Nitrous oxide sources and different nitrification pathways under various soil and environmental conditions

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Abstract

Nitrification is an important nitrogen transformation process in soils, including autotrophic and heterotrophic pathways. Autotrophic nitrification is mediated by microbial ammonia oxidizers, including ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB). The relative significance of heterotrophic and autotrophic nitrification in soils is still under debate, and the role of AOA and AOB in nitrification in different soil types and environmental conditions is poorly understood.

Nitrous oxide (N\textsubscript{2}O) is a by-product of nitrification and a potent greenhouse gas. N\textsubscript{2}O can also be produced from other pathways, such as denitrification. Nitrification is the predominant process of N\textsubscript{2}O production in many soils. More than 60% of the annual global N\textsubscript{2}O emissions are produced by microbes that live in the soil, while in Australia, 80% of those emissions are from agricultural soils. Understanding the microbial pathways contributing to N\textsubscript{2}O production is essential to manage nitrogen (N) nutrients in Australian agricultural soils. Moreover, many models of N\textsubscript{2}O emissions estimate the contribution of nitrification using a fixed ratio, such as, 0.2% (DNDC) and 2% (DAYCENT). To improve N\textsubscript{2}O emission modelling capability and to decrease its uncertainty, it is essential to know how much N\textsubscript{2}O comes from nitrification and what are the factors affecting N\textsubscript{2}O emission from this process.

The objectives of this research were to investigate the contribution of nitrification to N\textsubscript{2}O production, identify the key drivers which control this, assess the importance of heterotrophic nitrification to nitrification, determine the role of AOA and AOB in nitrification and N\textsubscript{2}O emission, and determine the relationship between N\textsubscript{2}O production and AOA and AOB populations.

The research comprised a series of laboratory incubation experiments to examine the above objectives. Top soils (0-10 cm) were collected from five different agricultural systems in Australia including cropping, sugarcane, vegetable production and pasture systems. After being air-dried, ground and sieved (< 2 mm), microcosm incubations were established under controlled conditions. In the past, \textsuperscript{15}N isotope techniques and nitrification inhibitors were widely used for distinguishing the source of N\textsubscript{2}O, but each method alone was unable to provide an unequivocal answer. Therefore the combined methods of \textsuperscript{15}N isotope, nitrification inhibitor
and molecular biology were used in my research. Firstly, the efficacy of nitrification inhibitors was tested in the study soils and the most effective inhibitor acetylene (C₂H₂) was selected. The contribution of nitrification to N₂O flux was measured by differently labelling ammonium (¹⁵NH₄⁺) and nitrate (¹⁵NO₃⁻) and comparing the ¹⁵N enrichment in N₂O, NH₄⁺ and NO₃⁻ pools at different sampling times. The relative importance of heterotrophic nitrification was studied by ¹⁵N dilution and C₂H₂ inhibition techniques and ¹⁵N abundance measured in different treatments. Using the microbial functional gene amoA, the abundance and composition of AOA and AOB were quantified in soils. Quantified amoA genes along with N₂O emission measurements under various conditions were used to investigate whether AOA and AOB population dynamics mirror N₂O emission changes under the controlled environment conditions.

Acetylene completely inhibited nitrification and performed better than the inhibitor 3, 4-dimethylpyrazole phosphate (DMPP) in all soils. DMPP was more effective in inhibiting nitrification in the neutral soil (93.5%) than in the alkaline soil (85.1%) and acid soil (70.5%). Neither DMPP nor C₂H₂ was a selective nitrification inhibitor in neutral and alkaline soils, in which both AOA and AOB were inhibited. Soil pH thus plays an important role in the effectiveness of DMPP and C₂H₂ in inhibiting nitrification and ammonia oxidizer populations.

Heterotrophic nitrification was the predominant NO₃⁻-N production pathway in an acid dairy soil with relative high organic C content (Longworry), while autotrophic nitrification was prevalent in another dairy soil (Glenormiston) with a higher pH and a lower organic C content, and in an acidic cropping soil (Hamilton).

The contribution of heterotrophic nitrification to total nitrification varied with temperature and soil moisture suggesting that these factors are important in controlling the significance of heterotrophic nitrification. More heterotrophic nitrification was observed in the acidic cropping soil (Hamilton) under lower temperature (15°C) conditions. Around 69% of the NO₃⁻ produced was a result of heterotrophic nitrification at 15°C and 50% WFPS, and 50% at 15°C and 70% WFPS. However at 25°C and 35°C nitrification was largely autotrophic regardless of moisture.

Nitrification was the main contributor of N₂O production, being responsible for 87%, 80% and 53% of total N₂O production at 25°C 50%, 70% and 85% WFPS respectively and for 86%,
74% and 33% at 35°C 50%, 70% and 85% WFPS respectively. The contribution of nitrification to N₂O production decreased as temperature and moisture increased. The proportion of nitrified N as N₂O (P₅₀ value) increased as temperature and moisture increased except at 85% WFPS. Compared to the dairy pasture, vegetable production and cropping soils, more N₂O was produced by nitrification from sugarcane soil under the incubation conditions.

