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The effect of nitrification inhibitor on N_2O, NO and N_2 emissions under different soil moisture levels in a permanent grassland soil

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Abstract

Emissions of gaseous forms of nitrogen from soil, such as nitrous oxide (N_2O) and nitric oxide (NO), have shown great impact on global warming and atmospheric chemistry. Although in soil both nitrification and denitrification could cause N_2O and NO emissions, most studies demonstrated that denitrification is the dominant process responsible for the increase of atmospheric N_2O, while nitrification produces mostly NO. The use of nitrification inhibitors (NIs) has repeatedly been shown to reduce both N_2O and NO emissions from agricultural soils; nevertheless, the efficiency of the mitigation effect varies greatly. It is generally assumed that nitrification inhibitors have no direct effect on denitrification. However, the indirect impact, due to the reduced substrate (nitrate) delivery to microsites where denitrification occurs, may have significant effects on denitrification product stoichiometry that may significantly lower soil-borne N_2O emissions. Soil-water status is considered to have a remarkable effect on the relative fluxes of nitrogen gases. The effect and mechanism of NI on N_2O, NO and N_2 emission under different soil water-filled pore space (WFPS) is still not well explored. In the present study, we conducted a soil incubation experiment in an automated continuous-flow incubation system under a He/O_2 atmosphere. Ammonium sulfate was applied with and without NI (DMPP) to a permanent UK grassland soil under three different soil moisture conditions (50, 65, and 80% WFPS). With every treatment, glucose was applied to supply enough available carbon for denitrification. Emissions of CO_2, N_2O, NO and N_2 were investigated. Additionally, isotopic signatures of soil-emitted N_2O were analyzed. Generally, higher WFPS led to higher N_2O and NO emissions, while N_2 emissions were only detected at high soil moisture condition (80% WFPS). Different processes were responsible for N_2O and NO emission in different phases of the incubation period. The application of DMPP did significantly reduce both N_2O and NO emissions at all three soil moisture conditions. Furthermore, DMPP application increased N_2 emissions and decreased the $N_2O/(N_2O+N_2)$ product ratio at 80% WFPS.

48

1. Introduction

Emissions of nitrogenous gases from agricultural soil, such as nitrous oxide (N_2O), nitric oxide (NO) and dinitrogen (N_2), represent a loss of N fertilizer and a reduction of plants N use efficiency (Bouwman et al., 2013). Grasslands, which are the dominant global ecosystem and cover 17% world surface, are also one of the main sources of N_2O and NO emissions (Cárdenas et al., 2007; Stehfest and Bouwman, 2006). Both N_2O and NO have great impact on global environmental change and atmospheric chemistry. Nitrous oxide has a global warming potential of about 300 times that of CO_2 and is considered as the major cause of ozone layer depletion in the 21st century (Bouwman et al., 2002; Ravishankara et al., 2009). Global anthropogenic N_2O emissions are estimated as approx. 6.5 Tg N yr^{-1} in 2010 (IPCC, 2013), of which soils are the largest source (Ciais et al., 2014). Although both nitrification and denitrification could produce N_2O in soil, recent studies suggested that denitrification is the dominant process responsible for the increase in atmospheric N_2O (Baggs, 2008). Denitrifying activity could be exhibited by both bacteria and fungi. However, fungal denitrification pathway, which recently has been found to be a major process in the nitrogen cycle, is not capable of reducing N_2O to N_2 (Laughlin and Stevens, 2002; Shoun et al., 2012; Sutka et al., 2008). Anthropogenic nitrogen oxide (NO_x =NO+NO_2) emissions were estimated as approx. 43 TgN yr^{-1} in 2010 globally (IPCC, 2013). The atmospheric lifetime of NO_x is relatively short (1-2 days), but as they are readily deposited on land and water surfaces (soil, plants, open waters), they lead to eutrophication and acidification of ecosystems (Crutzen, 1979). A recent study indicates that NO also plays an important role in haze formation of urban air pollution (Guo et al., 2014). In soil, NO can be produced by both nitrification and denitrification, as NO is not only a facultative by-product of the nitrification pathway, but also an obligatory intermediate of the denitrification pathway (Skiba et al., 1997). Nevertheless, nitrification is believed to be the main source of NO, as the diffusion of NO is restricted at high soil moisture

contents and NO produced from denitrification is reduced to N_2O before it escapes to the soil surface (Davidson, 1992; Firestone and Davidson, 1989; Skiba et al., 1997). Yet some studies showed that denitrification could also be a major source of NO emission from soils (Cárdenas et al., 1993; Loick et al., 2016; Pereira et al., 2010; Sanhueza et al., 1990).

Nitrification inhibitors (NIs) have been widely tested and studied for the purpose of decreasing nitrate leaching and mitigating greenhouse gas (GHG) emissions. Nitrification inhibitors are a group of chemical compounds that can reduce the bacterial oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) in the soil by inhibiting the activity of ammonia-oxidizing bacteria, e.g., of the genus *Nitrosomas*, in the soil (Zerulla et al., 2001). Most of NIs inhibit the first enzymatic step of nitrification, which is catalyzed by the enzyme ammonia monooxygenase (AMO) (Subbarao et al., 2006). A large number of NIs are known, but only a few of them, such as dicyandiamide (DCD) and 3, 4-Dimethylpyrazole phosphate (DMPP), have been widely and commercially used (Ruser and Schulz, 2015). The addition of NIs has been frequently reported to reduce both N_2O and NO emissions from agricultural soils, although their efficiency varies greatly in different environments (Pereira et al., 2010; Ruser and Schulz, 2015). Interestingly, some authors reported that the use of the NI reduced N_2O emission more effectively under higher soil moisture level, which is more favoured by denitrification (Di et al., 2014; Menendez et al., 2012). Although previous studies showed that most NIs did not have a direct effect on denitrification (Bremner and Yeomans, 1986; Müller et al., 2002), other studies suggested that denitrification-derived N_2O emission may also be affected by NIs indirectly via altering the product stoichiometry of denitrification (Hatch et al., 2005; Wu et al., 2017). As a key process of the global N cycle, denitrification leads to significant N losses from agricultural systems by converting NO_3^- and NO_2^- into NO, N_2O and N_2 (Bouwman et al., 2013). However, the product stoichiometry of denitrification, which is usually studied as $N_2O/(N_2O+N_2)$ product ratio, is affected by factors such as soil NO_3^-

concentration, water-filled pore space (WFPS), and soil available carbon (C) (Weier et al., 1993). The effects of these factors on the product ratio are still not well understood, as the direct and precise measurements of N_2 production via denitrification in soils are challenging due to the high N_2 abundance in the atmosphere.

