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Effects of the nitrification inhibitor acetylene on nitrous oxide emissions and ammonia-oxidizing microorganisms of different agricultural soils under laboratory incubation conditions

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4 **Title: Effects of the nitrification inhibitor acetylene on nitrous oxide emissions and**  
5 **ammonia-oxidizing microorganisms of different agricultural soils under laboratory**  
6 **incubation conditions**

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22

23 **Abstract**

24 Acetylene ( $C_2H_2$ ) is an effective nitrification inhibitor targeting autotrophic ammonia  
25 oxidizers, and has shown promise for improving nitrogen use efficiency by mitigating  
26 greenhouse gas nitrous oxide ( $N_2O$ ) emissions and reducing nitrate leaching. Its efficacy,  
27 however, varies considerably with edaphic and environmental conditions and remains largely  
28 less studied in dryland agricultural soils. Here we conducted two laboratory microcosm  
29 incubations to explore the efficacy of  $C_2H_2$  across various agricultural soils and under  
30 different conditions. The first incubation was with four agricultural soils at  $25^\circ C$  and 60%  
31 water-filled pore space (WFPS), and the second incubation included one cropping soil under  
32 a range of conditions ( $15^\circ C$ ,  $25^\circ C$ ,  $35^\circ C$  and 50%, 70% WFPS). Our results showed that  
33 incubation of soil with 1% v/v  $C_2H_2$  resulted in complete or partial inhibition of nitrification,  
34  $N_2O$  emission, and AOA or AOB growth under the experimental conditions. Acetylene can  
35 totally inhibit nitrification in acidic cropping and dairy pasture soils through retarding both  
36 AOA and AOB growth, while  $C_2H_2$  partly inhibited nitrification and  $N_2O$  emission in the  
37 alkaline vegetable soil through impeding only AOB growth. The highest inhibition effect of  
38  $C_2H_2$  was achieved at  $25^\circ C$  and 50% WFPS, while there was no inhibitory effect of  $C_2H_2$   
39 when soil was incubated at  $15^\circ C$  and 50% WFPS suggesting soil temperature may have a  
40 significant influence on  $C_2H_2$  effectiveness. The inhibition of  $C_2H_2$  on cumulative  $N_2O$   
41 emission increased with increasing temperature at 50% WFPS. In contrast, at 70% WFPS, the  
42 inhibition of  $C_2H_2$  on cumulative  $N_2O$  emission decreased with increasing temperature. Since  
43 the effect of  $C_2H_2$  varied with soils and environmental conditions, this highlights the  
44 assumption that  $N_2O$  production and nitrification can be affected by low concentrations of  
45  $C_2H_2$  may be not appropriate in some occasions.

46 **Keywords:** Acetylene; nitrification inhibitor; AOA; AOB; N<sub>2</sub>O emission; temperature;  
47 moisture

48

## 49 **1. Introduction**

50 Nitrogen (N) is an essential nutrient for food production, but the amount of applied fertilizer  
51 N used by crops rarely exceeds 40% (Chen et al., 2008), and can be as low as 20% in  
52 vegetable production systems in Australia (Suter et al., 2014). The majority of applied N is  
53 lost from agro-ecosystems through ammonia (NH<sub>3</sub>) volatilization, gaseous emission of  
54 nitrous oxide (N<sub>2</sub>O) and di-nitrogen (N<sub>2</sub>) and nitrate (NO<sub>3</sub><sup>-</sup>) leaching. Nitrous oxide is a  
55 potent greenhouse gas contributing significantly to global climate change with a 300-fold  
56 higher global warming potential than CO<sub>2</sub> (IPCC, 2007). It is also involved in the destruction  
57 of the protective ozone layer (Ravishankara et al., 2009). Soil ecosystems are the largest  
58 source of N<sub>2</sub>O, accounting for approximately 65% of the atmospheric N<sub>2</sub>O loading (IPCC,  
59 2007). The major pathways of N<sub>2</sub>O production in soils include microbial-mediated  
60 nitrification and denitrification (Hu et al., 2015; Zhang et al., 2015). Microorganisms, such as  
61 ammonia oxidizers and bacterial denitrifiers, involved in the N cycle can directly regulate  
62 N<sub>2</sub>O production and consumption from soils, and increased abundance and activity of these  
63 microorganisms may increase N<sub>2</sub>O emissions (Burger et al., 2005).