AOA were the dominant ammonia oxidizers in all studied soils, but the AOB population was significantly related to the amount of N₂O emitted from nitrification under 25°C, 35°C and 50%, 70% and 85% WFPS in the cropping soil (Hamilton). In the sugarcane soil, N₂O-yielding nitrification was strongly related with AOA abundance. Soil moisture and land-use had a greater effect than soil temperature on AOA and AOB populations.

This thesis contributed a number of important findings to improve the understanding of N₂O production from agricultural soils in Australia, and for identifying where and when nitrification is a significant N₂O source. This knowledge will enable informed land management to address N₂O emissions in agriculture. In addition, the inclusion of the microbial research, exploring the relation between N₂O production and AOA and AOB populations suggested that their populations’ dynamics can mirror N₂O emission changes. This knowledge sheds light on the role of microbial communities in N₂O production.

Keywords: Nitrification, N₂O emission, heterotrophic nitrification, autotrophic nitrification, ammonia-oxidizing archaea, ammonia-oxidizing bacteria
Declaration

This is to certify that:

(i) the thesis comprises only my original work towards the PhD except where indicated in the Preface,

(ii) due acknowledgement has been made in the text to all other material used, and

(iii) the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliography and appendices.

Rui Liu
June, 2015
Preface and Contribution of Authors

This thesis is comprised of eight chapters. The first chapter is the introduction, followed by the literature review which clearly details the related research questions and current knowledge gaps. A general methodology which gives detailed information on the materials and methods used in the PhD project is written as Chapter 3. The experimental chapters 4 to 7 are written in manuscript format according to the specific format of the individual journals. Finally, the general discussion and conclusions highlight the key findings of this thesis.

The candidate was the primary author of all manuscripts, and co-authors included Deli Chen, Helen Suter, Helen Hayden, Jim (Jizheng) He, Hangwei Hu and Pauline Mele. The candidate was responsible for designing and conducting the experiments, collecting and analyzing the data, and writing the manuscripts. The co-authors assisted in improving the experimental design, laboratory work management, analysis and the manuscripts.

The manuscripts included in this thesis are presented in the following order:

Chapter 4

Chapter 5.1

Chapter 5.2
Chapter 6

Chapter 7
Liu, R., Hangwei Hu, Hayden, H., Suter, H., He, J.Z., Mele, P.M., Chen, D.L. Impact of different land-use on soil nitrous oxide production and ammonia oxidizing microorganisms in Australian soils. Ready to submit to Environmental Research.
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Full title: The effect of nitrification inhibitors in reducing nitrification and the ammonia oxidizer population in three contrasting soils

Authors: Rui Liu • Helen Hayden • Helen Suter • Jizheng He • Deli Chen

Candidate's contribution (%): 70%

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Status: [ ] Accepted and In press [ ] Published Date accepted/ published: 3/2/2015

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Chapter 1

Introduction

1.1 Research background

One of the major challenges of the 21st century is to mitigate the effects of global environmental changes caused by increasing atmospheric greenhouse gas (GHG) emissions (Trivedi et al., 2013), particularly nitrous oxide (N\textsubscript{2}O). N\textsubscript{2}O is a potent GHG greatly contributing to global climate change, with a 300-fold higher global warming potential than CO\textsubscript{2} (IPCC, 2007), and is involved in the destruction of the protective ozone layer (Ravishankara et al., 2009). Soil ecosystems are a significant and the largest source of N\textsubscript{2}O, accounting for an estimated 65% of the atmospheric loading of this gas (IPCC, 2007). In Australia, agriculture is the 2\textsuperscript{nd} largest greenhouse gas emitter, accounting for 16% of total greenhouse gas emissions (Australian Greenhouse Office, 2007). Australian agriculture accounts for nearly 80% of the total N\textsubscript{2}O released (Australia Greenhouse Office, 2001), with 32% of this derived from N fertilizer (Dalal et al., 2003). Global N\textsubscript{2}O concentrations are likely to continue to rise in the coming decades (Reay et al., 2012) due to increasing application of nitrogen (N) fertilizer for food production to feed a growing population. In order to develop more effective mitigation strategies to counteract the steady increase in N\textsubscript{2}O emissions, a better understanding of the underlying mechanisms of soil N\textsubscript{2}O formation is needed. The major pathways of N\textsubscript{2}O production in soils include nitrification, denitrification, and nitrifier denitrification (Figure 1). However, the current N\textsubscript{2}O emission measurement from field sites, either by small chambers or large scale micro-meteorological techniques, do not distinguish between N\textsubscript{2}O sources. There is high uncertainty in estimation of N\textsubscript{2}O derived from nitrification due to lack of available data and many process-oriented biogeochemical models, such as, CENTURY, DAYCENT, DNDC, estimate the contribution of nitrification to N\textsubscript{2}O emission using a fixed ratio (Chen et al., 2008). Previous studies have shown that in Australia, based on model (WNMM) simulations, nitrification may account for up to 45% of N\textsubscript{2}O emitted from irrigated pasture and 70% of N\textsubscript{2}O emitted in soils in semi-arid regions (Chen et al., 2010; Li et al., 2012).

