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**Title:** The effect of temperature and moisture on the source of N<sub>2</sub>O and contributions from ammonia oxidizers in an agricultural soil

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## **ABSTRACT**

In recent years, identification of the microbial sources responsible for soil N<sub>2</sub>O production has substantially advanced with the development of isotope enrichment techniques, selective inhibitors, mathematical models, and the discoveries of specific N-cycling functional genes. However, little information is available to effectively quantify the N<sub>2</sub>O produced from different microbial pathways (e.g. nitrification and denitrification). Here, a <sup>15</sup>N-tracing incubation experiment was conducted under controlled laboratory conditions [50%, 70% and 85% water-filled pore space (WFPS) at 25°C and 35°C]. Nitrification was the main contributor to N<sub>2</sub>O production. At 50%, 70% and 85% WFPS, nitrification contributed 87%, 80% and 53% of total N<sub>2</sub>O production, respectively, at 25°C, and 86%, 74% and 33% at 35°C. The proportion of nitrified N as N<sub>2</sub>O (P<sub>N<sub>2</sub>O</sub>) increased with temperature and moisture, except for 85% WFPS, when P<sub>N<sub>2</sub>O</sub> was lower at 35°C than at 25°C. Ammonia-oxidizing archaea (AOA) were the dominant ammonia oxidizers, but both AOA and ammonia-oxidizing bacteria (AOB) were related to N<sub>2</sub>O emitted from nitrification. AOA and AOB abundance was significantly influenced by soil moisture, more so than temperature, and decreased with increasing moisture content. These findings can be used to develop better models for simulating N<sub>2</sub>O from nitrification to inform soil management practices for improving N use efficiency.

**Keywords** Nitrification, ammonia-oxidizing archaea, ammonia-oxidizing bacteria, <sup>15</sup>N isotope technique, model

## Introduction

Nitrous oxide ( $\text{N}_2\text{O}$ ) is a trace greenhouse gas that contributes to global warming and stratospheric ozone depletion. Soils are the largest source of  $\text{N}_2\text{O}$ , accounting for approximately 65% of the anthropogenic atmospheric loading of  $\text{N}_2\text{O}$  (IPCC 2007).  $\text{N}_2\text{O}$  is generated through several microbiological processes in soils including nitrification, denitrification, dissimilatory nitrate reduction to ammonium (DNRA) and nitrifier denitrification (Wrage et al. 2005; Li et al. 2014; Hu et al. 2015), though the primary  $\text{N}_2\text{O}$  sources are believed to be nitrification and denitrification. Ammonia oxidation, the first step of nitrification catalyzed by the *amoA* gene encoding the ammonia monooxygenase (AMO) enzyme, is performed by two distinct types of nitrifiers: ammonia-oxidizing bacteria (AOB) (Purkhold et al. 2000) and ammonia-oxidizing archaea (AOA) (Brochier-Armanet et al. 2008; Watanabe et al. 2011). Ammonia oxidation is the rate-limiting step of the nitrification process and thus is critical for nitrification-derived  $\text{N}_2\text{O}$  emissions. Denitrification is the process in which nitrate ( $\text{NO}_3^-$ ) is reduced to  $\text{N}_2$  under anaerobic conditions by denitrifiers, and  $\text{N}_2\text{O}$  is an intermediate.

There have been many studies to investigate and distinguish the  $\text{N}_2\text{O}$  production pathways by using stable isotope ( $^{15}\text{N}$  and  $^{18}\text{O}$ ) tracer technique (Wrage et al. 2005). Some of these studies used selective inhibitors (e.g. Bateman and Baggs 2005), and mathematical modelling (Müller et al. 2014). However, each methodology was used alone cannot provide a precise clarification of the pathways, and the microbial mechanisms and the relative contribution of each pathway are poorly understood as well (Hu et al. 2015).

Furthermore, there are multiple factors regulating  $\text{N}_2\text{O}$  emissions from soils, such as agricultural practices, climatic conditions and soil properties (Mørkved et al. 2007; Livesley

et al. 2008; Butterbach-Bahl et al. 2013; Yamamoto et al. 2014), adding uncertainty in predicting N<sub>2</sub>O production and consumption pathways in complex soil environments.

Soil water content and temperature are the predominant factors regulating N<sub>2</sub>O production and the contribution of nitrification and denitrification to N<sub>2</sub>O emission from soils (Cheng et al. 2014; Hu et al. 2015). It was reported that N<sub>2</sub>O emitted from fields amended with N fertilizer was affected mainly by soil moisture conditions (Zheng et al. 2000; Huang et al. 2004). Increasing N<sub>2</sub>O emissions with increasing temperature from 10°C to 15°C indicated that N<sub>2</sub>O production was sensitive to soil temperature (Lang et al. 2011). Allen et al. (2010) showed that N<sub>2</sub>O emissions were higher during wet and hot months than in cool and dry months. A higher temperature was favourable for nitrification within a certain range (25°C to 35°C) (Haynes 1986). Generally, the rate of N<sub>2</sub>O production is low below 40% water-filled pore space (WFPS), but increases rapidly from around 50% to 65% WFPS in the absence of other limiting factors, such as NH<sub>4</sub><sup>+</sup> supply (Dalal et al. 2003; Bateman and Baggs 2005; Mathieu et al. 2006. ) It is a common belief that nitrification is the principal source of N<sub>2</sub>O in aerobic conditions while denitrification the predominant contributor to N<sub>2</sub>O production in anaerobic conditions (Mathieu et al. 2006). It has been reported that nitrification could contribute around 80% of total N<sub>2</sub>O emissions depending on soil temperature and moisture (Godde and Conrad 1999). It is usually considered that the maximum N<sub>2</sub>O production from denitrification and/or nitrification will occur when soil moisture content falls within 55-85% WFPS (Granli and Bøeckman 1994). A higher soil water content stimulates the reduction of N<sub>2</sub>O to N<sub>2</sub> in denitrification, thus the ratio of N<sub>2</sub>/N<sub>2</sub>O increases with soil moisture content above 75% WFPS (Davidson 1992; Weier et al. 1993; Saggar et al. 2013). The effect of a single factor, temperature or moisture, on nitrification and denitrification sourced N<sub>2</sub>O emissions has been well documented (Liu et al.

2016). However, limited information is available on the interaction of these factors regulating N<sub>2</sub>O pathways and the correlations between nitrification-derived N<sub>2</sub>O and nitrifiers under different environmental conditions.