64 Nitrification inhibitors (NIs) can decelerate the rate of soil nitrification by deactivating the  
65 enzyme ammonia monooxygenase (AMO) which catalyses ammonia oxidation, the first and  
66 rate-limiting step of nitrification which is encoded by the *amoA* gene within ammonia-  
67 oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) (Zhang et al., 2012). NIs  
68 can block the growth of AOA and AOB (Di et al., 2010; Hink et al., 2016), and are widely  
69 used to improve N fertiliser efficiency, mitigate N<sub>2</sub>O emissions and reduce NO<sub>3</sub><sup>-</sup> leaching in

70 agricultural systems (Chen et al., 2008; Kelly et al., 2008; Chen et al., 2010; Di et al., 2010;  
71 Zhang et al., 2012; Hu et al., 2015). Acetylene ( $C_2H_2$ ) is an effective inhibitor of bacterial  
72 ammonia oxidation (Offre et al., 2009), acting with AMO as a suicide substrate. It is usually  
73 used as an autotrophic nitrification inhibitor in experimental studies (de Boer and  
74 Kowalchuk, 2001) and can inhibit nitrification at a low concentration (e.g. 10 Pa) in most  
75 soils under aerobic conditions (Hyman and Wood, 1985; Schmidt and Bock, 1998; De Boer  
76 et al., 1991; Offre et al., 2009). In contrast,  $C_2H_2$  does not efficiently inhibit ammonia  
77 oxidation by heterotrophic nitrifiers (Moir et al., 1996; Daum et al., 1998). Higher  
78 concentrations of  $C_2H_2$  (1–20 kPa) will inhibit the  $N_2O$  reductase of denitrifying  
79 microorganisms (Davidson et al., 1986; Klemetsson et al., 1988). As a result of this  $C_2H_2$   
80 has been used as a routine method to distinguish nitrification-related  $N_2O$  and denitrification-  
81 related  $N_2O$  in soils experiments based on the inhibition of soil ammonia oxidation (Bateman  
82 and Baggs, 2005; Butterbach-Bahl et al., 2013).

83 It is generally believed that low concentrations of  $C_2H_2$  (0.1–10 Pa) totally inhibit  
84 nitrification (Hynes and Knowles, 1978; Berg et al., 1982) by forming a reactive epoxide  
85 which then irreversibly inactivates the AMO enzyme (Hyman and Wood, 1985), however,  
86 this does not always occur and its efficacy is considerably varied. While it was previously  
87 reported that 10 Pa of  $C_2H_2$  totally inhibited nitrification (Wrage et al., 2004), Bremner and  
88 Blackmer (1979) found that 10 Pa only partially inhibited soil nitrification.

89 Acetylene was also widely used to determine the community compositions of nitrifiers in  
90 soils through laboratory work (Boyle-Yarwood et al., 2008; Scheer et al., 2014). Recent  
91 studies have shown however, that AOA and AOB may have a variety of responses to  $C_2H_2$   
92 application. Gubry-Rangin et al. (2010) and Offre et al. (2009) revealed that AOA growth  
93 was inhibited in  $C_2H_2$ -containing microcosms but not AOB growth. Liu et al. (2015a)  
94 demonstrated that both AOA and AOB were inhibited by  $C_2H_2$  in three Australian

95 agricultural soils. However, these results contrast with those of Jia and Conrad (2009), who  
96 found changes in the abundance of AOB *amoA* genes correlated best with nitrification rate  
97 rather than AOA *amoA*, and bacterial growth occurred only in actively nitrifying microcosms  
98 with added C<sub>2</sub>H<sub>2</sub>. Considering the various responses of AOA and AOB to C<sub>2</sub>H<sub>2</sub> addition, it is  
99 therefore necessary to find out how soil factors influence the response of AOA and AOB to  
100 C<sub>2</sub>H<sub>2</sub> addition and what are the key factors affecting the response of AOA and AOB to C<sub>2</sub>H<sub>2</sub>.

101 This study was designed to determine the impact of C<sub>2</sub>H<sub>2</sub> on N<sub>2</sub>O emissions, nitrification  
102 rates and the abundance of ammonia oxidizers in different agricultural soils under laboratory  
103 conditions. Microcosm incubation experiments were established under a set of controlled  
104 environment conditions with the following objectives: (i) to examine the effects of C<sub>2</sub>H<sub>2</sub> on  
105 N<sub>2</sub>O emissions and the abundances of AOA and AOB from different agricultural soils and (ii)  
106 to investigate the effects of C<sub>2</sub>H<sub>2</sub> on N<sub>2</sub>O emissions and the abundances of AOA and AOB  
107 under different temperature and soil water contents in one agricultural soil. We hypothesized  
108 that (i) C<sub>2</sub>H<sub>2</sub> would have significant inhibitory effects on nitrification and N<sub>2</sub>O production  
109 from soils with different physicochemical traits, and (ii) AOA and AOB would exhibit  
110 distinctly different responses to C<sub>2</sub>H<sub>2</sub>. This study represents comprehensive efforts to  
111 examine C<sub>2</sub>H<sub>2</sub> efficacy from different soils under controlled conditions, and the findings from  
112 the study can improve our understanding of the interactions between soil microbial  
113 communities and the nitrification inhibitor C<sub>2</sub>H<sub>2</sub> in different agricultural soils under  
114 laboratory controlled conditions.