Therefore, to improve modelling capability and understanding of N\textsubscript{2}O emissions and to develop more effective mitigation strategies to counteract the steady increase in N\textsubscript{2}O emissions,
it is essential to quantify how much N$_2$O comes from nitrification and what are the factors affecting N$_2$O emission from this process.

Both nitrification-related pathways (autotrophic and heterotrophic nitrification) contribute soil N$_2$O emissions (Zhu et al., 2013). In arable soils nitrification is mostly autotrophic, while heterotrophic nitrification is the dominant process in acid forest soils (Well et al., 2008). The relative contributions of autotrophic and heterotrophic nitrification to N$_2$O emissions and nitrification is still debatable for different soil types and ecosystems, particularly for forestry production systems. Kuroiwa et al. (2011) showed heterotrophic nitrification was the minor way of gross total nitrification in most forest mineral topsoils in Japan. However, Zhang et al. (2014) revealed that the contribution of heterotrophic nitrification to total nitrification was 93% when maize straw was used as a N substrate in acidic forest soil (pH 4.7). Islam et al. (2007) demonstrated heterotrophic nitrifiers can utilise both inorganic N and organic N. However, there is very limited information about whether soil and environmental conditions affect the contribution of heterotrophic nitrification.

Because of uncertainties about the role of heterotrophic and autotrophic pathways in nitrification, further investigation is needed to ascertain the relative contribution of each. Research on N$_2$O leakage from nitrification is further complicated because of the existence of different steps within the N cycle, including the ammonium (NH$_4^+$) to nitrite (NO$_2^-$) step of autotrophic and heterotrophic nitrification, and the reduction of NO$_2^-$ to N$_2$O from nitrification (nitrifier denitrification) (Figure 1).
Figure 1 Nitrogen pools and nitrous oxide production processes in soils (Zhang et al., 2015)

Burger et al. (2005) verified that microorganisms involved in the N cycle and N\textsubscript{2}O emission from soils can directly regulate N\textsubscript{2}O production and consumption, and N\textsubscript{2}O emission would increase with high abundance and activity of these microorganisms. Increasing evidence suggests that ammonia-oxidizing archaea (AOA) and bacteria (AOB) have the genetic potential to produce N\textsubscript{2}O, but their contributions to soil N\textsubscript{2}O emissions in natural environments and the relevant pathways remain unclear. Mitigating the steady increase in N\textsubscript{2}O concentrations is strongly hindered by lack of knowledge on microbial mechanisms underpinning the N\textsubscript{2}O producing processes (Hu et al., 2015). In order to better predict and mitigate future N\textsubscript{2}O emissions, it is essential to identify the dominant biological sources of soil N\textsubscript{2}O production and to characterize the environmental factors which influence their activity.

The activity and abundance of these communities can be influenced by environmental conditions, such as rainfall and temperature (Szukics et al., 2010). Therefore, it is necessary to investigate the dynamics of microbial community composition and abundance associated with N\textsubscript{2}O emission and the N cycle in response to environmental changes. Culture-independent techniques such as gene-based community structure or function studies reduce bias associated with medium selection. Functional genes that encode enzymes directly involved in N\textsubscript{2}O production are more commonly used to examine N cycle gene abundance or expression. For example, *narG*, which encodes the enzyme nitrate reductase, and *nosZ*, which encodes the
enzyme nitrous oxide reductase, can be examined in soil to indicate potential N₂O production and consumption in the denitrification pathway (Kandeler et al., 2006).

1.2 Conceptual framework

The research in this thesis investigated the effect of environmental conditions and soils on the contribution of nitrification to N₂O emission, and the contribution of heterotrophic nitrification to nitrification in Australia. The three objectives of the study were to: (i) quantify the contribution of heterotrophic nitrification to nitrification under different soils and environmental conditions; (ii) identify when and where nitrification is the significant source of N₂O and (iii) assess whether there is a relationship between N₂O production by nitrification and microbial communities (nitrifiers) involved in this process.

To achieve the first two objectives, three sets of incubation experiments were conducted. The first set of experiments examined the effectiveness of nitrification inhibitors, which can separate nitrification and denitrification, at reducing nitrification. This set of experiments examined the efficiency of the nitrification inhibitors DMPP and acetylene in reducing nitrification in a range of soils in order to select the most efficient inhibitor to be used for the following experiments. The responses of microbial communities involved in nitrification, including ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB), to the nitrification inhibitors were also investigated in this experiment. AOA and AOB are responsible for the first step of nitrification and the amoA gene, which encodes the active site of ammonia monooxygenase, an enzyme unique to this group of nitrifying microorganisms, is the target of nitrification inhibitors. The results of this experiment enabled selection of the most effective nitrification inhibitor for subsequent work.