The difference between ^{15}N at the central (α position) and the terminal N atom (β position) in the asymmetric N_2O molecule (^{15}N site preference, SP) has been shown as useful indicators of N_2O production and consumption processes in soils (bacterial nitrification: 34-37‰, bacterial denitrification: -10-0‰) (Sutka et al., 2008, 2006; Toyoda et al., 2005). The advantages of this isotopic technique are that it is a non-invasive, source-process tracking method, enabling convenient low-cost gaseous sampling, which facilitates investigation of both laboratory incubation and field-scale experiments (Decock and Six, 2013). The limitations of this technique have also been demonstrated, e.g., the uncertainties of N_2O source partitioning due to the overlapping or unknown SP signature of various pathways (Baggs, 2008; Decock and Six, 2013).

The first objective of this study was to examine the effectiveness of NI on mitigating N_2O and NO emissions at different soil moisture conditions in a UK grassland soil, as NIs have been widely used in grazed grassland. Furthermore, as the soil has been studied for different N pools that involved for nitrogenous gases emissions in our previous study, we further explored the effect of different soil moisture conditions on the fluxes, relationship and sources of N_2O, NO and N_2, in order to gain a better understanding of the different processes involved, thereby helping to develop better management strategy to further limit N_2O and NO emissions.

2. Material and methods

2.1 Soil

123 The soil was collected from a permanent grassland in North Wyke, Devon, UK (50° 46' 10"
124 N, 3° 54' 05" E) to a depth of 15 cm in November 2013. The soil was classified as clayey
125 pelostagnogley soil (Clayden and Hollis, 1985) (44% clay, 40% silt, 15% sand) and contained
126 0.5% total N and 11.7% organic matter, with a pH of 5.6. Root and plant residues were removed
127 and the soil was sieved to <2 mm and stored at 4 ° C since 7 days before rewetting.

128 2.2 Automated soil incubation experiment

129 The incubation experiment was carried out at Rothamsted Research, North Wyke, UK, in a
130 denitrification incubation system using a He/O_2 atmosphere (Cárdenas et al., 2003; Loick et
131 al., 2016). Soils were packed into 12 stainless steel vessels of 140 mm diameter at a bulk density
132 of 0.8 g cm^{-3}, which is similar to previous studies (Loick et al., 2016; Meijide et al., 2010). The
133 atmospheric N_2 was removed by flushing the soil core with a mixture of $He:O_2$ (80:20) in order
134 to measure N_2 fluxes. The experiment consisted of 6 treatments in total, i.e. soil amended with
135 mineral N fertilizer (ammonium sulfate) and glucose (AS), or NI (DMPP) mixed with
136 ammonium sulfate and glucose, at 50, 65, and 80% WFPS, respectively (AS50, DMPP50,
137 AS65, DMPP65, AS80, DMPP80). The incubation experiment was conducted in two
138 consecutive runs due to limited numbers of vessels. Prior to incubation, the soil was pre-
139 incubated for 7 days at the final WFPS to allow microbial activity to stabilize, taking the later
140 amendment into account. Ammonium sulfate was applied at a rate of 150 kg N ha^{-1} and glucose
141 was applied at a rate providing 400 kg C ha^{-1}. DMPP was added at rate of 1.5 kg ha^{-1}. The
142 amendment was dissolved in 50 ml water and added to each vessel. The temperature of the
143 incubation cabinet was set at 22 °C.

144

145 2.3 Measurement of trace gases

For online trace gas concentration analysis of N_2O and CO_2, gas samples from each incubation vessel were measured every two hours and quantified using a gas chromatograph (Clarus 500, Perkin Elmer Instruments, Beaconsfield, UK), fitted with a flame ionization detector (FID) and methanizer for the quantification of CO_2, and an electron capture detector (ECD) for N_2O. Nitric oxide (NO) emissions were quantified using a chemiluminescence analyzer (Sievers NOA280I. GE Instruments, Colorado, USA). Dinitrogen (N_2) emissions were measured by using a gas chromatograph fitted with a helium ionization detector (VICI AG International, Schenkon, Switzerland) and are presented as average fluxes per day. The flow rate from each incubation vessel's outlet was measured daily (Loick et al., 2016).

2.4 Isotopomer analysis

Gas samples for isotopic analysis were taken from each incubation vessel by attaching 120-mL serum bottles to the outlets in flow-through mode (with an inlet and an outlet needle) for approx. 1 hour during the incubation time. The N_2O $\delta^{15}N^{bulk}$ (i.e., the average $\delta^{15}N$ over the N_2O molecule), $\delta^{15}N_\alpha$ (i.e., $\delta^{15}N$ at the central position of the N_2O molecule), and $\delta^{18}O$ isotope signatures were then determined by analysing *m/z* 44, 45, and 46 of intact N_2O^+ molecular ions, and *m/z* 30 and 31 of NO^+ fragment ions (Toyoda and Yoshida, 1999) on an isotope ratio mass spectrometer (IsoPrime 100, Elementar Analysensysteme, Hanau, Germany). The $\delta^{15}N$ at the terminal position of the N_2O molecule, $\delta^{15}N_\beta$, was calculated according to $\delta^{15}N_\beta = 2 \cdot \delta^{15}N^{bulk} - \delta^{15}N_\alpha$. The details for correction and calibration are described in Heil et al. (2015). The isotope effects during N_2O reduction on N_2O SP values have been calculated using a Rayleigh-type model, assuming that isotope dynamics followed closed-system behaviour. The model can be described as follows:

$$SP_{N2O-r} = SP_{N2O-0} + \eta_r \ln\left(\frac{C}{C_0}\right)$$

In this equation, $SP_{N_2O\text{-}r}$ is the SP value of the remaining substrate (i.e. N_2O), $SP_{N_2O\text{-}0}$ is the SP value of the initial substrate, η_r is the net isotope effect (NIE) associated with N_2O reduction, and C and C_0 are the residual and the initial substrate concentration (i.e. C/C_0 expresses the $N_2O/(N_2O+N_2)$ product ratio). In this study an NIE of -4‰ was used based on previously reported average values (Lewicka-Szczebak et al., 2014).