## 115 **2. Materials and methods**

### 116 **2.1 Site description and soil sampling**

117 The soils used in this study were collected from four agricultural sites in Australia: vegetable  
118 soil at Boneo, VIC (38.3°S, 144.9°E), sugarcane soil at Bundaberg, QLD (24.8°S, 152.3°E),

119 dairy pasture soil at Glenormiston, VIC (38.2°S, 143°E), and cereal cropping soil at  
120 Hamilton, VIC (38.3°S, 142.7°E). At each site, 10 replicate samples of the top soil (0–10 cm)  
121 were collected, thoroughly homogenized, and transported on ice to the laboratory. Fresh soils  
122 were sieved through a 2.0 mm mesh, and root and leaf residues were removed with tweezers  
123 prior to the establishment of microcosms. Soil moisture contents were determined by oven-  
124 drying three subsamples (10 g of fresh soil) at 105°C for 48 h. Soil texture (sieve and  
125 hydrometer procedures), pH (1:5 soil/water), total carbon (Dumas method) and other soil  
126 properties were determined and are shown in Table 1.

## 127 **2.2 Soil microcosm incubations**

### 128 **2.2.1 The laboratory incubation with different agricultural soils**

129 Soil microcosms were established in 500 ml vials containing 60 g of soils (oven-dry  
130 equivalent). Distilled water was added to soil to just under the final moisture content (60%  
131 water-filled pore space, WFPS) and the microcosms were pre-incubated at 25°C for three  
132 weeks to stabilise soil microbial communities and minimise priming effects associated with  
133 wetting events. After pre-incubation, treatment was applied to each incubation vial to reach  
134 60% WFPS (Linn and Doran, 1984). The treatments contained 100 mg N kg<sup>-1</sup> soil as  
135 exchangeable NH<sub>4</sub><sup>+</sup>-N and 50 mg N kg<sup>-1</sup> soil as NO<sub>3</sub><sup>-</sup>-N, which were added to the soil as 1)  
136 NH<sub>4</sub>Cl + KNO<sub>3</sub>; and 2) NH<sub>4</sub>Cl + KNO<sub>3</sub> + C<sub>2</sub>H<sub>2</sub>. Five ml of C<sub>2</sub>H<sub>2</sub> (1% v/v) was injected into  
137 the headspace of the vials using an air-tight syringe. Aerobic conditions, soil moisture and  
138 C<sub>2</sub>H<sub>2</sub> contents in the vials were maintained every three days by opening microcosms and  
139 replenishing. Soil microcosms were incubated at 25°C in the dark for three weeks.

#### 140 **2.2.1.1 Gas sampling and analysis**

141 Gas samples were collected on days 0, 4, 8, 12, 16 and 20 after fertilizer application. Gas  
142 samples (20 ml) for N<sub>2</sub>O analysis were taken from the 500 ml vials using gas-tight syringes.

143 Prior to collection of gas samples, the vials were opened to ensure that N<sub>2</sub>O concentration in  
144 the headspace was at ambient levels. During each sampling, gas samples were collected at 0,  
145 8, 16, 24, 48 and 72 hours after vials closure. Before gas collection, 20 ml compressed zero  
146 air was injected into 500 ml vials to maintain the pressure in the vials and then 20 ml gas  
147 samples were collected into a pre-evacuated 12 ml exetainer (Exetainer®, Labco Ltd.,  
148 Lampeter, Ceredigion, UK). Samples were analysed for N<sub>2</sub>O concentration by a gas  
149 chromatograph (Agilent 7890A) using an ECD (N<sub>2</sub>O) detector.

### 150 **2.2.1.2 Soil Sampling and analysis**

151 Soils were destructively sampled for soil mineral N analysis on days 0, 7, 14 and 21  
152 immediately after gas sampling. There were four replicates at each sampling day. A  
153 subsample (2 g) of soil was taken from each vial for molecular analysis and stored in a -80°C  
154 freezer before DNA extraction. The remaining 50 g of soil in the vials was shaken with 250  
155 ml of 2 M KCl for 1 h at 200 rpm at room temperature, and the extract filtered through a  
156 qualitative filter paper (Whatman 42). The extracts (30 ml) were stored at -20°C prior to  
157 measurement of NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> concentrations on a segmented-flow analyzer (Skalar,  
158 SAN++).

### 159 **2.2.1.3 Soil DNA extraction and quantitative PCR (qPCR)**

160 The Power Soil DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) was  
161 used for DNA extraction following the manufacturer's instructions. Extracted DNA was  
162 quantified using a NanoDrop ND2000c spectrophotometer (NanoDrop Technologies,  
163 Wilmington, DE, USA) and the quality of extracted DNA was checked on a 1% agarose gel.  
164 The archaeal and bacterial *amoA* gene copy numbers were quantified from triplicate samples  
165 using qPCR. The primer sets were Arch-amoAF/Arch-amoAR (Francis et al., 2005) and  
166 amoA1F/amoA2R (Rotthauwe et al., 1997), respectively. Each qPCR reaction for the

167 archaeal *amoA* gene was performed in a 20 µl volume including 0.5 µM of each primer, 10 µl  
168 SensiFAST SYBR No-ROX reagent (Bioline, Sydney, Australia), and 2 µl of 10-fold dilution  
169 DNA template (1–10 ng). Each qPCR reaction for the bacterial *amoA* gene was performed in  
170 a 10 µl volume containing 0.6 µM of each primer, 5 µl iTaq Universal SYBR Green  
171 Supermix (Bio-Rad Laboratories, USA), and 2 µl of 10-fold dilution DNA template (1–10  
172 ng). Amplification programs for both AOA and AOB were as follows: 3 mins at 95°C, 40  
173 cycles of 5 s at 95°C, 30 s at 60°C, and 72°C for 45 s. A known copy number of plasmid  
174 DNA was used to create a standard curve for each AOA or AOB assay. For all assays, qPCR  
175 efficiency was 92.7–98.4% and  $r^2$  was 0.96–0.99.