The other two sets of experiments distinguished heterotrophic and autotrophic nitrification, and N₂O sources. The relative significance of heterotrophic and autotrophic nitrification to total nitrification and nitrification–sourced N₂O in different soil types and under different environmental conditions, was examined. A combination of ¹⁵N dilution techniques and nitrification inhibitors were used to separate autotrophic and heterotrophic nitrification, and ¹⁵N enrichment in the N₂O, NO₃⁻ and NH₄⁺ pools were used as indicators of when and where heterotrophic nitrification was the important pathway of nitrification, and nitrification was the predominant contributor of N₂O emissions.
To achieve the third objective, microcosms were established in the laboratory under controlled temperature and moisture conditions using molecular biology techniques to quantify the functional genes involved in nitrification and N$_2$O production. AOA and AOB carry the functional gene $amoA$, but they can be differentiated because of different sequences of archael and bacterial $amoA$ genes. $amoA$ can be detected and quantified without requiring laboratory culture. Therefore by using the molecular marker $amoA$ the abundance and composition of AOA and AOB can be detected accurately. Results of this experiment provided insight into the relationship between N$_2$O production by nitrification and the relative contributions of AOA and AOB. This information increased our knowledge on N$_2$O production processes and the role of microbial communities in N$_2$O production under different conditions.

The thesis concludes with an integrative discussion on the implications of the contribution of microbial pathways to N$_2$O production in Australian agricultural soils. These include: (i) the relative importance of heterotrophic nitrification; (ii) the key factors affecting N$_2$O emitted from nitrification; and (iii) the role of microbial communities in N$_2$O production.

1.3 Objectives of the study and organization of the thesis

Many studies have been undertaken to investigate global N$_2$O production and the contribution of denitrification. However, few studies have reported the production of N$_2$O due to nitrification and the role of microbial communities involved in this process.

This thesis begins with an Introductory Chapter 1, which covers the research background, conceptual framework, objectives and significance of the study. Chapter 2 presents the literature review on N$_2$O sources, the methods of separating N$_2$O pathways, the factors affecting nitrification sourced N$_2$O emission, the specific functional genes involved in N$_2$O production, and a summary of the current knowledge gaps. Chapter 3 provides details of the materials and methodologies used in the study. Chapters 4 to 7 are the experimental chapters and their specific objectives were to:

(i) Compare the efficacy of nitrification inhibitors on nitrification in the studied soils
and select the most effective one for subsequent studies (Chapter 4);

(ii) Determine the importance of AOA and AOB to nitrification (Chapter 4);

(iii) Investigate the relative importance of heterotrophic and autotrophic nitrification under different soil types and environmental conditions (Chapter 5);

(iv) Examine the effects of soil temperature, moisture and land-use on N₂O production from nitrification (Chapters 6 and 7); and

(v) Determine the relationship between N₂O emission from nitrification and AOA and AOB populations under various conditions. (Chapters 6 and 7).

This thesis concludes with Chapter 8, which summarises the major findings of the research and implications for future research.

1.4 Significance of the study

The results of this research will assist with improving the understanding of N₂O production from Australia agricultural soils, and identify where and when nitrification is a significant source of N₂O. This knowledge will enable informed land management to address N₂O emissions in agriculture. In addition, from the microbial aspect, exploring the relationship between N₂O production and AOA and AOB populations sheds light on the role of microbial communities in N₂O production, and whether their populations’ dynamics will mirror N₂O emission changes.

References


Zhu X, Burger M, Doane TA, Horwath WR (2013) Ammonia oxidation pathways and nitrifier denitrification are significant sources of N2O and NO under low oxygen availability. Proc Natl Acad Sci USA 110: 6328–6333
Chapter 2

Literature review

The continuous increase of the greenhouse gas nitrous oxide (N\textsubscript{2}O) in the atmosphere due to increasing anthropogenic nitrogen input in agriculture has become a global concern. In recent years, identification of the microbial sources responsible for soil N\textsubscript{2}O production has substantially advanced with the development of isotope enrichment and inhibition technologies, and the discoveries of specific functional genes. However, little information is available to effectively quantify the N\textsubscript{2}O sources. Therefore it is urgently necessary to improve the understanding of N\textsubscript{2}O formation and quantify the contribution of different pathways. Here, the latest knowledge is synthesized: (i) the key microbial pathways involved in N\textsubscript{2}O production and consumption in terrestrial ecosystems and the critical factors influencing their occurrence and contribution to N\textsubscript{2}O emissions, (ii) the methods to measure N\textsubscript{2}O sources, and (iii) the relative contributions of nitrifiers (AOA and AOB) to nitrification and soil N\textsubscript{2}O emissions and their regulating factors.