2.5 Analyses of soil

Soil samples were taken at the beginning and end of each incubation to determine the NH_4^+ and total oxidised N (TON= NO_3^-+NO_2^-) contents. It is assumed that total oxidised N is nearly exclusively made of NO_3^-, as NO_2^- contents in the soil samples are negligibly small (Burns et al., 1996). The soil samples were extracted with 2 M KCl by shaking for 1 h. The extracts were then filtered through Whatman 602 filter paper (Searle, 1984). The concentrations of NH_4^+ and NO_3^- in soil extracts were measured colorimetrically using a Skalar SANL^{PLUS} Analyser (Skalar Analytical B.V., Breda, Netherlands).

2.6 Calculations and statistical analysis

The total gas emissions were calculated by linear interpolation between measured fluxes. Emission rates are expressed as arithmetic means of the four replicates. Tukey's HSD post-hoc tests were used to reveal significant pairwise differences among treatments. Statistical analyses were done using R, with $P < 0.05$ used as the criterion for statistical significance.

3. Results

3.1 Gas fluxes

193 The incubation period was characterized by three phases with different nitrogen gas
194 emission patterns (Figs. 1, 2 and 3): phase I (0-5 days) with a sharp and high N_2O emission
195 peak, but low or no NO and N_2 emissions; phase II (5-20 days) with low or no N_2O and NO,
196 but relatively high N_2 emissions; and phase III (20-43 days) with slowly decreasing N_2
197 emission and slowly increasing N_2O and NO emissions.

198 Nitrous oxide emissions were consistently low at 50% WPFS during all three phases in both
199 AS and DMPP treatments (Fig. 1). Maximum average fluxes of 12.0±1.3 and 7.2±0.1 g N ha^{-1}
200 day^{-1} were observed at the end of phase III in AS and DMPP treatments at 50% WFPS,
201 respectively. At 65% and 80% WFPS, the first N_2O emissions peaks both occurred in phase I
202 about 1.5 days after amendment application. At 80% WFPS the peak was approx. 10-fold larger
203 than at 65% WFPS. The fluxes decreased drastically after the peak and showed constant low
204 emissions rates of approx. 10-15 g N ha^{-1} day^{-1} till the end of phase II. The fluxes then started
205 to increase gradually and peaked at the end of phase III. The second N_2O peak at 65% WFPS
206 was significantly larger than the first peak, while at 80% WFPS it was much lower than the
207 first one but lasted much longer. During the observation period the total N_2O emissions
208 increased with increasing WFPS, while DMPP significantly reduced total N_2O emissions
209 compared with the AS treatments at all three different soil moisture levels.

210 Fluxes of NO were much lower than those of N_2O (Fig. 2), and total NO emissions were
211 about 8% of total N_2O emissions. NO fluxes showed a gradually increasing trend in all
212 treatments during the 43 days incubation period. They were very low during phase I in all
213 treatments, then started to rise after phase I, with higher NO fluxes in the AS treatments
214 compared to the DMPP treatments (Fig. 2). In all treatments, NO emissions peaked closed to
215 the end of phase III. Larger average NO emissions were observed in treatments with higher
216 soil moisture. The application of DMPP significantly reduced NO emissions compared with
217 the AS treatments at all three soil moisture conditions.

218 Gaseous nitrogen (N_2) production occurred only at 80% WFPS, where higher N_2 fluxes
219 were observed in the DMPP treatment than in the mineral-N only treatment (Fig. 3). In phase
220 I, the first N_2 fluxes peaked at similar time to N_2O and then decreased until about day 4. In
221 phase II the N_2 fluxes rose again and showed another peak with a maximum at day 12 and then
222 started to decrease and stayed low till the end of the incubation. The cumulative N_2 emissions
223 were 16.4% higher (albeit not statistically significant) in the DMPP treatment compared with
224 the AS treatment.
225 Carbon dioxide emissions peaked at about 1-1.5 days after amendment application and
226 decreased immediately to about 10 kg C ha^{-1} day^{-1} after 5 days and stayed low for the rest of
227 the incubation for all treatments (Fig. S1).
228

229 3.2 NH_4^+ and NO_3^- concentrations in soil

230 Table 1 shows the concentrations of ammonium (NH_4^+) and nitrate (NO_3^-) in the soil before
231 and after the incubation. The initial soil NH_4^+ and NO_3^- content was 4.2±0.03 and 182.8±2.3
232 mg N kg^{-1} dry soil, respectively. At the end of the incubation, NO_3^- concentrations at 65%
233 WFPS and 80% WFPS in AS and DMPP treatments were significantly higher than the initial
234 NO_3^- concentration, while no significant difference was found between those at 50% WFPS
235 and the initial NO_3^- concentration. The NO_3^- concentrations at all three soil moisture levels
236 were significantly lower in DMPP treatments compared to those without inhibitor. Ammonium
237 contents at the end of the incubation were larger than at the beginning in all treatments, and
238 they were larger by 22, 89 and 108% in DMPP treatments compared to the AS treatments at
239 50, 65, 80% WFPS, respectively (although not statistically significant at 50 and 65% WFPS).
240

241 3.3 Isotopic signatures of soil-emitted N_2O

242 The SP values ranged from -6.4 to 41.0‰ in all treatments during the incubation period
243 (Table 2). At day 0, the N_2O SP values were lower in the higher WFPS treatments, indicating
244 a higher bacterial denitrification proportion of N_2O at these soil moisture levels. However, at
245 80% WPFS, where the highest N_2O peak occurred on day 1, the SP values were 24.4‰ and
246 35.4‰ in AS and DMPP treatments, respectively, indicating that other major sources
247 (nitrification or fungal denitrification) were involved in the N_2O production. During phase II
248 and phase III, the SP values at all treatments were relatively stable, ranging from 27.9 to 41.0‰
249 at 50% WFPS, from 26.7 to 32.9‰ at 65% WFPS, and from 19.3 to 27.7‰ at 80% WFPS.