### 176 **2.2.2 Soil microcosm incubation under different temperature and water contents with** 177 **one agricultural soil**

178 The cropping soil used for the second experiment was chosen from one of the four sites and  
179 collected from a cropping paddock in Hamilton, VIC (38.3°S, 142.7°E) (Table 1). The study  
180 site is in a high rainfall zone (688 mm per annum), and was chosen because the soil was  
181 subjected to two different land management practices, cropping and pasture. Field studies  
182 have shown that high N<sub>2</sub>O emissions occurred when the soil was converted from pasture to  
183 cropping. The laboratory soil incubation was carried out in 500 ml vials containing 60 g of  
184 soils (oven dry weight equivalent) to investigate the effects of C<sub>2</sub>H<sub>2</sub> on N<sub>2</sub>O emissions and  
185 the abundances of *amoA* genes under different soil temperature and moisture contents. The  
186 same treatments were established as the first experiment, though after the pre-incubation the  
187 samples were incubated at three temperatures (15°C, 25°C, 35°C) and two soil moisture  
188 levels (50% and 70% WFPS). During the 21-day incubation, soil, gas samples and *amoA*  
189 genes abundance were analysed as well.

### 190 **2.3 Calculations**

191 The equation developed by Persson and Wirén (1995) was used to calculate the net  
192 nitrification rates over the incubation time (21 days)

$$193 \quad n = [(\text{NO}_3^- \text{-N})_{d21} - (\text{NO}_3^- \text{-N})_{d0}] / 21$$

194 where  $(\text{NO}_3^- \text{-N})_{d0}$  and  $(\text{NO}_3^- \text{-N})_{d21}$  are the  $\text{NO}_3^- \text{-N}$  concentrations in the soil on days 0 and 21,  
195 respectively.

## 196 **2.4 Statistical Analyses**

197 Data were analysed using SPSS 19 and means were compared using one-way analysis of  
198 variance (ANOVA) between treatments to test the variance with a level of significance of  $P <$   
199 0.05. Spearman correlation analysis was performed to test the relationships between  $\text{N}_2\text{O}$ , the  
200 abundances of AOA and AOB under different conditions.

201

## 202 **3. Results**

### 203 **3.1 Soil microcosm incubation with different land-use agricultural soils**

204 The physical and chemical properties of the soil samples were highly variable across the  
205 different sampling sites (Table 1). Briefly, all soils except the vegetable soil were acidic ( $\text{pH}$   
206  $\leq 6.2$ ). The cropping soil had the highest organic C content (6.2%), while the vegetable soil  
207 had the lowest (0.8%). Nitrate-N ( $\text{NO}_3^- \text{-N}$ ) was the dominant inorganic nitrogen ranging from  
208 8.8 to 93  $\text{mg kg}^{-1}$  soil, with the highest value recorded in the cropping soil. Sugarcane and  
209 vegetable soils had a sandy texture, but cropping and dairy pasture soils were loam. The  
210 cropping soil has the highest clay content at 19%.

211 The highest cumulative  $\text{N}_2\text{O}$  emission was 3204.5 ( $\pm 52.5$ )  $\text{mg N kg}^{-1}$  soil in the cropping soil,  
212 compared to 122.4 ( $\pm 5.1$ )  $\text{mg N kg}^{-1}$  soil in the sugarcane soil, which was the lowest emission  
213 from fertilizer treatments (Table 2). Acetylene addition significantly reduced the cumulative

214 N<sub>2</sub>O emissions by 28.6% to 54.6% (Table 2). The efficacy of C<sub>2</sub>H<sub>2</sub> on reducing cumulative  
215 N<sub>2</sub>O emissions was ordered by dairy pasture (28.6%) < sugarcane (37.6%) < vegetable  
216 (44.9%) < cropping (54.6%).

