after day 5. Conversely, the lowest SP values in
285 the presence of glucose were observed before day 5 except for pH 7.1, which was observed on day
286 9. The addition of glucose increased the SP values on and after day 5 compared to the groups free
287 of glucose, and the differences between groups with and without glucose were higher for a lower
288 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
289 SP values were followed by lower pH conditions.

290 *3.4. The relationship between isotopocules and gaseous nitrogen fluxes*

291 Pearson correlation analysis were used to identify isotopocules of N_2O linked to N_2O and N_2
292 flux (Table 3). For all groups, the $\delta^{15}N^{bulk}$, $\delta^{18}O$ and SP values of N_2O were all significantly

correlated with N_2O flux, with positive correlations between $\delta^{15}N^{bulk}$ and N_2O and between SP and N_2O ($p < 0.01$) and negative correlations between $\delta^{18}O$ and N_2O ($p < 0.01$) in the absence of glucose. Additionally, a higher correlation coefficient was found between $\delta^{15}N^{bulk}$ and N_2O at pH 8.7, a negative correlation was found between SP and N_2O in all soils, and a positive correlation was found between $\delta^{18}O$ and N_2O at pH 6.2 after adding glucose. In the absence of glucose, no significant correlation was observed between N_2 flux and isotopocules of N_2O at pH 8.7 and pH 7.1 except for $\delta^{18}O$ and N_2 at pH 7.1. Positive correlations were found between $\delta^{15}N^{bulk}$ and N_2 and between SP and N_2 ($p < 0.01$), and a negative correlation was found between $\delta^{18}O$ and N_2 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 $\delta^{15}N^{bulk}$ and N_2, followed by negative correlations between $\delta^{18}O$ and N_2 and between SP and N_2.

3.5. Source partitioning

The mapping approach based on $\delta^{18}O$/SP plots for soil-derived N_2O was used for source partitioning (Fig. 1). $\delta^{18}O$ values were highest in natural soils with glucose, coupled with the SP values. These individuals were outside the vector that is interpretable by mixing N_2O 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 N_2O to N_2 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 $\delta^{18}O$ and of 7.61‰ to 22.93‰ for SP, compared to groups after adding glucose (18.48‰ to 34.28‰ for $\delta^{18}O$ and 6.80‰ to 25.73‰ for SP).

315 Based on the endmember signatures, f_{FD} calculated from Equation (5) ranged from 23% to 76%
316 (Table S1). The highest SP values were measured in individuals at pH 6.2 with glucose, and closer
317 SP values were observed from samples in groups without carbon sources, especially natural soils.
318 Using the mean values for the SP and $\delta^{18}O$ values of fungal denitrification and bacterial
319 denitrification (i.e., center values of endmember areas in Fig. 1), the contribution of fungal
320 denitrification to N_2O production was 45% for pH 8.7, 48% for pH 7.1 and 49% for pH 6.2 and was
321 lower for pH 8.7 (37%) and pH 7.1 (43%) and higher for pH 6.2 (57%) after adding carbon sources.
322 From the calculated SP (or SP_0) values estimated by the isotopocule map (calculated SP values)
323 and the measured SP values directly measured from the groups with C_2H_2 (measured SP values), the
324 contribution of fungal denitrification-derived N_2O based on the above two approaches was
325 calculated by using the end-members mixing model (the calculated and measured f_{FD} were estimated
326 by the above calculated and measured SP values, respectively). Hence, we compared the calculated
327 and measured f_{FD} and found good agreement between them with a significant fit to the 1:1 line (Fig.
328 4), especially for the group in the absence of glucose (R^2 = 0.81). The mean absolute difference
329 between the measured and calculated f_{FD} was 0.11 in the absence of glucose and 0.04 in the presence
330 of glucose. The mean relative error in the determination of the contribution of fungal denitrification
331 to N_2O production was 28% in the absence of glucose and 9% in the presence of glucose. A better
332 fit was not obtained when the calculated and measured f_{FD} for individual pH values were determined
333 and applied separately. From the correlation tested among different pH levels, we found that only
334 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
335 and calculated f_{FD} correlated. In the presence of glucose, although no correlation was observed for
336 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_{FD} values were associated with the incubation conditions.