250

251 **4. Discussion**

252 4.1 Tracing N_2O, N_2 and NO emissions pathways under different WFPS conditions

253 Soil moisture is a key factor that determines N cycle in soils (Galloway et al., 2004). Several
254 studies found that soil N mineralization rate increased with increasing soil moisture (Bengtson
255 et al., 2005; Zaman and Chang, 2004), while N immobilization was less sensitive to soil
256 moisture (Booth et al., 2005). Nevertheless, compared to N mineralization and immobilization,
257 nitrification rate is more sensitive to moisture, and is believed to increase with increasing soil
258 moisture to a certain content and decline when moisture is above it (Manzoni et al., 2012). It
259 is generally accepted that under oxic conditions nitrification is the main process for N_2O
260 production, while denitrification dominates N_2O production under anoxic conditions. In our
261 study higher soil moisture levels led to higher N_2O emissions, which is in agreement with an
262 earlier study by Davidson et al. (2000), who demonstrated that the highest N_2O fluxes should
263 be expected when denitrification dominates at 60-90% WFPS. We assume that the much higher
264 N_2O emissions at 80% WFPS compared with lower soil moisture treatments in phase I were
265 due to enhanced denitrification, which was triggered by the addition of glucose, oxygen

depletion, and the soil residual NO_3^- (Fig. 1). This is supported by the initial peaks of N_2 emissions at 80% WFPS in both AS and DMPP treatments, and the absence of N_2 emission in the lower soil moisture treatments (Fig. 3). Furthermore, the smaller SP values observed on day 0 (Table 2) at higher soil moisture also indicated that a larger proportion of N_2O was initially derived from bacterial denitrification (Sutka et al., 2006). Although the smaller SP values might also be interpreted as nitrifier denitrification, it is unlikely the case for our study due to the high available C and high soil moisture condition in phase I (Kool et al. 2011). It should be noted that in our experiment the nitrate concentration in the initial soil was quite high, probably due to the mineralization during pre-incubation. The high nitrate content may have affected the N_2/N_2O ratio towards higher N_2O portions in phase I (Senbayram et al., 2012). Therefore, the results of the same experiment using a soil with lower nitrate content might be quite different.

According to the SP values (Table 2), the major source of the N_2O peak in phase I at WFPS 80% could have been either nitrification or fungal denitrification, as the overlapping SP signature between the processes makes it impossible to distinguish these two N_2O production pathways (Sutka et al., 2008). However, the fact that the NI showed no effect on the first N_2O emissions peak suggested that the source was unlikely nitrification (Fig. 1). Much larger N_2 emissions occurred at 80% WFPS in phase II, which is in line with Davidson et al. (2000), who suggested N_2 will become the main end product of denitrification when soil moisture is above 80% WFPS. It has been found that nitrate can inhibit N_2O reduction to N_2 and the reduction process only occurs when nitrate content in soil is low (Cleemput, 1998; Senbayram et al., 2012). Therefore, in phase II the observed much larger N_2 emissions at WPFS 80% indicated the soil NO_3^- content may have fallen below a threshold value at the denitrifying microsites (Fig. 3). At this high soil moisture level, and in combination with the abundant available C and low NO_3^- concentration, this would lead to a low $N_2O/(N_2O+N_2)$ product stoichiometry of

291 denitrification (Senbayram et al., 2012). The N_2O reduction process was likely conducted by
292 bacterial denitrification, as most of the fungal denitrification systems seem to lack N_2O
293 reductase, leaving N_2O as the final product (Shoun et al., 2012). The large decrease of N_2 fluxes
294 after phase II can be explained by the depleted available C as shown by the smaller CO_2
295 emissions compared to phase I.

296 An increasing trend of N_2O fluxes was observed in every treatment in phase III (Fig. 1).
297 This increase is probably due to the slowly growing nitrifying bacteria, as the grassland soil
298 used in the current study has not been fertilized for over 20 years. A similar delay in N_2O
299 emission after fertilization was observed by Brümmer et al. (2008) for a previously unfertilized
300 agricultural soil in Burkina Faso after adding ammonium nitrate to the soil. In fact, at the end
301 of phase III, emissions had still not gone down to background levels. Nevertheless, the
302 emissions were smaller, slower and of longer duration compared to the first peak. The
303 incubation was therefore stopped as the system seemed to have reached steady state. This may
304 affect the estimation of the NI's reduction potential, but should have no significant effect on
305 our final conclusion.

306 In our study the high average N_2O SP values observed at all three soil moisture conditions
307 during phase III indicated that N_2O emissions mainly originated from nitrification or fungal
308 denitrification (Table 2). It could be assumed that the larger N_2O emissions observed at high
309 soil moisture condition were possibly produced through denitrification (Bollmann and Conrad,
310 1998). However, in our study the lower NH_4^+ at the end of the experiment with rising soil
311 moisture content indicated nitrification was likely also enhanced by higher soil moisture (Table
312 1). Although the high soil moisture is generally believed to favor denitrification, it could also
313 accelerate nitrification if the conditions are still oxic, which might occur through diffusion of
314 atmospheric oxygen from the headspace in our study (Cheng et al., 2014; Chen et al., 2015;
315 Loick et al., 2016). Furthermore, the fact that the NI significantly decreased N_2O emission in

316 this phase at all three soil moisture conditions would indicate that nitrification is an important
317 process in regulating N_2O emissions. The marginal N_2 fluxes and the smaller SP values
318 observed at WFPS 80% during phase III indicate that very likely bacterial denitrification was
319 also involved. Thus, we conclude that both nitrification and denitrification were responsible
320 for the observed larger N_2O emissions at 80% WFPS soil moisture condition.