217 Changes in the NO<sub>3</sub><sup>-</sup>-N concentrations of the four soils during the incubation period are  
218 shown in Figure 1. The NO<sub>3</sub><sup>-</sup>-N concentrations showed an increasing trend in all soils from  
219 fertilized treatments. While C<sub>2</sub>H<sub>2</sub> addition significantly reduced the NO<sub>3</sub><sup>-</sup>-N concentrations in  
220 all the soils ( $P < 0.05$ ), the efficacy of C<sub>2</sub>H<sub>2</sub> varied among soils. For example, the average net  
221 nitrification rates over 21 days in the dairy pasture soil were 1.8 ( $\pm 0.1$ ) mg NO<sub>3</sub>-N kg<sup>-1</sup> soil  
222 day<sup>-1</sup> for the fertilized treatment and 0 mg NO<sub>3</sub>-N kg<sup>-1</sup> soil day<sup>-1</sup> for the fertilizer plus C<sub>2</sub>H<sub>2</sub>  
223 treatment, while in the vegetable soil they were 2.1 ( $\pm 0.4$ ) mg NO<sub>3</sub><sup>-</sup>-N kg<sup>-1</sup> soil day<sup>-1</sup> for the  
224 fertilized treatment and 1.5 ( $\pm 0.3$ ) mg NO<sub>3</sub><sup>-</sup>-N kg<sup>-1</sup> soil day<sup>-1</sup> for the fertilizer plus C<sub>2</sub>H<sub>2</sub>  
225 treatment (Table 2).

226 The abundance of AOB before adding fertilizer varied greatly from  $2.7 \times 10^5$  copies g<sup>-1</sup> soil  
227 in the dairy pasture soil to  $2.6 \times 10^6$  copies g<sup>-1</sup> soil in the vegetable soil, and was evidently  
228 higher in the vegetable soil than in other soils (Figure 2). The addition of fertilizer  
229 significantly increased AOB abundance in all soils on day 7, while C<sub>2</sub>H<sub>2</sub> significantly  
230 decreased the AOB abundance in cropping, sugarcane and vegetable soils on day 7. In the  
231 dairy pasture soil, the abundance of AOB significantly decreased compared to the fertilizer  
232 treatment on day 14 ( $P < 0.05$ ). Before fertilizer addition, the AOA abundance was highest in  
233 the vegetable soil up to  $1.5 \times 10^8$  copies g<sup>-1</sup> soil and much lower in the cropping, sugarcane,  
234 and dairy pasture soils ranging between  $5.8 \times 10^6$  and  $1.1 \times 10^7$  copies g<sup>-1</sup> soil (Figure 3).  
235 Addition of fertilizer significantly increased AOA abundance in all soils except the vegetable  
236 soil. There was a decreasing trend in AOA abundance during incubation in the vegetable soil.  
237 AOA growth was inhibited by C<sub>2</sub>H<sub>2</sub> addition in cropping, sugarcane and dairy pasture soils,

238 however, there was no significant difference in the AOA abundance between treatments  
239 during the incubation in the vegetable soil (Figure 3).

### 240 **3.2 Soil microcosm incubation under different temperature and soil water contents**

241 The  $\text{NO}_3^-$ -N concentrations in fertilized treatments increased under 50% WFPS with  
242 increasing soil temperature except at 35°C (Figure 4). At 70% WFPS, the soil  $\text{NO}_3^-$ -N  
243 concentrations decreased with increasing temperature over the incubation period. The  
244 concentrations of  $\text{NO}_3^-$ -N remained largely unchanged in the fertilizer plus  $\text{C}_2\text{H}_2$  treatments at  
245 different conditions except at 35°C with 70% WFPS. Under 35°C with 70% WFPS,  $\text{NO}_3^-$ -N  
246 concentration significantly ( $P < 0.05$ ) decreased after day 14 in the fertilizer plus  $\text{C}_2\text{H}_2$   
247 treatment. Acetylene addition substantially reduced the nitrification rate by 53% - 100%,  
248 suggesting  $\text{C}_2\text{H}_2$  was capable of inhibiting nitrification under all conditions (Table 3), with  
249 the highest inhibition effect found at 25°C and 50% WFPS. The efficacy of  $\text{C}_2\text{H}_2$  on  
250 nitrification decreased with increasing soil temperature and moisture except at 15°C.

251 Total  $\text{N}_2\text{O}$  emission increased with increasing temperature and moisture (Table 3). When  
252  $\text{C}_2\text{H}_2$  was applied into soil microcosms, cumulative  $\text{N}_2\text{O}$  emission was reduced by 0–86.7%.  
253 There was no difference in cumulative  $\text{N}_2\text{O}$  emission between the fertilizer and fertilizer plus  
254  $\text{C}_2\text{H}_2$  treatments at 15°C and 50% WFPS. The inhibition of  $\text{C}_2\text{H}_2$  on cumulative  $\text{N}_2\text{O}$   
255 emission increased with increasing temperature at 50% WFPS. In contrast, when soil was  
256 wetted to 70% WFPS, the inhibition of  $\text{C}_2\text{H}_2$  on cumulative  $\text{N}_2\text{O}$  emission decreased with  
257 increasing temperature. At the same soil temperature, the efficacy of  $\text{C}_2\text{H}_2$  on total  $\text{N}_2\text{O}$   
258 emission decreased with increasing moisture except at 15°C.