4. Discussion

It is well established that soil pH is a main controlling factor for N_2O 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_2SO_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_2O and (N_2O+N_2) emissions in response to pH amendments, and distinguished the fraction of N_2O 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_2O reduction process (Weier et al., 1993; Azam et al., 2002). Soil acidification resulted in this C effect, which did not occur in neutral and alkaline soils.

4.2. N_2O and N_2 fluxes

The results showed that soil acidification was associated with both increasing denitrification rates and the $N_2O/(N_2O+N_2)$ 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_2O 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_2O production with more substrates due to the partial blockage of bacterial denitrifiers by acidic environments. Furthermore, a lower soil pH increased the $N_2O/(N_2O+N_2)$ product ratio due to the lower tolerance of N_2O reductase to

381 acidic soil than the other denitrification reductases (Knowles, 1982), also leading to the promotion
382 of N_2O release (Šimek and Cooper, 2002; Saggar et al., 2013).

383 *4.3. Isotopocules of N_2O*

384 The isotopocule characteristics of produced N_2O reflect multiple sources that are influenced
385 by microbial mixing processes, the extent of the N_2O reduction process and the variation of the
386 relevant isotope effects (Lewicka-Szczebak et al., 2015). The trends of the isotopocule
387 characteristics of produced N_2O were associated with those of the N_2O and (N_2O+N_2) product rates.
388 The results from the isotopocule data (Table 2, 3 and Fig. 3) showed that the addition of glucose
389 promoted the reduction of N_2O to N_2 and the enrichment of ^{15}N and ^{18}O of residual N_2O (Weier et
390 al., 1993; Azam et al., 2002). The complicated oxygen isotope fractionation during microbial N_2O
391 production by denitrification has led $\delta^{18}O$ to be a poor tracer of microbial origins in numerous
392 previous studies (Kool et al., 2007; Ostrom et al., 2007; Kool et al., 2009). Nonetheless, a
393 combination of $\delta^{18}O$ and SP has been used to correct N_2O reduction in this study, indicating a viable
394 approach to resolve the confusion for N_2O source partitioning (Deppe et al., 2017; Lewicka-
395 Szczebak et al., 2017).

396 Lower $\delta^{18}O$ values in the absence of C_2H_2 were found in acidified soils, which were caused by
397 the reduced N_2O reduction resulting from the inhibition of the N_2O reduction by lower pH (Ostrom
398 et al., 2007), consistent with the product ratio of $N_2O/(N_2O+N_2)$. In addition, the lower $\delta^{18}O$ values
399 could also be attributed to the greater O-exchange for lower pH since soil acidification may have a
400 partial blockage effect on bacterial denitrification but favors fungal denitrification (Sahrawat and
401 Keeney, 1986; Laughlin and Stevens, 2002; Chen et al., 2015), during which increased O exchange
402 may occur than in bacterial denitrification (Rohe et al., 2017). Higher $\delta^{18}O$ values in the presence

403 of C_2H_2 were also found after soil acidification, which could be interpreted as increased N_2O
404 reduction leading to a greater enrichment of heavy isotopes of ^{15}N and ^{18}O. Thus, the inhibition of
405 N_2O reduction by C_2H_2 has a larger negative effect on the $\delta^{15}N^{bulk}$ and $\delta^{18}O$ values of the produced
406 N_2O in higher pH soils with higher N_2O reduction. In addition, other co-occurring microbial
407 processes, i.e., DNRA and anammox, may also be involved in the above process.

408 For each pH level, generally, the mean SP values in the absence of glucose decreased compared
409 to the groups with glucose. The results in this study visually showed that there was a transition
410 between fungal denitrification-derived and bacterial denitrification-derived N_2O release during the
411 whole incubation. Relative to the whole incubation, higher SP values were generally observed in
412 the first 3 days in the absence of glucose (18.91‰ to 21.55‰ for pH 6.2, 19.94‰ to 22.93‰ for
413 pH 7.1, 12.37‰ to 17.04‰ for pH 8.7; Fig. 3). These values come closer to the expected SP values
414 derived from fungal denitrification (Sutka et al., 2008; Rohe et al., 2014a), and a higher contribution
415 to N_2O production was also observed (45% to 66% for pH 6.2, 45% to 54% for pH 7.1, 36% to 48%
416 for pH 8.7, derived from the measured f_{FD}; Fig. 4). SP continued to decrease for the first 5 days at
417 all pH levels, which is in line with the N_2O product rates, suggesting the transition of moving N_2O
418 production processes closer to bacterial denitrification and farther from fungal denitrification and
419 that the higher N_2O release in the first 5 days was associated with fungal denitrification, especially
420 for the two lower pH values. The higher correlation coefficient between the N_2O product rates and
421 SP values at the two lower pH values (r = 0.82 for pH 7.1, r = 0.79 for pH 6.2) also demonstrated
422 the above since soil acidification may favor the existence of fungal denitrifiers but partially inhibits
423 bacterial denitrifiers (Sahrawat and Keeney, 1986; Laughlin and Stevens, 2002; Chen et al., 2015).
424 The SP values in natural soils were generally lower than 15‰, indicating a smaller contribution of