321 It was suggested that the highest NO fluxes should be expected at 30-60% WFPS, when
322 nitrification dominates, as the NO can diffuse out of the soil before it is consumed, whereas at
323 high soil moisture, when gas diffusion is lower, NO emission should be low, as it is reduced to
324 N_2O before escaping the soil (Bollmann and Conrad, 1998; Davidson et al., 2000; Skiba et al.,
325 1997). In the present study, however, the NO emissions significantly increased with increasing
326 WFPS from 50% to 80%, which therefore suggests that the larger amounts of NO at 80% WFPS
327 are probably produced through denitrification (Fig. 2). Although many studies did suggest that
328 emitted NO is mainly produced by nitrification (Scheer et al., 2008; Skiba et al., 1997, 1993),
329 several studies have challenged this assumption and found denitrification could also be a major
330 source of NO emission from soils (Cárdenas et al., 1993; Loick et al., 2016; Pereira et al., 2010;
331 Sanhueza et al., 1990). To distinguish the relative contributions of nitrification and
332 denitrification to NO and N_2O production, the N_2O/NO emission ratio has been proposed as a
333 useful indicator. When the N_2O/NO emission ratio is <1, soil conditions are favourable for
334 nitrification, whereas emission ratios >10 are associated with denitrification and restricted
335 aeration (Lipschultz et al., 1981; Skiba et al., 1993). During the first phase of our incubation
336 experiment, the average N_2O/NO ratios in AS treatments were 70, 151, and 383 at 50, 65, 80%
337 WFPS, respectively. This clearly reinforced our assumption that N-fluxes were mainly
338 associated with denitrification in phase I, when increasing soil moisture increased the
339 contribution of denitrification. In phase II and III, when NO emissions increased sharply, the
340 average N_2O/NO ratios were 18, 22, and 7 at 50, 65, 80% WFPS, respectively. The significantly

lower ratios at 80% WFPS confirm our hypothesis that the higher NO emissions at 80% WFPS might be caused by a higher nitrification rate, as mentioned previously, although both nitrification and denitrification were likely involved. Similarly, Cheng et al. (2014) reported NO and N_2O emissions of a forest soil that were favoured at high soil moisture (up to 90% WHC), whereas both NO and N_2O emissions showed a positive relationship with gross nitrification rates, indicating that nitrification was likely the dominant process. Furthermore, the significant mitigation effect of NI on NO emissions at all three soil moisture conditions also suggests the importance of nitrification as an important pathway in our study.

4.2 Effect of NI on N_2O, NO and N_2 emissions

Nitrification inhibitor application significantly reduced total N_2O emissions during observation period at all three soil moisture conditions. This agrees with recent review and meta-analysis studies which suggested that NIs are highly effective for reducing N_2O emissions at various soil conditions (Gilsanz et al., 2016; Qiao et al., 2015; Ruser and Schulz, 2015). In our study, the NI showed no significant effect on N_2O and N_2 emission in phase I, in line with previous reports which showed that NIs did not have a direct effect on denitrification (Bremner and Yeomans, 1986; Müller et al., 2002). However, the $N_2O/(N_2+N_2O)$ product ratios in the NI treatments were much smaller than the ratios in the AS treatments (Fig. 3). We assume this is because the use of NI limited the NO_3^- supply to the soil microsites, the lower NO_3^- concentration and available C would therefore decrease the $N_2O/(N_2+N_2O)$ ratio due to the competitive effect of NO_3^- and N_2O as terminal electron acceptors during denitrification (Senbayram et al., 2012 ; Wu et al., 2017).

The assumption that NIs could reduce N_2O emission under denitrification conditions by decreasing the $N_2O/(N_2+N_2O)$ ratio has been brought forward by several authors, but has still not been directly proven (Ruser and Schulz, 2015; Wu et al., 2017). Hatch et al. (2005) found

366 that two slurry treatments with NIs (DCD and DMPP) could significantly increase N_2 emissions
367 and reduce $N_2O/(N_2+N_2O)$ ratios compared with slurry-only treatment. However, the results
368 were observed in an incubation experiment conducted under anoxic conditions (100% helium
369 atmosphere). In the present study, although the soil moisture was high, the atmosphere of the
370 soil surface was kept oxic (20% oxygen and 80% helium), which is more comparable with the
371 field condition. To the best of our knowledge, our study is the first one showing that NI could
372 promote N_2 emissions under oxic conditions.

373 Most studies investigating the use of NIs did not consider the mitigation effect on NO
374 emissions, which can be significant after fertilization (Pereira et al., 2015). Several recent
375 studies reported a wide range of NO mitigation effects ranging from 35 to 80% when the NI
376 was applied with mineral fertilizer N or slurry (Akiyama et al., 2010; Pereira et al., 2015, 2010
377). In our study, application of the NI significantly reduced NO emissions at all three soil
378 moisture conditions, which is likely due to the inhibition effect of NI on nitrification process,
379 indicating that the overlooked mitigation effect of NI on NO emissions should be taken into
380 account when evaluating NI's mitigation effect on GHG emissions.

381 In this study the effect of NI on NH_3 volatilization was not evaluated, nevertheless, it should
382 be noted that the beneficial effect of NI application in decreasing N_2O and NO emissions might
383 be overestimated by the potentially increased NH_3 volatilization, especially when applied with
384 ammonium-based fertilizer (Kim et al., 2012; Lam et al., 2017).