2.1 Sources of N\textsubscript{2}O production in soils

Globally, soil ecosystems are the largest contributor of N\textsubscript{2}O emission comprising around 65% of total N\textsubscript{2}O emitted into the atmosphere (Figure 1) (IPCC, 2007). It is predicted that agricultural soils will contribute up to 59% of total N\textsubscript{2}O emissions in 2030 (Figure 1). Several biological processes can produce N\textsubscript{2}O in soil systems, such as nitrification (autotrophic and heterotrophic), denitrification, dissimilatory nitrate reduction to ammonium (DNRA), nitrifier denitrification and non-biological chemodenitrification (Figure 2) (Butterbach-Bahl et al., 2013; Wrage et al., 2001; Hu et al., 2015; Zhang et al., 2015). Nitrification and denitrification are recognised as the principal N\textsubscript{2}O production pathways in soils and can occur simultaneously. To date, the quantification of these varied N\textsubscript{2}O production pathways and the identification of the role of microbial communities involved in N\textsubscript{2}O emission are difficult to determine. In addition, this lack of knowledge has led to the reliance on assumptions of the contribution of different pathways which is then as a fixed ratio for modeling N\textsubscript{2}O emission from nitrification. There is an urgent need to quantify N\textsubscript{2}O sources to develop a more confident simulation of future N\textsubscript{2}O emissions from terrestrial ecosystems, and thus more targeted mitigation strategies for use in agricultural systems.
Figure 1 Global N$_2$O emissions from various sources in 1995, 2005 and 2030. ‘Other energy sources’ here includes waste combustion, fugitives from solid fuels, natural gas and oil systems. ‘Other industrial processes sources’ includes metal production, solvent and other product use. ‘Other agricultural sources’ includes field burning of agricultural residues and prescribed burning of savannas. ‘Other waste sources’ includes miscellaneous waste handling processes (From Hu et al., 2015, Figure 1).

Figure 2 Outline of sources of N$_2$O from nitrification, nitrifier denitrification, denitrification and dissimilatory nitrate reduction to ammonium (DNRA).

2.1.1 Nitrification

Nitrification, or ammonia oxidation, is the aerobic oxidation of ammonium (NH$_4^+$) to nitrate
(NO$_3^-$) via nitrite (NO$_2^-$), which is carried out by microbial nitrifiers, and is a principal part of the global N cycle. It also determines the balance between reduced and oxidized forms of N. Nitrification has two steps, with each step performed by a specialized group of prokaryotes (Figure 3). The first step is NH$_3$ oxidation, which is the rate-limiting step for the whole nitrification process (Kowalchuk and Stephen, 2001), and is critical for production of the nitrification-sourced N$_2$O. Ammonia oxidation is catalyzed by the amoA gene encoding ammonia monooxygenase (AMO) and is performed by two distinct types of microbes: ammonia-oxidizing bacteria (AOB) (Purkhold et al., 2000), and ammonia-oxidizing archaea (AOA) (Brochier-Armanet et al., 2008). The second step, the conversion of NO$_2^-$ to NO$_3^-$, is regulated by the nxrB gene encoding nitrite oxidoreductase within nitrite-oxidizing bacteria (NOB) (Freitag et al., 1987). Nitrification can be either autotrophic or heterotrophic (Figure 3). In autotrophic nitrification, the energy released during the oxidation of inorganic N compounds can be used by AOA, AOB and NOB organisms as the sole energy source for carbon dioxide (CO$_2$) fixation, and their growth (Hooper et al., 1997). The key enzyme for autotrophic nitrification is AMO, with ammonia (NH$_3$), rather than ammonium (NH$_4^+$) being the substrate (Suzuki, 1974). Heterotrophic nitrification is carried out by heterotrophic bacteria or fungi. Heterotrophs utilise organic C as their energy source. The intermediates and products of both autotrophic and heterotrophic nitrification are the same. Whilst there are many studies on autotrophic nitrification, there are only a limited number that focus on the heterotrophic nitrification pathway because the mechanism remains unclear.

![Autotrophic and heterotrophic nitrification process](image)

**Figure 3** Autotrophic and heterotrophic nitrification process

Autotrophic nitrification can be inhibited by some chemicals (nitrification inhibitors)
such as Nitrapyrin, acetylene (C$_2$H$_2$) and 3, 4-dimethylpyrazole phosphate (DMPP). C$_2$H$_2$ is an effective inhibitor of nitrification (Bremner and Blackmer, 1978). Moreover, C$_2$H$_2$ is bacteriostatic (Juliette et al., 1993) and commonly inhibits autotrophic nitrifiers at a low concentration (e.g. 10 Pa) (De Boer and Kowalchuk, 2001). In contrast to autotrophic nitrification, heterotrophic nitrification is unaffected by nitrification inhibitors, even the highly effective C$_2$H$_2$, but the reason for this remains unclear (Moir et al., 1996). Due to the different effect of nitrification inhibitors on the autotrophic and heterotrophic nitrifiers, they can be used to distinguish these nitrification pathways. It is now known that autotrophic nitrifiers can make use of only inorganic N to produce N$_2$O, while some heterotrophic nitrifiers, such as Alcaligenes faecalis can utilize both organic and inorganic N compounds and produce N$_2$O in culture (Islam et., 2007; Zhang et al., 2014). Moreover, many studies have suggested that soil pH is the important factor determining the prevalence of heterotrophic nitrification in the nitrification process in soil (Zhang et al., 2011).