There is emerging evidence that the soil microbial communities (AOA and AOB) play a vital role in determining the consumption and production of N<sub>2</sub>O (Di et al. 2009; Jia and Conrad 2009; Leininger et al. 2006; Mertens et al. 2009; Offre et al. 2009; Tourna et al. 2008; Zhang et al. 2012; Akiyama et al. 2014). However, there are some controversial conclusions on the contributions of AOA and AOB in nitrification. Di et al. (2010) demonstrated that N<sub>2</sub>O flux was associated with the dynamics of AOB in N-rich grassland soils. Jia and Conrad (2009) reported that AOB was more active in ammonia oxidation than AOA in agricultural soils even though the AOA populations were much more abundant than the AOB populations. However Andert et al. (2011) did not observe a significant relationship between AOA or AOB abundance and N<sub>2</sub>O emission in boreal peat soils. Therefore, more research is required, particularly under variable conditions, to improve our understanding of possible impacts on AOA and AOB abundance dynamics and N<sub>2</sub>O production in agricultural soils.

The objectives of this study were: 1) to investigate how soil moisture and temperature affect the contribution of nitrification and denitrification to N<sub>2</sub>O emissions and the abundance of AOA and AOB, and 2) to explore the relationship between N<sub>2</sub>O emission and AOA and AOB abundance.

## **Materials and methods**

### Site description and soil sampling

Surface soil (0-10 cm) was collected from the Victorian Department of Economic Development, Jobs, Transport and Resources research farm 9 km south of Hamilton, Victoria, Australia (38.32° S, 142.07° E). The study site is in a high rainfall zone (688 mm per annum) and subjected to two land management practices: cropping and pasture. Previous studies at the site found that high N<sub>2</sub>O emissions occurred when the soil was converted from pasture to cropping (Officer et al. 2008, 2012). We collected soil from the cropping system after harvest (January 2012) when wheat-stubble covered the soil surface. The soil is classified as a Chromosol (Isbell 1996), with an acidic gravelly loam topsoil, and contained 0.52% total N, 5.2% organic C, 19% clay, 38% silt and 43% sand. The CEC was 9.42 meq/100g and the soil pH (H<sub>2</sub>O) was 4.5. The original exchangeable NH<sub>4</sub><sup>+</sup>-N and NO<sub>3</sub><sup>-</sup>-N were 13 and 93 mg kg<sup>-1</sup> soil, respectively.

## Soil incubations

### Design of soil microcosms

Collected soil was air-dried and remaining roots and leaves were removed with tweezers. The soil was then ground and sieved (< 2 mm) prior to establishment of the microcosm incubation. Subsamples were taken to determine gravimetric moisture content and for chemical analyses. Replicated samples of soil (60g oven-dry equivalent) were placed into 500 ml incubation vials. Distilled water was added to soil to just under the final moisture content (50%, 70% and 85% WFPS) and the microcosms were pre-incubated at 25°C and 35°C for 3 weeks to stabilise the soil microbial communities and prevent priming effects associated with wetting events. After pre-incubation, 2 ml solution were added to each incubation vessel to reach the targeted soil moisture contents. The treatments contained 100 mg N kg<sup>-1</sup> as exchangeable NH<sub>4</sub><sup>+</sup>-N and 50 mg N kg<sup>-1</sup> as NO<sub>3</sub><sup>-</sup>-N, which were added to the soil as 1) <sup>15</sup>NH<sub>4</sub>Cl (10 atom%

$^{15}\text{N}$ ) +  $\text{KNO}_3$  and 2)  $\text{NH}_4\text{Cl}$  +  $\text{K}^{15}\text{NO}_3$  (10 atom%  $^{15}\text{N}$ ). The soils were incubated for 3 weeks under 50%, 70% and 85% WFPS at 25°C and 35°C. The soil samples were aerated by removing the caps for 30 min and the headspace was flushed with fresh air every 3 days and water was replenished every 3 days.

### Gas sampling and analysis

Gas samples (20 ml) for  $\text{N}_2\text{O}$  and  $\text{CO}_2$  analysis were taken from the headspace of the vials on 0, 4, 7, 12, 15 and 21 days after fertilizer application. Before each gas collection, the vials were opened, flushed with ambient air to ensure ambient  $\text{N}_2\text{O}$  and  $\text{CO}_2$  concentrations in the headspace. The vials were sealed with gas collection caps and gas samples (20 ml) were taken using gastight syringes at 0, 8, 24, 48 and 72 hours after vial closure, to determine the  $\text{N}_2\text{O}$  and  $\text{CO}_2$  flux, with each sample transferred into a pre-evacuated 12 ml exetainer (Exetainer®, Labco Ltd., Lampeter, Ceredigion, UK). The 20 ml gas samples were analyzed for  $\text{N}_2\text{O}$  and  $\text{CO}_2$  by gas chromatography (Agilent 7890A) using an ECD ( $\text{N}_2\text{O}$ ) and TCD ( $\text{CO}_2$ ) detector, respectively. A single 70 ml gas samples was collected and injected into a 60 ml evacuated vial for  $^{15}\text{N}$ - $\text{N}_2\text{O}$  by Isotope Ratio Mass Spectrometry (IRMS) (Hydra 20–20, SerCon, Crewe, UK) on days 0, 4, 7 and 15 following flux sample collection (72 hours).

### Soil Sampling and analysis

Soils were destructively sampled for soil mineral N analysis on days 0, 7, 15 and 21 and for  $^{15}\text{N}$  on days 0, 7, 15 immediately after gas sampling. Two grams of soil was also collected at each sampling time for DNA extraction. Soil (50 g) was shaken with 250 ml 2M KCl for 1 h at 200 rpm at room temperature, and the extracts were filtered through a Whatman 42 qualitative filter paper and analysed for mineral N (exchangeable  $\text{NH}_4^+$  and  $\text{NO}_3^-$ ), using a

segmented-flow analyzer (Skalar, SAN++), and  $^{15}\text{N}$  abundance in mineral N. The  $^{15}\text{N}$  enrichment of exchangeable  $\text{NH}_4^+$  and  $\text{NO}_3^-$  was determined by a micro-diffusion method as described by Saghir et al. (1993), with the modification that an acidified filter paper disc was used instead of the petri dish of acid to absorb  $\text{NH}_3$ , and analysed by the IRMS (Hydra 20–20, SerCon, Crewe, UK).

#### Soil DNA extraction and quantitative PCR analysis

The PowerSoil DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) was used for DNA extraction, following the manufacturer's instructions, of soil samples collected on days 0, 7, and 15. The quantity and quality of the DNA extracted was assessed using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The *amoA* gene copy numbers were quantified from triplicate samples using quantitative PCR (qPCR) with the universal primer sets to target AOA (Francis et al. 2005) and AOB (Rotthauwe et al. 1997). Each AOA *amoA* qPCR reaction was performed in a 20  $\mu\text{l}$  volume containing 10  $\mu\text{l}$  SensiFAST SYBR green (Bio-Rad Laboratories, USA), 0.5  $\mu\text{M}$  of each primer and 2  $\mu\text{l}$  of 10-fold dilution DNA template (1-10 ng). Amplification conditions were as follows: 95°C for 3 mins, 40 cycles of 5 s at 95°C, 30 s at 60°C, and 45 s at 72°C. Each AOB *amoA* qPCR reaction was performed in a 10  $\mu\text{l}$  volume containing 5  $\mu\text{l}$  iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, USA), 0.6  $\mu\text{M}$  of each primer and 2  $\mu\text{l}$  of 10-fold dilution DNA template (1-10 ng). Amplification conditions were the same as the AOA qPCR assay. A known copy number of plasmid DNA for AOA or AOB was used to create a standard curve. For all assays, qPCR efficiency was 90-100% and  $r^2$  was 0.96-0.99.