385 **5. Conclusions**

386 The combination of the measurement of N_2O, NO and N_2 fluxes and N_2O isotopomer
387 analysis provided insight into the different pathways involved in the production of nitrogen
388 gases in soil at different soil moisture conditions. Our study showed that higher soil moisture
389 in a grassland soil was associated with higher N_2O, NO and N_2 emissions, and those different

processes were responsible for N_2O and NO emissions in three phases of the incubation period. To the best of our knowledge, our study is the first showing that NI could indirectly affect the product stoichiometry of denitrification under oxic conditions. The fact that the NI significantly reduced both N_2O and NO emissions at all three soil moisture conditions suggests that NIs could be used as an effective approach to mitigate GHGs emissions at various soil moisture conditions.

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595

596 **Figures and Tables**

597

598 **Figure 1.** Fluxes of N_2O of soil with only mineral-N at 50% WFPS (AS-50), or mineral-N+
599 nitrification inhibitor at 50% WFPS (DMPP-50), or only mineral-N at 65% WFPS (AS-65), or
600 mineral-N+nitrification inhibitor at 65% WFPS (DMPP-65), or only mineral-N at 80% WFPS
601 (AS-80)), or mineral-N+nitrification inhibitor at 80% WFPS (DMPP-80), during the 43 days
602 of the incubation experiment. Error bars show the standard error of the mean of each treatment
603 (n = 3).

604 **Figure 2.** Fluxes of NO of soil with only mineral-N at 50% WFPS (AS-50), or mineral-N+
605 nitrification inhibitor at 50% WFPS (DMPP-50), or only mineral-N at 65% WFPS (AS-65), or
606 mineral-N+nitrification inhibitor at 65% WFPS (DMPP-65), or only mineral-N at 80% WFPS
607 (AS-80), or mineral-N+nitrification inhibitor at 80% WFPS (DMPP-80), during the 43 days of
608 the incubation experiment. Error bars show the standard error of the mean of each treatment (n
609 = 3).

610

611 **Figure 3.** Fluxes of N_2O, NO and N_2 of soil with only mineral-N at 80% WFPS (AS-80), or
612 mineral-N+ nitrification inhibitor at 80% WFPS (DMPP-80) during the 43 days of the
613 incubation experiment. Error bars show the standard error of the mean of each treatment (n =
614 3).

615

Table 1 Nitrate (NO_3^-) and ammonium (NH_4^+) at the end of the experiment of soil with only mineral-N at 50% WFPS (AS-50), or mineral-N+nitrification inhibitor at 50% WFPS (DMPP-50), or only mineral-N at 65% WFPS (AS-65), or mineral-N+nitrification inhibitor at 65% WFPS (DMPP-65), or only mineral-N at 80% WFPS (AS-80), or mineral-N+nitrification inhibitor at 80% WFPS (DMPP-80), during the 43 days of the incubation experiment. Means denoted by a different letter in the same column differ significantly according to the Tukey's HSD post-hoc tests at alfa=0.05. The capital letters indicate comparison among different soil moisture levels, while the small letters indicate comparison between treatments with or without NI at the same soil moisture level.

Parameter	NO_3^- (mg N kg^{-1} dry soil)	NH_4^+ (mg N kg^{-1} dry soil)
Initial	182.8±2.3	4.18±0.03
AS-50	222.0±10.1 $^{A\,a}$	249.7±63.3 $^{A\,a}$
DMPP-50	167.7±2.5 $^{A\,b}$	305.0±35.4 $^{A\,a}$
AS-65	420.5±21.2 $^{B\,a}$	87.5±56.1 $^{B\,a}$
DMPP-65	332.4±16.7 $^{B\,b}$	165.4±65.9 $^{B\,a}$
AS-80	383.3±3.0 $^{B\,a}$	64.0±11.2 $^{B\,a}$
DMPP-80	277.9±10.4 $^{B\,b}$	139.2. ±14.2 $^{B\,b}$

637 **Table 2** Site preference (SP) values (‰) of N_2O of soil with only mineral-N at 50% WFPS
638 (AS-50), or mineral-N+ nitrification inhibitor at 50% WFPS (DMPP-50), or only mineral-N at
639 65% WFPS (AS-65), or mineral-N+nitrification inhibitor at 65% WFPS (DMPP-65), or only
640 mineral-N at 80% WFPS (AS-80)), or mineral-N+nitrification inhibitor at 80% WFPS (DMPP-
641 80), during the 43 days of the incubation experiment. Symbol "-" represents SP values that
642 were not measured at that day, while "*" indicates missing or out of range values due to
643 analytical reasons; the standard error was not given if the replicates were less than three.

644

	Phase I			**Phase II**		**Phase III**			
Date	Day 0	Day 1	Day 3	Day 13	Day 20	Day25	Day 30	Day 34	Day 43
AS-50	20.7±8.4	-	-	-	38.2±3.8	31.6±0.7	30.3±0.7	-	27.9±0.2
DMPP-50	*	-	-	-	*	41.0	38.0	-	*
AS-65	11.3±6.0	-	-	-	*	32.5±1.0	28.7±1.0	-	30.9±0.8
DMPP-65	*	-	-	-	*	32.9	26.7	-	28.4
AS-80	2.3±0.7	24.4±3.7	26.5±4.2	23.8±2.6	-	-	-	27.7±0.9	26.2±2.0
DMPP-80	-6.4	35.4±2.7	31.7±6.8	19.3±0.5	-	-	-	26.9±1.2	24.7±1.5