259 The changes in abundances of AOB and AOA *amoA* genes are shown in Figures 5 and 6. In  
260 the fertilized treatment, AOA and AOB *amoA* gene copy numbers ranged from  $1.0 \times 10^7$  to  
261  $3.3 \times 10^8$  and  $1.8 \times 10^6$  to  $1.7 \times 10^7$  copies  $\text{g}^{-1}$  soil, respectively, and increased with

262 increasing soil moisture. However, both AOA and AOB abundance decreased with increasing  
263 soil temperature. AOA *amoA* genes at different soil temperatures and moistures were 3.3-107  
264 times more abundant than AOB *amoA* genes. AOA *amoA* gene abundance comprised 93–  
265 96% and 76–92% of the total *amoA* gene abundance at 15°C to 35°C, respectively. The ratio  
266 of AOA to AOB decreased significantly with increasing soil temperature ( $P < 0.05$ ).  
267 Acetylene application significantly ( $P < 0.05$ ) decreased AOA abundance by 48% (15°C and  
268 70% WFPS) and reduced AOB abundance by 93% (25°C and 70% WFPS) relative to that in  
269 the fertilizer treatments on day 21. However, no significant effect of C<sub>2</sub>H<sub>2</sub> addition on AOA  
270 or AOB *amoA* gene abundances was observed at 35°C, 50% WFPS and 15°C, 70% WFPS,  
271 respectively.

272

#### 273 **4. Discussion**

274 This study investigated the changes in N<sub>2</sub>O production, nitrification rates, and abundance of  
275 ammonia oxidizers after addition of the nitrification inhibitor C<sub>2</sub>H<sub>2</sub> at various conditions. Our  
276 experiments showed that incubation of soil with 1% v/v C<sub>2</sub>H<sub>2</sub> resulted in complete or partial  
277 inhibition of nitrification, N<sub>2</sub>O emission, and AOA or AOB growth in all conditions.

##### 278 **4.1 Inhibitory effects of C<sub>2</sub>H<sub>2</sub> in different soils.**

279 The results demonstrate that C<sub>2</sub>H<sub>2</sub> has different efficacy in inhibiting nitrification and N<sub>2</sub>O  
280 emission in different soils. Nitrification can be inhibited by C<sub>2</sub>H<sub>2</sub>, implying AMO-dependent  
281 (and presumably autotrophic) nitrification, as frequently observed in many acidic soils (De  
282 Boer and Kowalchuk, 2001). In this study, C<sub>2</sub>H<sub>2</sub> was much more effective in inhibiting  
283 nitrification and cumulative N<sub>2</sub>O emission in acidic soils than the alkaline soil, and the acidic  
284 cropping soil had the highest inhibitory effect among the four soils. Soil pH might be a key  
285 factor in C<sub>2</sub>H<sub>2</sub> inhibitory effects because pH is a strong environmental determinant of AOA

286 and AOB abundance. A number of studies have shown niche separation based on pH with  
287 AOA favoured in acidic soils and AOB in alkaline. The abundances of AOA and AOB in our  
288 acidic and alkaline soils supported this observation (as reviewed in He et al., 2012). Our  
289 findings are consistent with these previous studies, and supported the clear niche separation  
290 between AOA and AOB shaped by soil pH. We suggest that in the alkaline soil, nitrifiers can  
291 be protected within microenvironments due to the high amounts of carbonates present in the  
292 alkaline condition.

293 Besides soil pH, other soil physicochemical traits may also have the potential effects on C<sub>2</sub>H<sub>2</sub>  
294 inhibitory efficacy. The dairy pasture soil had the lowest inhibition by C<sub>2</sub>H<sub>2</sub> on cumulative  
295 N<sub>2</sub>O emission (28.6%) while there was a complete inhibition (100%) on nitrification. It is  
296 possibly that because the dairy pasture soil had the highest ratio of C to NO<sub>3</sub><sup>-</sup>, that there might  
297 be a loss in inhibitory efficiency on N<sub>2</sub>O production. Previous studies have shown that the use  
298 of C<sub>2</sub>H<sub>2</sub> to inhibit N<sub>2</sub>O production and reduction has been problematic for soils with a very  
299 high ratio of C to NO<sub>3</sub><sup>-</sup> (Davidson et al., 1986). Another potential explanation for low  
300 inhibition of C<sub>2</sub>H<sub>2</sub> on cumulative N<sub>2</sub>O emission is that the release of N<sub>2</sub>O was due to  
301 processes other than nitrification, such as denitrification and heterotrophic nitrification,  
302 rendering the C<sub>2</sub>H<sub>2</sub> unable to effect N<sub>2</sub>O emitted from either denitrification or heterotrophic  
303 nitrification. The dairy pasture soil had a high organic carbon content which might result in  
304 high denitrification-related N<sub>2</sub>O because of the high availability of soil organic C for  
305 denitrifiers. Wan et al. (2009) also demonstrated that more N<sub>2</sub>O was released from  
306 denitrification than from nitrification in a soil with high organic carbon. Regarding the  
307 highest inhibitory effect on nitrification in dairy pasture soil, this may be due to nitrification  
308 in the dairy pasture soil being largely autotrophic. This can be supported by our previous  
309 study (Liu et al., 2015b) where we indicated that nitrification was primarily autotrophic with  
310 heterotrophic nitrification accounting for only 20%. In the alkaline sandy soil, C<sub>2</sub>H<sub>2</sub>