#### Calculations

N<sub>2</sub>O fluxes were calculated according to the following equation (Lan et al. 2013):

$$F = \rho \times \frac{V}{A} \times \frac{\Delta c}{\Delta t} \times \frac{273}{273 + T} \quad (1)$$

where F is the gas flux in  $\mu\text{g N}_2\text{O-N m}^2 \text{ d}^{-1}$ ,  $\rho$  is the density of N<sub>2</sub>O under the standard state ( $\text{kg m}^{-3}$ ), V is the volume of the head space ( $\text{m}^3$ ), A is the area of the vial ( $\text{m}^2$ ),  $\frac{\Delta c}{\Delta t}$  is the change in gas concentration (c) per unit of time (t) in  $\text{ppb d}^{-1}$ , and T is the air temperature within the vessel ( $^{\circ}\text{K}$ ).

The gross nitrification rate (n) was determined by the <sup>15</sup>N dilution technique (Kirkham and Bartholomew 1954; Barraclough and Puri 1995).

The relative contributions of denitrification (Cd) and nitrification (Cn) to N<sub>2</sub>O production were calculated using the method of Stevens et al. (1997). Briefly, if there is only one denitrifying pool of NO<sub>3</sub>, simultaneous nitrification and denitrification can be confirmed by examining the distribution of <sup>15</sup>N atoms within the N<sub>2</sub>O molecules, particularly in the treatment pair where NO<sub>3</sub> is labelled. When the NH<sub>4</sub> pool is at natural abundance and the NO<sub>3</sub> pool is enriched with <sup>15</sup>N, nitrification will produce N<sub>2</sub>O at natural abundance while denitrification will produce N<sub>2</sub>O of the same enrichment as the NO<sub>3</sub> pool from which it was derived. The relative contributions of two processes can be calculated following the equations:

$$\text{Cd} = (a_{\text{N}_2\text{O}} - a_{\text{NH}_4}) / (a_{\text{NO}_3} - a_{\text{NH}_4}) \quad (2)$$

$$\text{Cn} = 1 - \text{Cd} \quad (3)$$

where  $a_{\text{N}_2\text{O}}$  is the <sup>15</sup>N atom% enrichment of N<sub>2</sub>O,  $a_{\text{NO}_3}$  is the <sup>15</sup>N atom% enrichment in NO<sub>3</sub><sup>-</sup> pool, and  $a_{\text{NH}_4}$  is the <sup>15</sup>N atom% enrichment in NH<sub>4</sub><sup>+</sup> pool. Based on Stevens et al. (1997), the relative contributions of nitrification and denitrification to N<sub>2</sub>O emission were calculated from the <sup>15</sup>N-NO<sub>3</sub> treatment as follows;

N<sub>2</sub>O production from nitrification (N<sub>2</sub>O<sub>n</sub>) was calculated as:

$$\text{N}_2\text{O}_n = \text{Cn} \times \text{N}_2\text{O}_T \quad (4)$$

where  $N_2O_T$  is the total  $N_2O$  emission

$N_2O$  production from denitrification ( $N_2O_d$ ) was calculated as:

$$N_2O_d = Cd \times N_2O_T \quad (5)$$

The proportion of nitrified N emitted as  $N_2O$  ( $P_{N_2O}$ ) was calculated as:

$$P_{N_2O} = N_2O_n / n \quad (6)$$

## Statistical analyses

Data were analysed using SPSS 19 (IBM, USA) and means were compared using one-way ANOVA between treatments to test the variance with a level of significance of  $P < 0.05$ . Significant differences in soil properties and microbial gene abundance levels over time and between treatments were examined by ANOVA. Relationships between measures were assessed by correlation analysis, using Pearson's  $r$  if data were normally distributed and Spearman's  $\rho$  if data were not normally distributed (normality assessed by Shapiro-Wilk's  $W$ ), in both Stata12/SE (StataCorp, USA) and the statistical software package PAST (v2.17; Hammer et al. 2001).

## Results

### $N_2O$ emission under different environmental conditions

There was no significant difference ( $P > 0.05$ ) in  $N_2O$  produced between the  $^{15}NH_4^+$  and  $^{15}NO_3^-$  treatments throughout the incubation period under any conditions (Figure 1).  $N_2O$  production rates increased over the first 7 days and then decreased before stabilising.  $N_2O$  production reached a peak flux on day 4 at 70% and 85% WFPS at 25°C, and on day 3 at 35°C for all moisture treatments (Figure 1). The largest  $N_2O$  peak (48.92 mg  $N_2O$  kg $^{-1}$  d $^{-1}$ ) was detected at 85% WFPS and 25°C. A significantly ( $P < 0.05$ ) higher level of  $N_2O$  was

emitted at each temperature at 70% or 85% WFPS than 50% WFPS. The N<sub>2</sub>O production rates were significantly higher ( $P < 0.05$ ) when the soil temperature was 35°C compared to 25°C, except for the 85% WFPS treatments (Figure 1).

#### Sources of N<sub>2</sub>O under different environmental conditions

The <sup>15</sup>N enrichment of the N<sub>2</sub>O pool consistently remained between the <sup>15</sup>N enrichment of the NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> pools under all conditions (Figures 2 and 3), suggesting that N<sub>2</sub>O was produced by both nitrification and denitrification. In the <sup>15</sup>NH<sub>4</sub>Cl + KNO<sub>3</sub> treatment, the enrichment of the <sup>15</sup>NO<sub>3</sub><sup>-</sup> pool increased during the incubation due to nitrification (Figure 2). In the NH<sub>4</sub>Cl + K<sup>15</sup>NO<sub>3</sub> treatment, the enrichment of the <sup>15</sup>NH<sub>4</sub><sup>+</sup> pool remained close to natural abundance throughout the incubation period and <sup>15</sup>N abundance in the NO<sub>3</sub><sup>-</sup> pool decreased due to dilution by <sup>14</sup>NO<sub>3</sub><sup>-</sup> from nitrification (Figure 3).