In previous studies, which analysed soils sampled from geographically different locations, it was found that autotrophic nitrification was the dominant form of nitrification, even in areas when the substrate (NH$_3$) available for autotrophic nitrifiers was relatively low (Paavolainen and Smolander, 1998; Well et al., 2008). However, many studies have proposed that heterotrophic nitrification is the dominant form of nitrification in acid soils (Wood, 1990) because the autotrophic nitrifiers are sensitive to soil pH (Weber and Gainey, 1962). Whereas, De Boer and Kowalchuk (2001) and He et al. (2012) stated that active nitrification in acid soils could also be attributed to acid-tolerant autotrophs. Recently, studies of heterotrophic nitrification have been focussed either on the relative importance of heterotrophic and autotrophic nitrification (Kuroiwa et al., 2011; Zhang et al., 2013) or the substrates of heterotrophic nitrifiers (Islam et al., 2007). These studies have suggested that soil pH is the important factor affecting the role of heterotrophic nitrification, and that heterotrophic nitrifiers can utilise both NH$_3$ and organic N (Zhang et al., 2011). Kuroiwa et al. (2011) showed that heterotrophic nitrification was a minor pathway for gross nitrification in acid (pH 4.1-5.6) forest topsoils in Japan. De Boer et al. (1992) suggested nitrification in acid soils was autotrophic after testing the impact of C$_2$H$_2$ on four acid (pH 4-6) forest soils. Killham (1987) also found that nitrification in an arable acid (pH 4.4) grassland soil was largely autotrophic. Using a combination of $^{15}$N and nitrapyrin-inhibition methods for the measurement of heterotrophic nitrification in an acid (pH 3.8) woodland soil in the laboratory, Barraclough and Puri (1995) showed that heterotrophic nitrification accounted for only 8% of observed
Islam et al. (2007) found that heterotrophic nitrification accounted for 19% and 7% of total nitrification in two acidic (pH 4.8-5.3) pasture soils in Australia. However, Zhang et al. (2011) demonstrated that the contribution of heterotrophic nitrification was higher than that of autotrophic nitrification in an acid (pH 4.3) coniferous forest soil and was governed by soil pH and organic matter content. Zhang et al. (2013) showed that heterotrophic nitrification was the predominant nitrification pathway in an acid (pH 4.5) coniferous forest soil in subtropical China. Zhang et al. (2014) revealed that the contribution of heterotrophic nitrification to total nitrification was 93% in acidic (pH 4.7) forest soil. These conflicting reports regarding the importance of heterotrophic nitrification to total nitrification in acidic soils indicates that there are other factors affecting the significance of heterotrophic nitrification, although little information exists on this topic. Therefore, determining the relative roles of heterotrophic and autotrophic nitrification in different soil types is needed in order to develop models for nitrification which will guide soil management to improve N use efficiency.

N₂O is produced as a by-product of nitrification. It has been indicated that almost 80% of soil N₂O production could be attributed to nitrification if the correct soil ecosystem types, temperature and moisture existed (Godde and Conrad, 1999). Maggs and Vinther (1996) identified that the ratio of N₂O to NO₃⁻ varied with O₂ content and soil environmental conditions, soil pH and substrate availability (Chen et al., 2010; Zhao et al., 2007). For example N₂O increased with a decrease in partial pressure of O₂, or an increase in soil moisture and temperature within the favourable range. Moreover, Davidson (1992) also suggested there was a positive relationship between N₂O emission from nitrification and the nitrification rate. However the commonly used models worldwide all estimate and predict the amount of nitrification–oriented N₂O using a fixed ratio, such as DNDC (0.24%) and the DayCent Model (2%) (Li, 2000; Parton et al., 1996). The results based on model predictions using empirical factors are inconsistent with the facts, since N₂O production from soil is highly variable both temporally and spatially. Lan et al. (2013) showed that the proportion of N emitted as N₂O from nitrification varied with soil temperature and moisture ranging from 0.05% to 0.16%. Zhang et al. (2011) indicated that the ratio of N₂O emitted from nitrification ranged from 0.05% to 26% depending on soil types.

Because of culture-dependent technique development, increasing evidence has been presented that pure AOB cultures could directly contribute to soil N₂O emissions (Shaw et al., 2006). The main N₂O-yielding pathway in AOB is proposed to be related to the conversion of
NH$_2$OH to NO$_2^-$, the second substep of ammonia oxidation under oxic conditions (Ritchie and Nicolas, 1972). However, the mechanism of N$_2$O production from NH$_2$OH is not completely characterized (Schreiber et al., 2012). The prevailing view that AOB are strongly correlated with ammonia oxidation and thus the nitrification-oriented N$_2$O production has been challenged because of the discovery of amoA gene in AOA strains (Lund et al., 2012), and by the demonstration of the N$_2$O production capacity of AOA enriched in agricultural soils and marine environments (Jung et al., 2011; Loscher et al., 2012; Jung et al., 2014). These observations suggested that AOA have a potential role in nitrification-related N$_2$O emissions. However, the mechanisms for AOA or AOB N$_2$O production are unclear and still in debate. In addition, it is still not clear whether N$_2$O emissions from AOA and/or AOB are affected by nitrification.