425 fungal denitrification (Zou et al., 2014), and Fig. 4 shows the lower contribution derived from fungal
426 denitrifiers for soils of pH 8.7 (36% for pH 8.7) than the two other soils (42% for pH 7.1, 43% for
427 pH 6.2).

428 *4.4. Source partitioning*

429 The combination of $\delta^{18}O$ and SP has only recently been applied for correcting N_2O reduction
430 (Deppe et al., 2017; Lewicka-Szczebak et al., 2017), indicating a viable approach to resolve the
431 confusion regarding N_2O source partitioning.

432 This study was conducted at a relatively higher soil moisture favoring denitrification; therefore,
433 it is undisputable to consider oxygen isotope exchange with soil water for the analysis of $\delta^{18}O$ of
434 N_2O (Lewicka-Szczebak et al., 2016). Rohe et al. (2017) reported that the extent of oxygen exchange
435 varied between 83% and 94% for fungal denitrification and between 17% and 73% for bacterial
436 denitrification under the conditions tested. The $\delta^{18}O$ values used for the endmember values of fungal
437 denitrification (Fig. 1) were derived from pure culture studies (Sutka et al., 2008; Rohe et al., 2014a),
438 which excluded extreme values from pure cultures that were not considered representative of soil-
439 produced N_2O since they showed more variable oxygen exchange than that of soil incubations. The
440 lower $\delta^{18}O$ values on the left side of the mixing line might be interpreted as the $\delta^{18}O$ (-11.52‰)
441 values of H_2O used in this study being lower than in studies applied to determine endmember values
442 (-8.9‰; (Rohe et al., 2014b)). Additionally, the pure culture used by Rohe et al. (2014) was
443 conducted at pH 7.4, which is slightly higher than the two soils with lower pH values (pH 6.2 and
444 pH 7.1) used in this study. Although fungi can contribute to N_2O release at low pH (Yanai et al.,
445 2007), some fungi prefer neutral to weakly alkaline environments for N_2O release (Burth and Ottow,
446 1983; Shoun et al., 1992). It was assumed that the slight pH difference between the two studies may

447 also be one of the reasons that some points with lower $\delta^{18}O$ and higher SP values were outside the
448 vector. Soil acidification resulted in increasing N_2O release associated with fungi-mediated
449 denitrification, leading to higher SP values derived from fungal denitrification (Maeda et al., 2015).
450 The isotopocule characteristics (SP, $\delta^{15}N^{bulk}$, $\delta^{18}O$) for the analysis of the produced N_2O are
451 informative. The isotopocule map used for the correction of N_2O reduction based on SP/$\delta^{18}O$
452 somehow made a difference for N_2O source partitioning, especially in the absence of glucose. The
453 validation shows a certain agreement between the calculated and measured f_{FD}, indicating that the
454 SP and $\delta^{18}O$ values were sufficient within soil incubation experiments for calculating f_{FD} using the
455 two end-member mixing model. Although a slightly weaker fit was observed in the presence of
456 glucose ($R^2 = 0.40$), there was also some significance of the calculated f_{FD} relative to the measured
457 f_{FD}. A weaker fit to the 1:1 line in the presence of glucose had a lower absolute error than the group
458 free of glucose. Summarizing the results of the validation between the calculated and measured f_{FD},
459 we can conclude that this research can provide certain references for the correction of N_2O reduction
460 and N_2O source partitioning, although there is some bias between the calculated and measured f_{FD}.
461
462 **5. Conclusion**
463 This study observed an accordant variation in soil pH with the addition of KNO_3 comparable
464 to field conditions and confirmed the hypothesis in a field study that fungal denitrification greatly
465 contributes to N_2O production.
466 The facilitation of soil acidification by fungal denitrification-derived N_2O and N_2O release
467 from soil has been confirmed. In addition, acidified soils have a negative effect on the reduction of
468 N_2O to N_2. Although the addition of glucose weakened this negative effect, a higher $N_2O/(N_2O+N_2)$