Total N<sub>2</sub>O emission (N<sub>2</sub>O<sub>d</sub> + N<sub>2</sub>O<sub>n</sub>) increased with temperature and moisture except for 85% WFPS. At 85% WFPS there was higher total N<sub>2</sub>O production at 25°C than at 35°C, suggesting an interactive effect of temperature and moisture on the relative contribution of nitrification and denitrification to N<sub>2</sub>O emission (Table 1). The contribution of nitrification (C<sub>n</sub>) to N<sub>2</sub>O production decreased with increasing temperature and moisture, while the contribution of denitrification (C<sub>d</sub>) was the reverse (Table 1). Nitrification contributed more to N<sub>2</sub>O emission than denitrification when soil moisture content was 50% and 70% WFPS. At the first incubation week under 85% WFPS, the contribution of denitrification was approximately equal to (35°C) or less than (25°C) that from nitrification. On average across all sample days, nitrification was responsible for 87.2%, 79.8% and 52.9% of the N<sub>2</sub>O production at 50%, 70%, 85% WFPS respectively at 25°C, and for 86.3%, 73.9% and 33.1% of total N<sub>2</sub>O production at 50%, 70%, 85% WFPS respectively at 35°C. N<sub>2</sub>O from

nitrification ( $N_2O_n$ ) increased as the soil moisture content increased from 50% to 85% WFPS, with the greatest production detected at 25°C and 85% WFPS in the first week.

The percentage of nitrified N emitted as  $N_2O$  ( $P_{N_2O}$ ) decreased over the incubation time regardless of soil moisture and temperature (Table 1). For the period of day 7 to day 15,  $P_{N_2O}$  at 50% and 70% WFPS at 25°C (0.0012% and 0.106%, respectively) were lower than those at 50% and 70% WFPS at 35°C (0.0068% and 0.663%, respectively). However, at 85% WFPS,  $P_{N_2O}$  was higher when the soil temperature was 25°C (0.614%) compared to 35°C (0.0901%).

#### The dynamics of ammonia oxidizer populations

The abundance of both AOA and AOB responded to the changes in soil temperature and moisture over the incubation period after the application of the treatments (Figure 4). At day 0, at the same soil moisture content, AOA *amoA* gene copies were higher at 35°C compared to 25°C, but AOB *amoA* gene copies were higher at 25°C than at 35°C. Before incubation commencement at day 0 the initial abundances of archaeal and bacterial nitrifiers were greater under higher WFPS conditions (85%) but there were no statistically significant differences. Following application of fertilizers, both AOA and AOB *amoA* gene abundance significantly increased ( $P < 0.05$ ), with the greatest increase observed for AOB. Over the incubation period AOA *amoA* gene copy numbers increased from day 0 to 7 and then declined for the remaining period, whereas AOB *amoA* gene copy numbers increased continually over the incubation period from day 0 to 15 (Figure 4).

Soil moisture had a significant ( $P < 0.05$ ) influence on AOA and AOB abundance (Figure 4). After the treatments were applied, AOA *amoA* gene copy numbers declined as WFPS

increased. There was a modest effect of temperature on the abundance of AOA and AOB populations with a similar pattern of change in AOA and AOB *amoA* gene copies observed at 25°C and 35°C.

#### Correlation between nitrification-sourced N<sub>2</sub>O and AOA and AOB populations

There was a significant correlation between AOA-*amoA* gene abundance and nitrification-sourced N<sub>2</sub>O regardless fertilizer addition or not ( $P < 0.05$ ) (Table 2). Before the fertilizer application, there was no significant relationship between nitrification-derived N<sub>2</sub>O and AOB-*amoA* gene copies. However, after fertilizer application, AOB-*amoA* gene copies showed a strong significant relationship with N<sub>2</sub>O production through nitrification (N<sub>2</sub>O<sub>n</sub>) ( $P < 0.001$ ). AOA and AOB abundances had a strong significant correlation with NO<sub>3</sub><sup>-</sup> concentration suggesting both AOA and AOB were responsible for nitrification.

## Discussion

### Source of N<sub>2</sub>O

From both previous studies and the current study, soil water content and temperature were the predominant factors regulating N<sub>2</sub>O emission from soils. Kool et al. (2010) found that emitted N<sub>2</sub>O was mainly related to soil moisture conditions. An increase in soil water content due to irrigation and rainfall can stimulate nitrification and denitrification (Hu et al. 2014), and also can promote N<sub>2</sub>O production (Hofstra and Bouwman 2005). The increase in gross nitrification rate (*n*) with soil moisture content (Table 1) is consistent with previous studies (Maag and Vinther 1996; Garrido et al. 2002). In N fertilizer amended soil, N<sub>2</sub>O emission has been found to be highly correlated with WFPS, with the highest emission under around 70% WFPS levels, which was attributed to a combination of nitrification (35–53%), and

denitrification (only 2–9%) (Huang et al. 2014). In sandy loam soils, when moisture status was sub-optimal for denitrification (50% and 70% WFPS), nitrification was the significant contributor (around 29%) to N<sub>2</sub>O emissions (Kool et al. 2011), but in wetter soils (-0.1 kPa) nitrification contributed less than 3% (Webster and Hopkins 1996). Well et al. (2008) revealed that 88% of total N<sub>2</sub>O emission was attributed to nitrification at 45% WFPS. This suggests that favourable conditions for N<sub>2</sub>O production from nitrification occur within the range of 30% to 70%, whereas denitrification dominates N<sub>2</sub>O production in wet soils with >80–90% WFPS (Braker and Conrad 2011; Huang et al. 2014), which is consistent with our observation that nitrification was the main source of N<sub>2</sub>O production at 50% and 70% WFPS (Table 1) and denitrification dominated N<sub>2</sub>O production at 85% WFPS soils. At 35°C, and 70% and 85% WFPS the peak N<sub>2</sub>O flux occurred 3 days after N was applied, while at 25°C, and 70% and 85% WFPS the peak N<sub>2</sub>O flux occurred at day 4 (Figure 1), suggesting increasing soil temperature can promote N<sub>2</sub>O emission.

Soil water content and temperature not only determine the availability of O<sub>2</sub>, but also influence transport of nutrients within the soil matrix and the metabolic activity of microbial cells (Hu et al. 2014), which could confound the relationships between soil temperature and WFPS and rates of N<sub>2</sub>O emissions (Hu et al. 2015). The effect of WFPS on the relative contributions of nitrification and denitrification to N<sub>2</sub>O emissions is much more complex due to the heterogeneity of the soil environment where both anaerobic and aerobic conditions can exist within the soil at the same time, and cannot be as clearly depicted as changes in O<sub>2</sub> concentrations with changing moisture content (WFPS) (Hu et al. 2015).