2.1.2 Denitrification

Denitrification is the process whereby NO$_3^-$ is reduced to N$_2$ under anaerobic conditions by denitrifiers, and N$_2$O is a regular intermediate (Philippot et al., 2007). The enzymes involved in denitrification are nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase (Hochstein and Tomlinson, 1988) (Figure 4). These reductases are encoded by distinct functional genes like $narG$ or $napA$ genes (encoding nitrate reductase), $nirK$ or $nirS$ genes (encoding two entirely different types of nitrite reductase), $cnorB$ or $qnorB$ genes (encoding nitric oxide reductase) and $nosZ$ gene (encoding nitrous oxide reductase) (Philippot et al., 2007; Jones et al., 2013). Nitrous oxide reductase activity can be retarded by low soil pH and low O$_2$ levels (Wrage et al., 2001), blocking formation of N$_2$, and increasing N$_2$O emissions from denitrification. Therefore N$_2$O production from denitrification can be higher from acid soils than from neutral or alkaline soils (Knowles, 1982).

Denitrification can lead to N loss of up to 30% of applied fertilizers, thereby influencing the efficiency of N fertilizer use (Ambus and Zechmeister-Boltenstern, 2007). Denitrification also can act as a sink for N$_2$O, as N$_2$O can be reduced to N$_2$ under strong denitrifying conditions (Zhang et al., 2009). There is evidence that 30–80% of the N$_2$O produced from deeper soil layers may be reduced to N$_2$ before diffusion into the atmosphere (Clough et al., 2005). C$_2$H$_2$ is the only chemical identified as an inhibitor of denitrification, and can prevent reduction of N$_2$O to N$_2$ at 10 kpa addition, leading to the accumulation of N$_2$O (Bateman and Baggs, 2005). In general, substantial N$_2$O can be released as an intermediate of denitrification if the
environment lacks oxygen and has abundant NO$_3^-$ and organic C. Although we have a conceptual understanding of how soil parameters such as soil moisture, texture, soil C, and pH influence the N$_2$O:N$_2$ ratio, the quantitative information required to develop algorithms and parameters required by process models is lacking (Chen et al, 2008a). In addition to bacteria, denitrification also can be catalyzed by fungi in a wide variety of soils (Thamdrup, 2012), but the primary product of fungal denitrification is N$_2$O (Baggs, 2011). This may be because fungi generally lack the nosZ gene required for the reduction to N$_2$ (Philippot et al., 2011).

![Figure 4](image)

**Figure 4** The denitrification process and the enzymes involved in each step

2.1.3 Other important microbial sources of soil N$_2$O

Ammonium can be produced by mineralization and also by dissimilatory nitrate reduction to ammonium (DNRA) in a highly reduced system (Yin et al., 2002). DNRA is the process where NO$_3^-$ is reduced to NH$_4^+$ by fermentative bacteria (Figure 5) under conditions similar to those required for denitrification (Laughlin and Stevens, 2002). This pathway has been recently implicated as a source of N$_2$O in soils (Rutting et al., 2011), but previously it has been frequently ignored in many studies and models (Baggs, 2011). DNRA activity is regulated by many factors, including C:N conditions, Eh, soil pH, bulk density, sand content and NO$_3^-$ concentrations in temperate soils (Schmidt et al., 2011; Silver et al., 2001; Tiedje et al., 1982; Yin et al., 2002). Based on these findings, Baggs (2011) indicated that DNRA was an important source of N$_2$O emission in the rhizosphere where root-derived C resources and transient high demand for O$_2$ and NO$_3^-$ might favour the growth of DNRA microorganisms. Moreover, Yin et al. (2002) demonstrated that less than 5% of added NO$_3^-$ was reduced to N$_2$O or ammonium by DNRA under anoxic conditions without exogenous C addition. When soil samples were pre-incubated with exogenous C, or C was added during incubation, more than 5% of added NO$_3^-$ was reduced via DNRA (Corrêa and Germon, 1991). Because similar environments are required for both denitrification and DNRA, distinguishing the two processes in soils is a challenge.
Nitrifier denitrification is another N$_2$O-yielding process (Shaw et al., 2006; Kool et al., 2010). In this pathway, NH$_3$ is oxidized to NO$_2^-$, followed by reduction of NO$_2^-$ to NO by nitrite reductases and further reduction to N$_2$O by NO reductases, with the whole process carried out solely by microorganisms. Nitrifier denitrification accounts for a large fraction of the N$_2$O loss from dry soil (matric potential -1.0 kPa) (Webster and Hopkins, 1996). Autotrophic nitrification and nitrifier denitrification are initiated by autotrophic nitrifiers and both can be inhibited by nitrification inhibitors (Kool et al., 2010). Nitrifier denitrification has been suggested to be an important source of N$_2$O under low organic C contents, low O$_2$ levels and low pH (Wrage et al., 2001), while the autotrophic nitrification pathway for N$_2$O is favoured by high ammonia contents, low nitrite concentrations and high nitrification rates (Wunderlin et al. 2012). Webster and Hopkins (1996) measured the contribution of nitrifier denitrification to N$_2$O emissions, using the inhibitors C$_2$H$_2$ and O$_2$ and showed that there was no N$_2$O production from nitrifier denitrification in a wet soil (matric potentials of -0.1kPa). Whilst a number of laboratory studies have concluded that nitrifier denitrification is not a major pathway of N loss (e.g. Kester et al., 1997; Dundee and Hopkins, 2001; Kesik et al., 2006).