ratio occurred in more acidic soils. Therefore, the addition of glucose promoted both N_2O reduction and production. The mapping approach provided rational results for correcting N_2O reduction in comparison with the acetylene inhibition method and indicated a reasonably large contribution of fungal denitrification to total N_2O production in acidified soils. However, there is a gap between the mapping approach and the C_2H_2 inhibition method for correcting N_2O 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 N_2O reduction since it offsets the deficiency of the acetylene inhibition method in field research, as well as in quantifying and qualifying N_2O 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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Figures

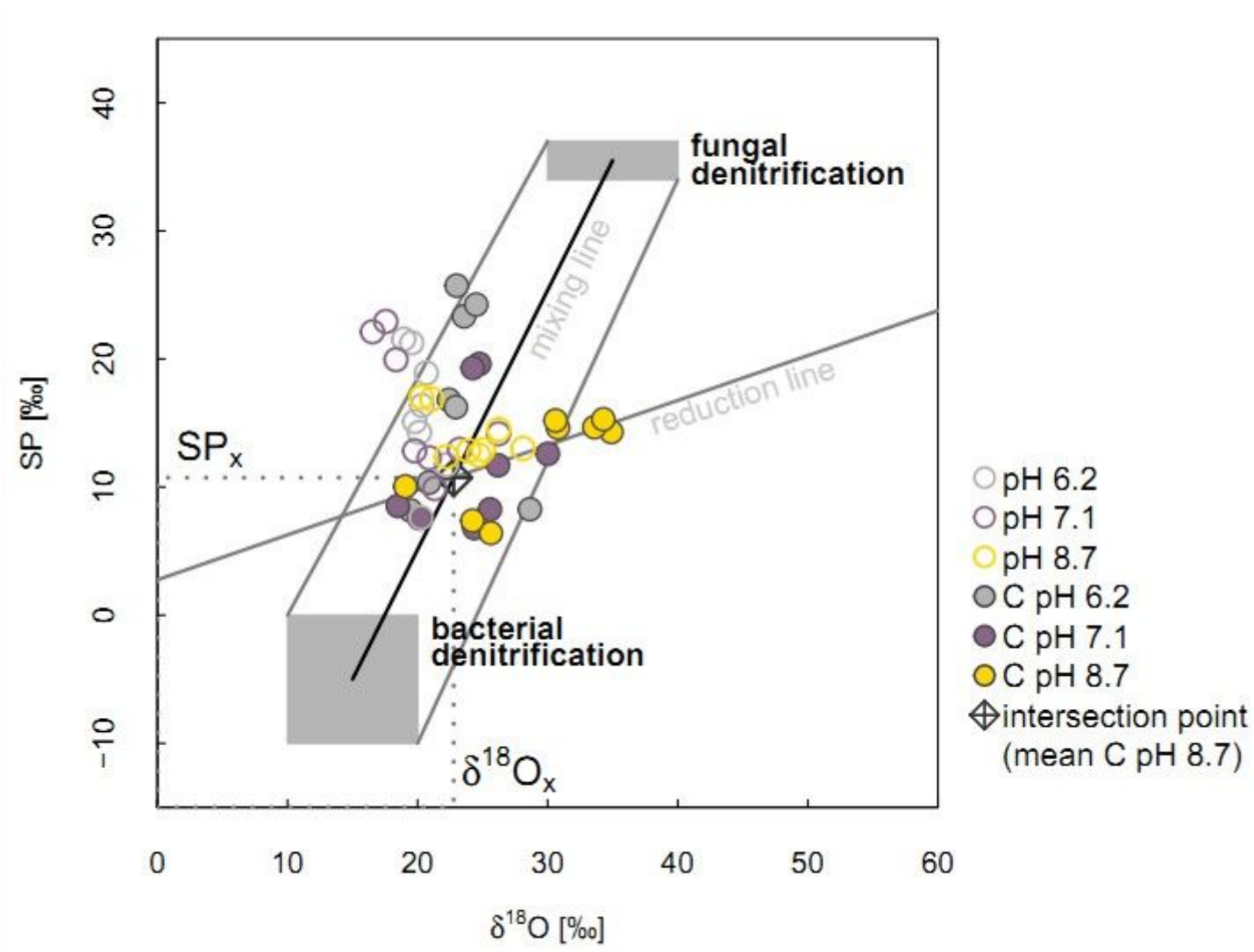

Figure 1

Isotopocule mapping approach to determine the source partitioning of N2O derived from bacterial denitrification versus fungal denitrification used mean 15N site preference (SP) and δ18O values of N2O. The linear mixed model approach was based on Deppe et al. (2017) and Lewicka-Szczebak et al. (2017). Top and