This is likely due to the effect of soil moisture content on the growth of nitrifiers. When soil moisture content was 85% WFPS, N<sub>2</sub>O<sub>n</sub> was higher at 25°C than at 35°C. It is also

possible that the supply of  $\text{NO}_3^-$  became limiting for denitrification during the first few days under 25°C and 85% WFPS (gross nitrification rate was lower than that of 35°C) (Ciarlo et al. 2008; Harris et al. 2013). But we hypothesis that denitrification was driven to  $\text{N}_2$  in the 35°C 85% WFPS treatment. This can be supported by the total  $\text{N}_2\text{O}$  production being reduced by around 90% during the second incubation week compared to the first week incubation at 35°C 85% WFPS (Table 1).

Dalal et al. (2003) reported that when soil moisture content was above 80-90% WFPS, both  $\text{N}_2\text{O}$  and  $\text{N}_2$  production occurred. The  $\text{N}_2\text{O}:\text{N}_2$  ratio has often been found to decrease with increasing soil water content (Colbourn and Dowdell 1984; Davidson 1992; Rudaz et al. 1999), particularly when the soil water content exceeds 75% WFPS (Davidson 1992; Weier et al. 1993). Likewise, the measured  $\text{N}_2\text{O}:\text{N}_2$  ratio was highest ( $\geq 1$ ) under dry conditions during summer and early autumn when denitrification was relatively inactive in a study on three pasture soils in New Zealand (Ruz-Jerez et al. 1994). Friedl et al. (2016) revealed that  $\text{N}_2$  emissions exceeded  $\text{N}_2\text{O}$  emissions by a factor of  $8 \pm 1$  at 80% WFPS and a factor of  $17 \pm 2$  at 100% WFPS. In our study, the average proportion of nitrified N as  $\text{N}_2\text{O}$  ( $P_{\text{N}_2\text{O}}$ ) was 0.007% to 0.7% at 35°C across the three moisture contents, which is higher than the range of 0.02% to 0.2% reported in previous studies in agricultural soils under aerobic conditions (Tortoso and Hutchinson 1990; Magg and Vinther 1996; Garrido et al. 2002; Zhu et al. 2011). However, Khalil et al. (2004) demonstrated a higher ratio of 0.16% to 1.48% when  $\text{O}_2$  concentration was reduced from 20.4 to 0.76 kPa. The  $P_{\text{N}_2\text{O}}$  in our study varied significantly with temperature and moisture. Higher temperature increased  $P_{\text{N}_2\text{O}}$ , except at 85% WFPS, which was also noted by Goodroad and Keeney (1984). This suggests that models such as DNDC (Li et al. 2000) and CENTURY (Mathieu et al. 2006), which use fixed ratios to estimate  $P_{\text{N}_2\text{O}}$ , may not accurately simulate the relative contribution of nitrification and

denitrification to N<sub>2</sub>O production.

## AOA and AOB

AOA were the predominant ammonia oxidizers (Figure 4) in the acidic soil, which is consistent with other research (Schleper et al. 2005; Zhang et al. 2012). The activity of nitrification enzymes, including AMO, can vary with environmental conditions (Niklaus et al. 2001; Zak et al. 2000). Several studies have reported significant increases in AOA and AOB abundance in response to N fertilization (Mendum et al. 1999; Hermansson and Lindgren 2001; Okano et al. 2004), which is consistent with our observations. Soil moisture and temperature have also been found to affect N<sub>2</sub>O emission by nitrification indirectly through changes in the abundance of ammonia oxidizers (Avrahami and Bohannan 2009; Szukics et al. 2010). However compared to soil moisture, we found soil temperature only had a modest effect on the abundance of AOB (Figure 4), in agreement with the results in Horz et al. (2004). At day 0 the initial abundances of archaeal and bacterial nitrifiers were greater under higher WFPS conditions (85%). The population dynamic had changed however 7 days after fertilizer application with the greatest *amoA* copy numbers observed at 50% WFPS for both AOA and AOB. Our results demonstrated that increasing moisture content decreased AOA and AOB abundance. This may be attributed to a reduction in oxygen (O<sub>2</sub>) availability in the soil. However, previous studies showed that the effect of soil moisture content on AOB abundance could be positive or negative. Hastings et al. (2000) found that elevation of soil moisture increased AOB abundance by reducing water stress. Belser (1979) indicated that further increases in soil moisture can decrease abundance of nitrifiers due to decreased diffusion of O<sub>2</sub> into the soil. Another possibility is that elevated CO<sub>2</sub> due to increasing moisture and temperature altered the competition for resource substrates between AOA, AOB and heterotrophic microbes (Horz et al. 2004) and therefore both AOA and AOB abundance

decreased.

Before application of fertilizers, only AOA abundance were significantly related with  $N_2O_n$  emission but after N was applied, both AOA and AOB played an important role (Table 2). It suggests that AOA may be particularly adapted to unfavourable and low fertility environments (Schauss et al. 2009; Di et al. 2009), while AOB require high nutrient availability conditions to flourish (Di et al. 2009). Therefore, the present study demonstrated that AOB was only playing a limited role in  $N_2O$  emissions depending on N conditions. Di et al. (2010) reported that the total  $N_2O$  emissions from grazed grasslands rich in  $NH_4^+$  were significantly related to the *amoA* gene copy numbers from the AOB community. This suggests that the AOB were responsible for  $N_2O$  emissions from the N-rich soils, which is in agreement with our study. However, Andert et al. (2011) found that there was no relationship between the abundance of AOB and AOA communities and  $N_2O$  emissions from a peat soil. Therefore, future study needs to be done with wide range of soils.

We measured the *amoA* gene abundance at different conditions based on soil DNA, giving insights into community size and potential contribution to activity, however, measurements of active community based on soil RNA are highly desirable in future studies. It is not possible to accurately determine the relative contribution of AOA and AOB to  $N_2O$  emissions, because the assumptions were made that all AOA and AOB produced the same yield of  $N_2O$  per unit of ammonia oxidized. However, a large body of previous literature stated that this was not the case (Stieglmeier et al. 2014). Furthermore, the interpretation of the relative contributions of AOA and AOB to  $N_2O$  emissions cannot be made clearly and the underlying mechanism may need to be studied further using more advanced molecular techniques. 1-octyne, a recently reported AOB selective inhibitor, can be used to separate

AOA-related N<sub>2</sub>O and AOB-related N<sub>2</sub>O and specifically inhibited AOB growth, activity and N<sub>2</sub>O production (Hink et al. 2016). Therefore, it is essential to make use of AOA or AOB selective inhibitor to give an explicit interpretation on the relative role on nitrification-sourced N<sub>2</sub>O.