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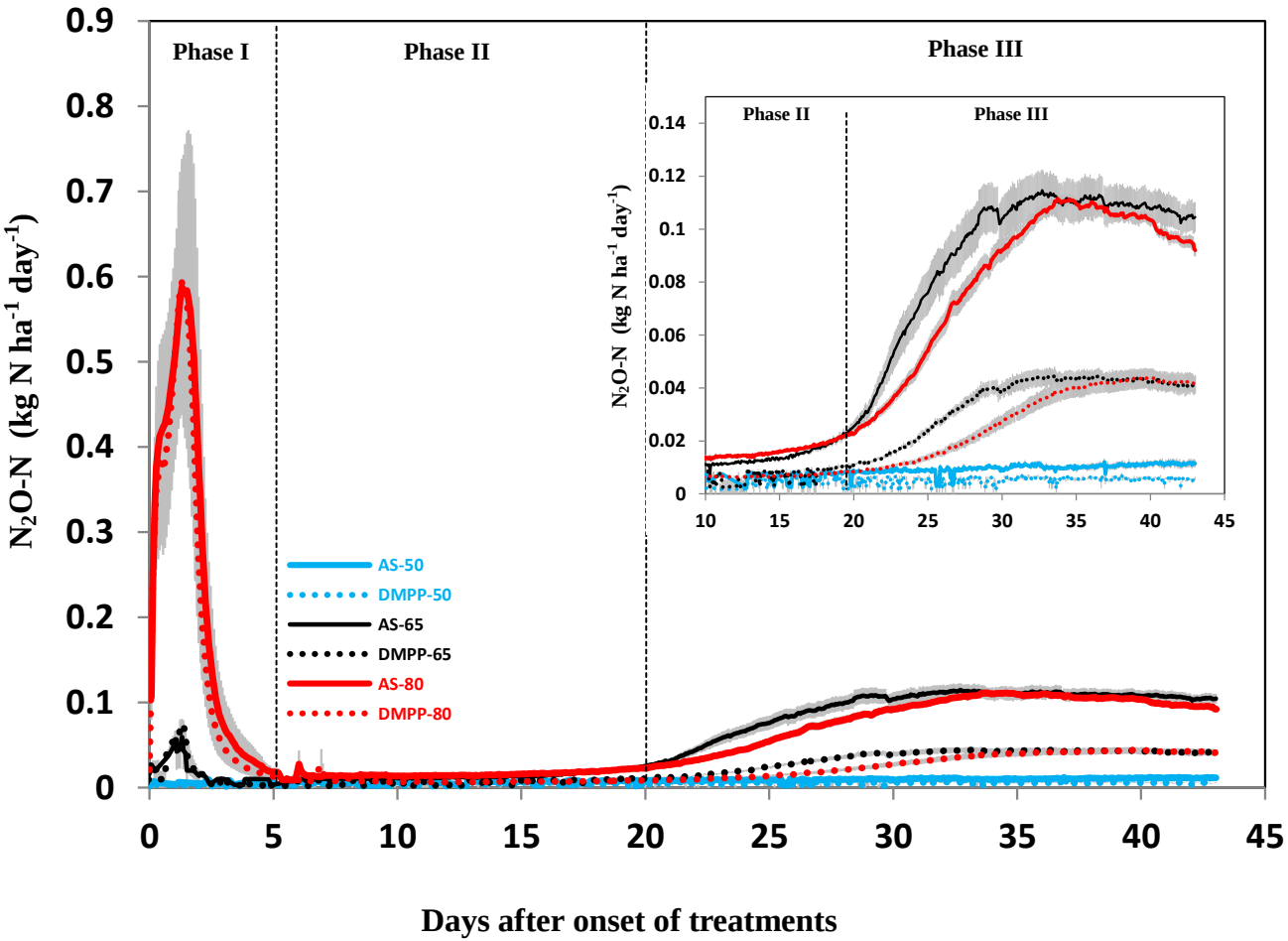
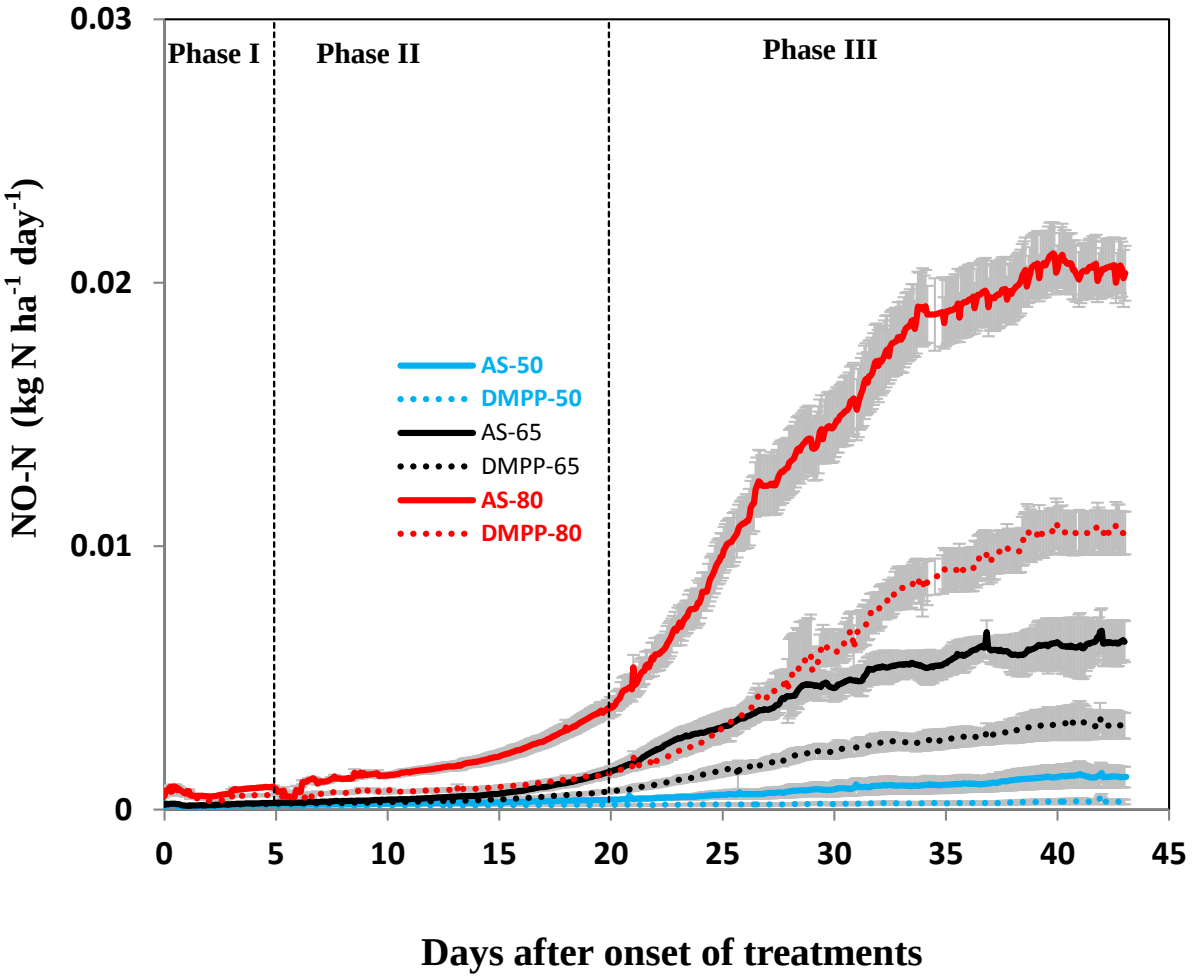
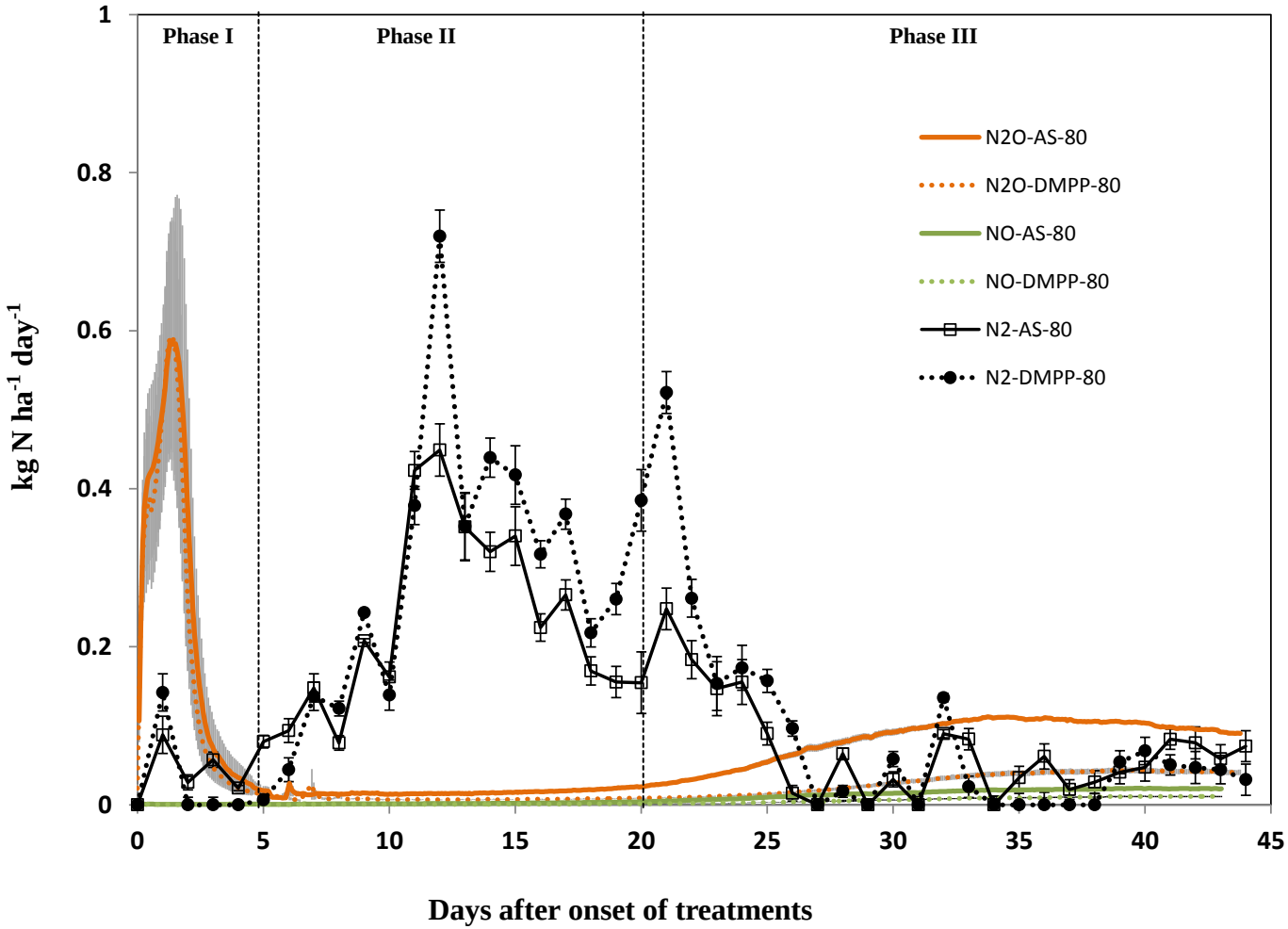
647


Phase I
Phase II
Phase III
N2O-N (kg N ha-1 day-1)
Days after onset of treatments
AS-50
DMPP-50
AS-65
DMPP-65
AS-80
DMPP-80
Phase II
Phase III
N2O-N (kg N ha-1 day-1)


Phase I
Phase II
Phase III
AS-50
DMPP-50
AS-65
DMPP-65
AS-80
DMPP-80
NO-N (kg N ha-1 day-1)
0.03
0.02
0.01
0
0
5
10
15
20
25
30
35
40
45
Days after onset of treatments


Phase I
Phase II
Phase III
N2O-AS-80
N2O-DMPP-80
NO-AS-80
NO-DMPP-80
N2-AS-80
N2-DMPP-80
kg N ha-1 day-1
Days after onset of treatments
0
0.2
0.4
0.6
0.8
1
0
5
10
15
20
25
30
35
40
45