bottom boxes indicate the expected ranges for fungal denitrification and bacterial denitrification (references see text in section 2.6.4). The mixing lines were drawn between mean values, minimum and maximum values for both SP and δ18O values of the respective processes. 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 was then placed through the respective sample values (in this scheme the average value for special samples (out of the vector) of the pH 8.7 without glucose) to calculate the point of intersection with the mean mixing line that represents values for N2O before reduction. Open circles showed the data points in the absence of glucose, and closed circles

showed the data points in the presence of glucose. “C” shown in the legend referred to the carbon source, i.e., glucose.

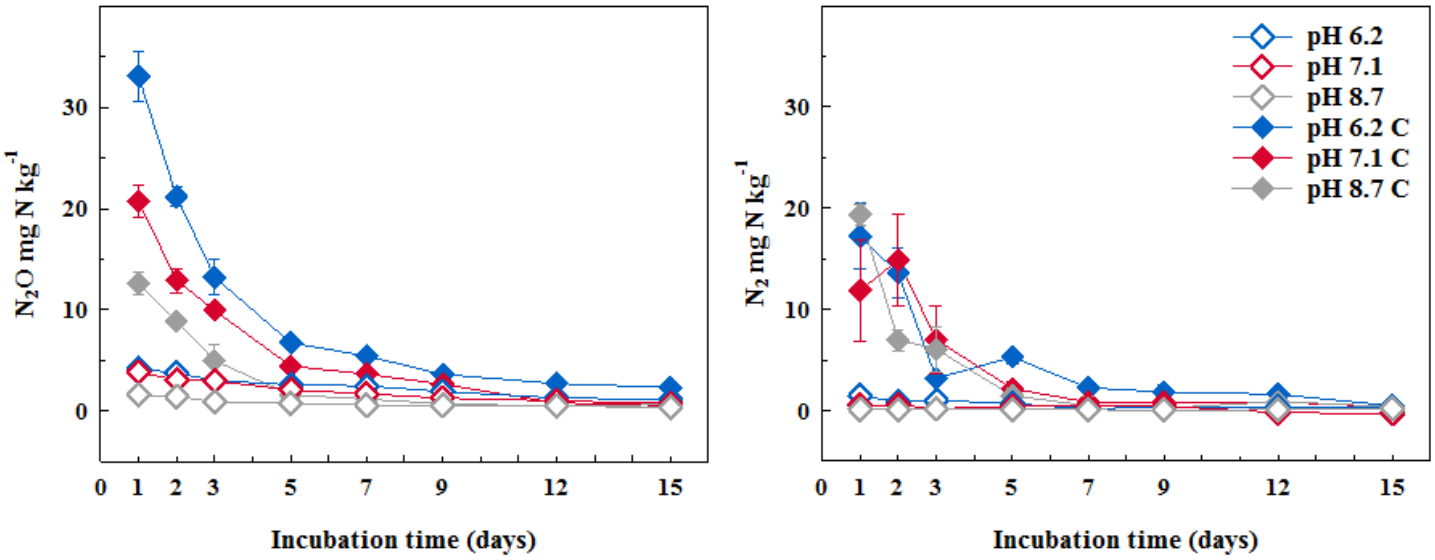

Figure 2

N2O fluxes at different sampling dates. Net N2O production (pN2O) are the N2O production determined in non-acetylated treatments. N2O reduction rates (rN2O) are the difference of N2O between acetylated and non-acetylated treatments. Mean values and standard deviations (n = 4) are shown.