## **Conclusion**

We have identified that soil water, temperature, and their interaction significantly affect N<sub>2</sub>O emissions, P<sub>N<sub>2</sub>O</sub>, and AOA and AOB abundance. Soil temperature and moisture decreased the contribution of nitrification to N<sub>2</sub>O emission. Nitrification occurred even at 85% WFPS in this study. Compared to soil temperature, soil moisture had a significant influence on the abundances of AOA and AOB population. In our study, a significant relationship was found between both AOA and AOB populations and N<sub>2</sub>O emitted from nitrification. Our study provides information on the variable contributions of nitrification and denitrification to N<sub>2</sub>O emissions. This information can be used to inform appropriate N<sub>2</sub>O mitigation strategies and improve the model simulation of N<sub>2</sub>O emissions. Further work needs to be done for validation of these findings under field conditions to account for the heterogeneity that exists and for expression of the functional genes which are involved in N<sub>2</sub>O consumption and production.

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**Figure 1** N<sub>2</sub>O production rate from the two treatments under different incubation conditions: 25°C + 50% WFPS (a), 25°C + 70% WFPS (b), 25°C + 85% WFPS (c), 35°C + 50% WFPS (d), 35°C + 70% WFPS (e), and 35°C + 85% WFPS (f). Error bars are the standard deviation of four replicates.

**Figure 2** <sup>15</sup>N enrichment of N<sub>2</sub>O, NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> in the <sup>15</sup>N labelled NH<sub>4</sub><sup>+</sup> treatment during the incubation. a, b and c represent 50%, 70% and 85% WFPS at 25°C, respectively. d, e and f represent 50%, 70% and 85% WFPS at 35°C, respectively. Error bars are the standard deviation of four replicates.

**Figure 3** <sup>15</sup>N enrichment of N<sub>2</sub>O, NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> in the <sup>15</sup>N labelled NO<sub>3</sub><sup>-</sup> treatment during the incubation. a, b and c represent 50%, 70% and 85% WFPS at 25°C, respectively. d, e and f represent 50%, 70% and 85% WFPS at 35°C, respectively. Error bars are the standard deviation of four replicates.

**Figure 4** AOA and AOB *amoA* gene copy numbers in <sup>15</sup>NH<sub>4</sub> treatment at 25°C and 35°C during the incubation period. Error bars are the standard deviation of four replicates.