311 inhibition of  $\text{NO}_3^-$  production was only 29%, thus 1% v/v of  $\text{C}_2\text{H}_2$  may be insufficient to  
312 inhibit nitrification completely in this soil, nevertheless, most studies have indicated that this  
313 partial pressure of  $\text{C}_2\text{H}_2$  is clearly sufficient for a complete inhibition. This result contrasts  
314 with our previous study (Liu et al., 2015a) where we found that  $\text{C}_2\text{H}_2$  addition produced  
315 100% nitrification inhibition in an alkaline clay loam soil. The difference between these two  
316 studies may be because the two alkaline soils differed in soil texture.

317 Overall, the four soils chosen from different land uses had very differing physicochemical  
318 properties. Of the properties characterised for the four soils, soil pH, texture, organic C and  
319  $\text{NO}_3^-$  content might be the key factors influencing the effectiveness of  $\text{C}_2\text{H}_2$  on nitrification  
320 and cumulative  $\text{N}_2\text{O}$  emission. Many recent studies also showed that soil physicochemical  
321 properties affected the efficacy of other NIs, such as DMPP and DCD, with their efficacy  
322 diminished with the addition of soil organic matter (Fisk et al., 2015) and decreased with  
323 higher clay content (Marsden et al., 2016). Although it is not possible to clearly discern the  
324 effects of land use from this experimental design, we speculate that land use may affect the  
325 efficacy of  $\text{C}_2\text{H}_2$  to inhibit nitrification and  $\text{N}_2\text{O}$  emissions. However, a multiple regression  
326 including more soil physicochemical properties is still necessary.

327  $\text{C}_2\text{H}_2$  addition was able to inhibit nitrification and  $\text{N}_2\text{O}$  emission in all soils, albeit to varying  
328 degrees. It is unclear however if this reduction in  $\text{N}_2\text{O}$  emission and nitrification is linked to  
329 soil microbial communities due to adding  $\text{C}_2\text{H}_2$  and further investigation is needed to  
330 examine if  $\text{C}_2\text{H}_2$  stimulates changes in microbial community population and activity.

331 As observed previously (He et al., 2007; Shen et al., 2008; Levc̃nik-Höfferle et al., 2012;  
332 Liu et al., 2015a), AOA grow better in acidic soils, while AOB thrive better in alkaline soils,  
333 which was supported by our study. In our study, the acidic sugarcane soil had the highest  
334 AOA abundance, while the highest AOB population was found in the alkaline vegetable soil.

335 Growth of AOB, and not of AOA, has been linked to soil nitrification activity with high  
336 levels of ammonium (Di et al., 2009; Jia and Conrad, 2009). In contrast, growth of AOA is  
337 associated with nitrification in soils with a continual supply of ammonia at low concentration  
338 through the mineralisation of organic matter (Offre et al., 2009). The difference in substrate  
339 preferences could affect the distribution of AOA and AOB in soils and further affect the  
340 efficacy of C<sub>2</sub>H<sub>2</sub> across different soils. Our study indicated that the application of C<sub>2</sub>H<sub>2</sub> could  
341 block AOA and AOB growth but to different extents. In acidic cropping, sugarcane and dairy  
342 pasture soils, C<sub>2</sub>H<sub>2</sub> addition significantly decreased AOA and AOB abundance, suggesting  
343 both AOA and AOB mediated nitrification in these soils and were sensitive to C<sub>2</sub>H<sub>2</sub>. The  
344 observed significant decrease in N<sub>2</sub>O emission and nitrification by the addition of C<sub>2</sub>H<sub>2</sub> in the  
345 cropping and sugarcane soils is mostly likely caused by the inhibitory effect of C<sub>2</sub>H<sub>2</sub> on both  
346 AOA and AOB growth. However, there was no inhibitory effect on AOA in vegetable soil  
347 and AOB on day 7 in dairy pasture soil, suggesting that AOB rather than AOA were involved  
348 in nitrification in the alkaline vegetable soil while in the dairy pasture soil AOB might be less  
349 important in nitrification than AOA. Moreover, it is possible that C<sub>2</sub>H<sub>2</sub> application may also  
350 change active strains of ammonia oxidizers and different effectiveness possibly due to  
351 different sensitive strains appearing in soils (Belser et al., 1980), and this needs further  
352 investigation.