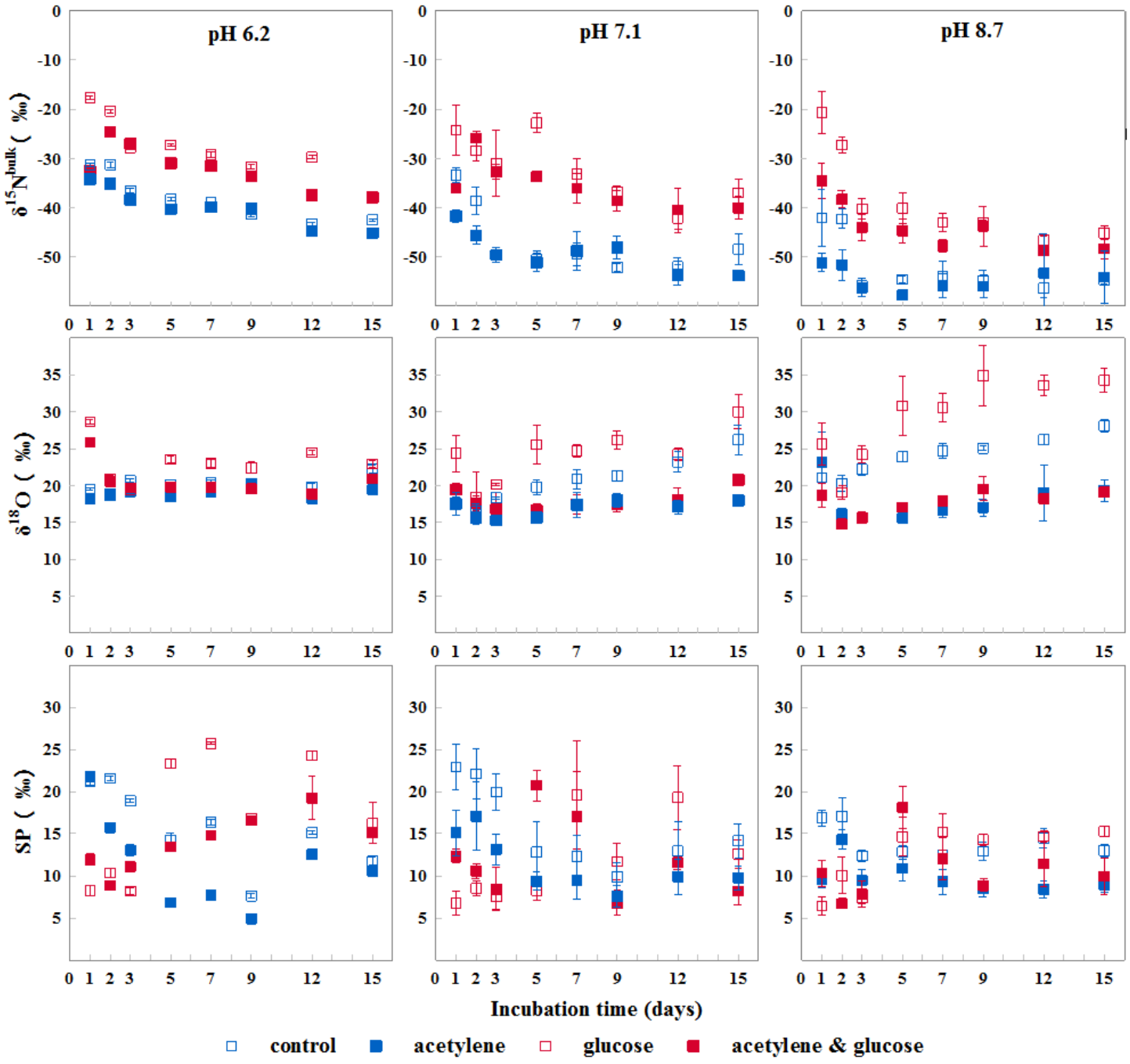

Figure 3

Here we show all the detailed results of N2O isotopoclues of this experiment incubated under both acetylated or non-acetylated treatments and with or without glucose. Mean values and standard deviations (n = 4) are shown.