Figure 1

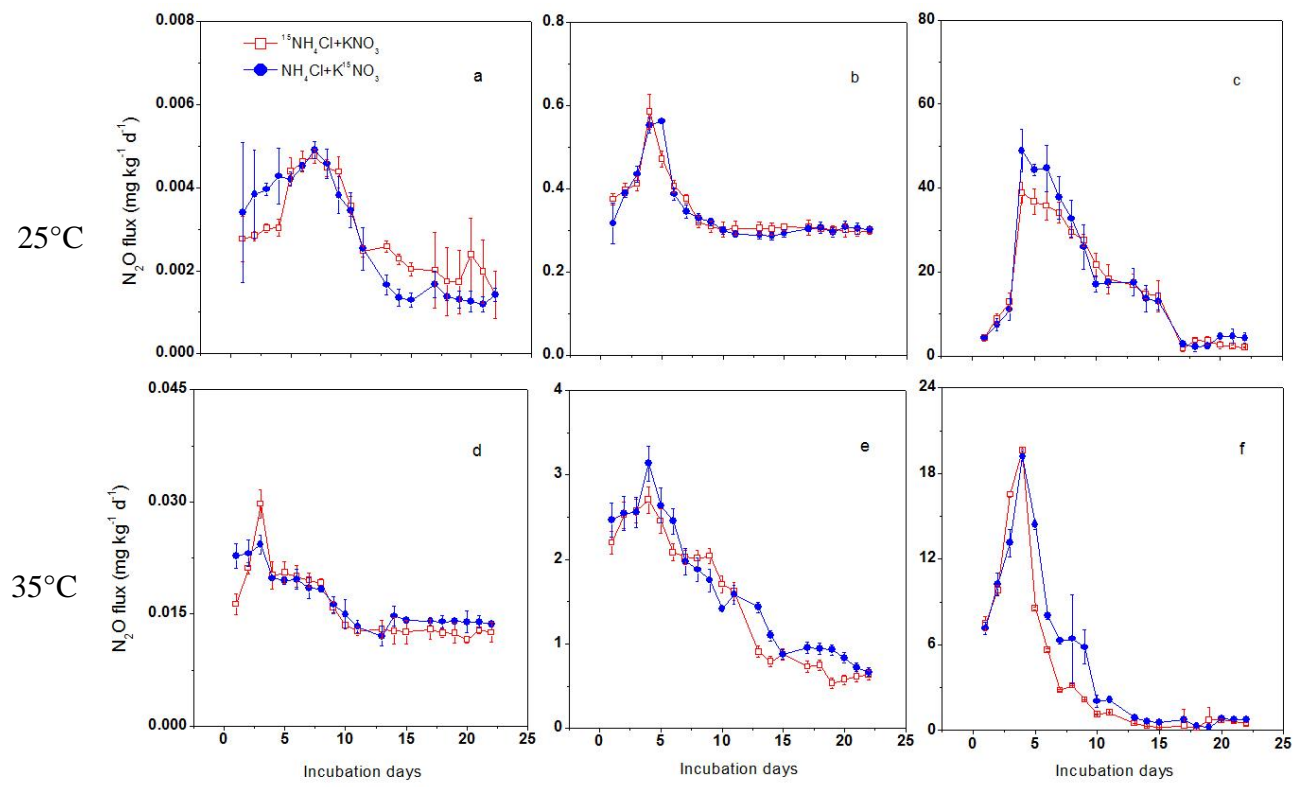


Figure 2

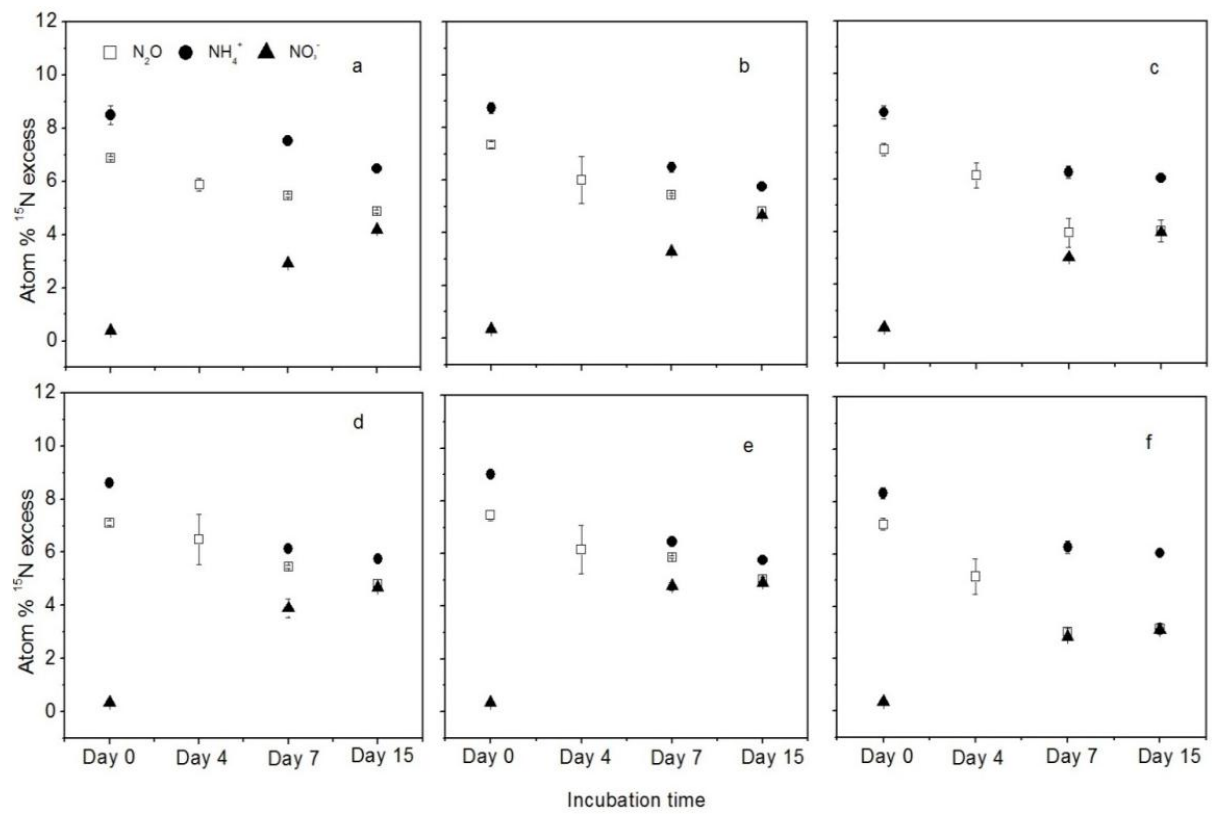


Figure 3

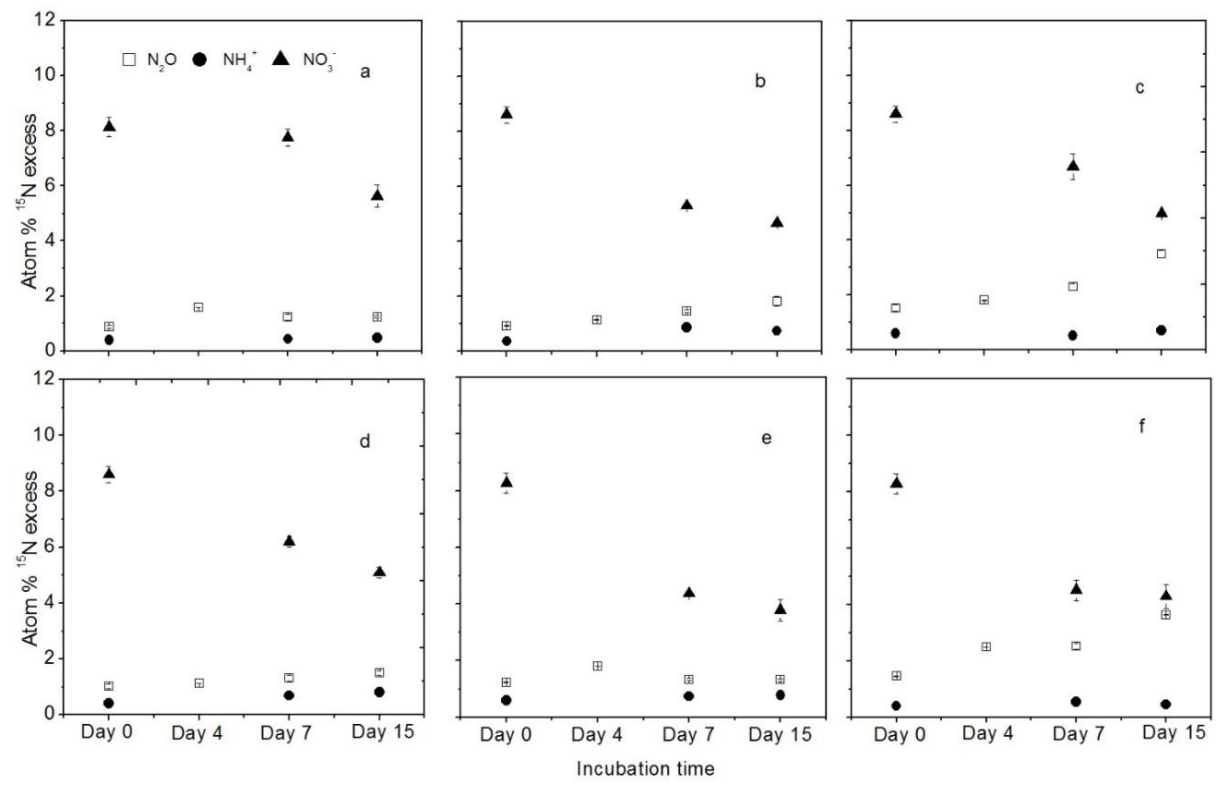
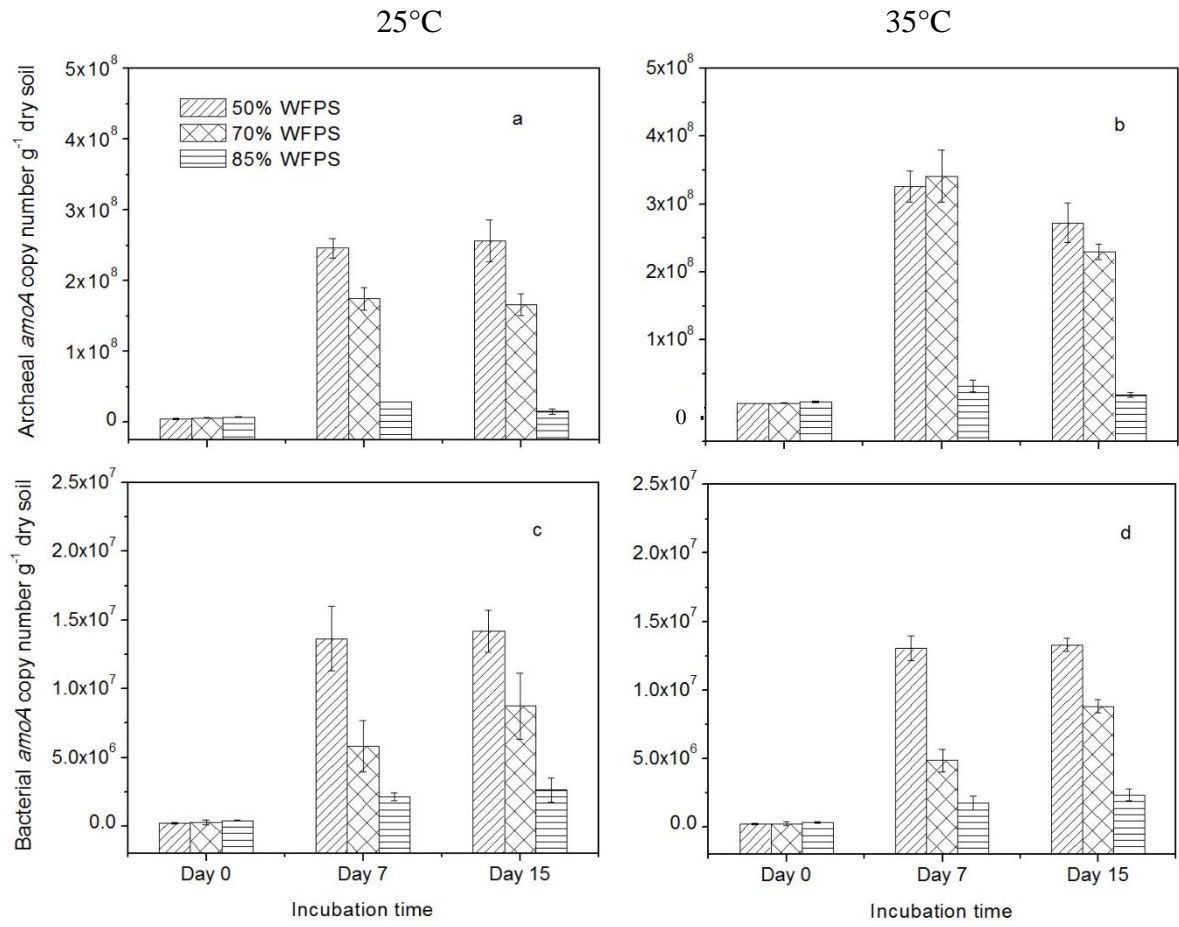