#### 353 **4.2 Inhibitory effects of C<sub>2</sub>H<sub>2</sub> at different incubation conditions.**

354 From both previous studies (Kool et al., 2010; Liu et al., 2017) and the current study, soil  
355 water content and temperature were the predominant factors regulating N<sub>2</sub>O emission from  
356 soils. The cumulative N<sub>2</sub>O emissions increased with increasing WFPS and temperature in the  
357 cropping soil (Table 3). The significant increase in emissions at 35°C between 50% and 70%  
358 WFPS is probably because these incubation conditions favoured nitrifying and denitrifying  
359 enzyme synthesis (Liu et al., 2017), and 35°C at 70% WFPS is the most suitable condition for

360 microbial activity. The degree of inhibition of cumulative N<sub>2</sub>O emission and nitrification by  
361 C<sub>2</sub>H<sub>2</sub> was shown to vary across the different incubation conditions, indicating that  
362 temperature and moisture content significantly affect C<sub>2</sub>H<sub>2</sub> efficacy. At lower soil  
363 temperature (15°C) and in drier soil (50% WFPS), C<sub>2</sub>H<sub>2</sub> addition had no inhibitory effect on  
364 the total N<sub>2</sub>O emissions, suggesting that nitrification may be heterotrophic under these  
365 conditions in this soil. This result is supported by Liu et al. (2015c) where under 15°C and  
366 50% WFPS incubation, nitrification was found to be predominantly heterotrophic. The low  
367 efficacy of C<sub>2</sub>H<sub>2</sub> to inhibit N<sub>2</sub>O emission under wetter soils (70% WFPS) is probably because  
368 more N<sub>2</sub>O was produced from denitrification. Another possibility for the low efficacy of  
369 C<sub>2</sub>H<sub>2</sub> at the lower soil temperature of 15°C is that C<sub>2</sub>H<sub>2</sub> may be unable to form a reactive  
370 epoxide to inactivate the AMO enzyme (Hynes and Knowles, 1978).

371 Compared to 70% WFPS, the inhibitory effect of C<sub>2</sub>H<sub>2</sub> was much greatest at inhibiting  
372 nitrification and cumulative N<sub>2</sub>O emission at 50% WFPS except at 15°C. This is possibly  
373 because 25°C and 35°C with 50% WFPS are more suitable for nitrification to occur (Garrido  
374 et al., 2002; Huang et al., 2014). During incubation, the nitrification rates in the fertilizer  
375 treatments were similar at low water content (50% WFPS) regardless of soil temperature,  
376 while the effectiveness of C<sub>2</sub>H<sub>2</sub> on inhibiting nitrification was different (Table 3). This  
377 possibly indicates that concentrations of C<sub>2</sub>H<sub>2</sub> may need to be adjusted for optimal inhibition  
378 under different soil temperature and moisture.

379 AOA and AOB reduced in abundance with varying degrees after C<sub>2</sub>H<sub>2</sub> addition under  
380 different environmental conditions, which might be attributed to the differential sensitivity of  
381 AOA and AOB to C<sub>2</sub>H<sub>2</sub> under different conditions. Under 15°C, 50% and 70% WFPS, C<sub>2</sub>H<sub>2</sub>  
382 showed no effect on AOB populations. One possible explanation is that under lower soil  
383 temperature (15°C), the active strains of AOB were less abundant than under higher  
384 temperature resulting in less sensitivity to C<sub>2</sub>H<sub>2</sub> addition. Another possible explanation is

385 AOA may prefer lower a soil temperature and be more involved in nitrification at 15°C than  
386 AOB.

387 The underlying mechanism of how C<sub>2</sub>H<sub>2</sub> targets AOA remains largely unknown. Acetylene  
388 might suppress the growth of *amoA*-containing archaea by inactivating the archaeal AMO  
389 protein, as demonstrated in AOB (Hyman and Wood 1985). However, Offre et al. (2009)  
390 demonstrated that the enzymes and metabolic pathways of AOA might differ significantly  
391 from those of AOB, in which C<sub>2</sub>H<sub>2</sub> interferes with the AMO protein. Future studies based on  
392 soil RNA and pure cultures are therefore necessary to elucidate the mechanism by which  
393 C<sub>2</sub>H<sub>2</sub> blocks the growth of AOA.

394 In the first incubation, we found that different soils have different C<sub>2</sub>H<sub>2</sub> efficacy. Therefore,  
395 we cannot ascertain whether the findings of the incubation study at different temperatures and  
396 moistures can be extended to other soils. Future work with alkaline soils or a larger range of  
397 soil properties is definitely needed to clarify this question.

398

## 399 **5. Conclusions**

400 In conclusion, C<sub>2</sub>H<sub>2</sub> was more effective in inhibiting nitrification and N<sub>2</sub>O emissions in acidic  
401 soils and under drier condition (50% WFPS). Soil pH, C content, texture, temperature,  
402 moisture and land use might be important factors affecting the efficacy of C<sub>2</sub>H<sub>2</sub>. The various  
403 C<sub>2</sub>H<sub>2</sub> inhibitory effects on nitrification and N<sub>2</sub>O production were also linked to different  
404 responses of the ammonia oxidizers to C<sub>2</sub>H<sub>2</sub>. Therefore, since the effect of C<sub>2</sub>H<sub>2</sub> varied with  
405 soils and environmental conditions, the assumption that N<sub>2</sub>O production and nitrification can  
406 be affected by low concentrations of C<sub>2</sub>H<sub>2</sub> may need verification for specific soils and  
407 conditions of interest.

408

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414

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