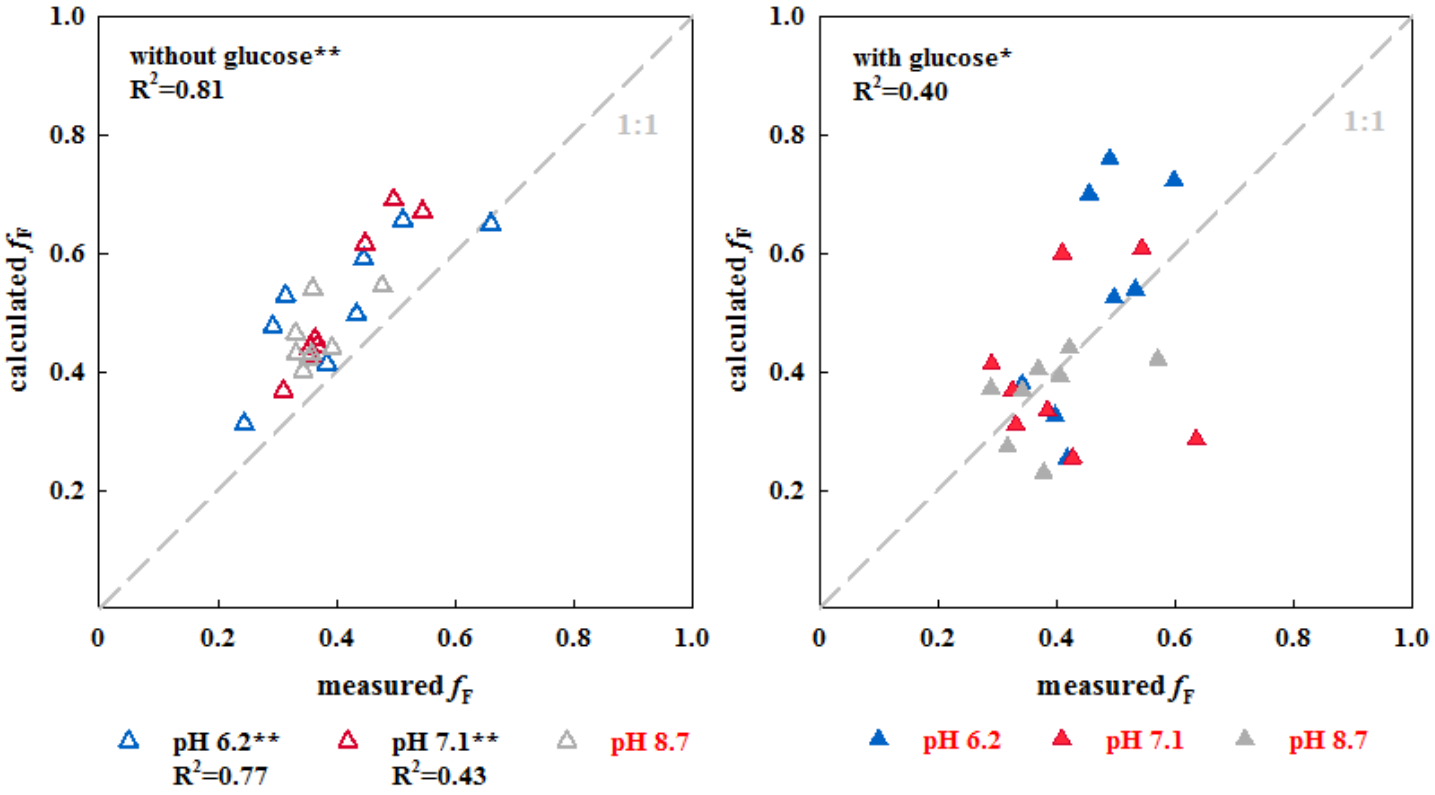

Figure 4

fFD calculated based on the linear mixed model approach and measured with independent method was compared. Open circles with different colors for groups without glucose and closed circles with different colors for groups with glucose were shown, respectively. R2 is used for expressing goodness of fit to the 1:1 line, and Pearson correlation coefficient was used to identify the statistical significance between the calculated and measured fFD for α = 0.05 with * $p < 0.05$ and ** $p < 0.01$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementarymaterials.docx
- Tables.docx