Figure 4



**Table 1** Gross nitrification rate, the relative contribution of denitrification ( $C_d$ ) and nitrification ( $C_n$ ) to  $N_2O$  production,  $N_2O$  derived from denitrification ( $N_2O_d$ ) and nitrification ( $N_2O_n$ ), and the proportion of  $N_2O$  to gross nitrification ( $P_{N_2O}$ ) under the different environmental conditions. Numbers in bracket represent the standard deviation of four replicates.

Temperature	Moisture	Time	Gross nitrification rate mg N kg <sup>-1</sup> d <sup>-1</sup>	Relative contribution %		$N_2O_d^c$ mg N <sub>2</sub> O-N kg <sup>-1</sup> d <sup>-1</sup>	$N_2O_n^d$ mg N <sub>2</sub> O-N kg <sup>-1</sup> d <sup>-1</sup>	$P_{N_2O}^e$ %
				$C_d^a$	$C_n^b$			
25°C	50% WFPS	d0-d7	1.97 (0.32)	10.68 (1.22)	89.32 (1.98)	0.0002 (2.3E5)	0.0017 (2.3E5)	0.0015 (0.0004)
		d7-d15	5.26 (1.57)	14.85 (1.92)	85.15 (3.92)	0.0002 (3.0E5)	0.0013 (1.1E5)	0.0012 (0.0006)
	70% WFPS	d0-d7	8.32 (0.69)	13.08 (2.45)	86.92 (1.68)	0.048 (0.009)	0.317 (0.009)	0.249 (0.103)
		d7-d15	2.37 (0.24)	27.42 (1.89)	72.58 (4.64)	0.055 (0.004)	0.147 (0.004)	0.106 (0.012)
	85% WFPS	d0-d7	3.84 (0.78)	29.09 (4.050)	70.91 (6.98)	10.979 (1.53)	26.76 (1.53)	18.101 (5.22)
		d7-d15	6.65 (1.51)	65.21 (2.90)	34.79 (5.49)	1.902 (0.085)	1.036 (0.086)	0.614 (0.06)
35°C	50% WFPS	d0-d7	5.39 (0.16)	11.14 (2.11)	88.86 (3.46)	0.0024 (0.0005)	0.019 (0.0005)	0.0161 (0.013)
		d7-d15	3.43 (0.41)	16.13 (1.24)	83.87 (2.37)	0.017 (0.001)	0.0086 (0.0009)	0.0068 (0.01)
	70% WFPS	d0-d7	7.05 (0.33)	16.21 (2.58)	83.79 (8.99)	0.320 (0.024)	1.952 (0.039)	1.970 (1.93)
		d7-d15	2.35 (0.76)	18.02 (1.93)	81.98 (4.51)	0.172 (0.018)	0.785 (0.014)	0.663 (0.27)
	85% WFPS	d0-d7	11.17 (2.24)	50.10 (4.20)	49.90 (6.78)	3.152 (0.264)	3.139 (0.109)	2.343 (0.19)
		d7-d15	0.97 (0.36)	83.63 (9.11)	16.37 (6.24)	0.624 (0.068)	0.122 (0.028)	0.0901 (0.07)

<sup>a</sup> The relative contribution by denitrification ( $C_d$ ) to  $N_2O$  production

<sup>b</sup> The relative contribution by nitrification ( $C_n$ ) to  $N_2O$  production

<sup>c</sup>  $N_2O$  production from denitrification ( $N_2O_d$ )

<sup>d</sup>  $N_2O$  production from nitrification ( $N_2O_n$ )

<sup>e</sup> The proportion of nitrified N emitted as  $N_2O$  ( $P_{N_2O}$ )

**Table 2** Correlations between soil mineral N levels, N<sub>2</sub>O<sub>n</sub><sup>a</sup> and AOA and AOB abundances.

Time	Factor	AOA (Log number)		AOB (Log number)	
		R	P-Values	R	P-Values
Before fertilizer applied	Exchangeable NH <sub>4</sub> <sup>+</sup> concentration	0.454	0.138	0.089	0.779
	NO <sub>3</sub> <sup>-</sup> concentration	0.217	0.499	0.063	0.846
	N <sub>2</sub> O <sub>n</sub>	0.615	0.033	0.517	0.085
After fertilizer applied	Exchangeable NH <sub>4</sub> <sup>+</sup> concentration	0.623	5.00E-05	0.535	0.008
	NO <sub>3</sub> <sup>-</sup> concentration	-0.622	5.07E-05	-0.369	0.027
	N <sub>2</sub> O <sub>n</sub>	0.346	0.038	0.578	0.0002

<sup>a</sup> means N<sub>2</sub>O production from nitrification