Chemodenitrification is a non-biological process producing di-nitrogen (N$_2$) or mono-nitrogen oxides (NO$_x$: the NO, and NO$_2$) through decomposition of the intermediate substrates from the oxidation of NH$_4^+$ to NO$_3^-$ or NO$_2^-$ with organic or inorganic compounds (Wrage et al., 2001). Because the occurrence of chemodenitrification is always associated with nitrification, it is difficult to determine from which of these two pathways the N$_2$O or NO$_x$ originated (Martikainen and De Boer, 1993).

### 2.1.4 Current understanding of N$_2$O production pathways

Although the N$_2$O production pathways are quite well known (nitrification, denitrification,
nitrifier denitrification, DNRA and chemodenitrification), the underlying mechanisms leading to N₂O emission from soil ecosystems, and the relative contribution of each pathway are poorly understood. Furthermore, studies of the microbial composition of soils, particular for nitrifiers, has often been inconclusive in explaining the relative contribution of nitrification–related N₂O production from soils (Hu et al., 2015). To fully understand the processes involved in the production and consumption of N₂O from soil, it is necessary to investigate the factors affecting N₂O fluxes from soils, including i) physical, chemical and biological parameters, ii) the preferred production pathway, and iii) the interactions between environmental conditions and the observed soil-atmosphere exchange of N₂O at various spatial and temporal scales (Chen et al., 2008a).

2.2 Factors controlling N₂O production from soils

Agricultural practice, climatic conditions and soil properties all influence N₂O emission from soil. These include soil moisture and temperature (Livesley et al., 2008), aeration, ammonium and nitrate concentration (Jørgensen and Elberling, 2012) and pH (Mørkved et al., 2007). Therefore, in order to better predict and mitigate N₂O emissions, it is essential to identify the key environmental factors that can regulate microbial N₂O sources.

2.2.1 The effect of soil moisture on N₂O emission

Soil water content is one of the predominant factors regulating N₂O emission from soils. Kool et al. (2010) found that emitted N₂O was mainly related to soil moisture conditions. Generally, the N₂O production rate is low below 40% water-filled pore space (WFPS), but it increases rapidly from around 50% to 65% WFPS, in the absence of other limiting factors, such as NH₄⁺ supply (Bateman and Baggs, 2005; Mathieu et al., 2006, Dalal et al., 2003). Increasing soil water content due to wetting-up events such as irrigation and rainfall can stimulate nitrification and denitrification (Hu et al., 2014), and can promote N₂O production (Hofstra and Bouwman, 2005). At higher water content (> 70% WFPS) aeration will be suppressed, which leads to oxygen diffusion limitation, and denitrification becomes the dominant source of N₂O production (Bateman and Bags, 2005). In N fertilizer amended soil, N₂O emission has been found to be highly correlated with WFPS, with the highest emission under 70% WFPS coming from both nitrification (35-53%) and denitrification (44-58%) pathways (Huang et al., 2014). Eighty-eight percent (88%) of total N₂O emission was attributed to NH₃ oxidation at 45%
WFPS (Well et al., 2008). This may suggest that favourable conditions for N\textsubscript{2}O production from nitrification occur within the range of 30%-70% WFPS (Hu et al., 2015), whereas denitrification dominates N\textsubscript{2}O production in wet soils with >80-90% WFPS (Braker and Conrad, 2011; Huang et al., 2014). Soil moisture has also been found to affect N\textsubscript{2}O emission by nitrification indirectly through changes in the abundance of ammonia oxidizers (Avrahami and Bohannan, 2009; Szukics et al., 2010). Moreover, soil water content not only determines the availability of O\textsubscript{2}, but also influences diffusion and transport of nutrients within the soil matrix and the metabolic activity of microbial cells (Hu et al., 2014), which could confound the relationships between WFPS and rates of N\textsubscript{2}O emissions (Hu et al., 2015). Bergstermann et al. (2011) demonstrated that antecedent moisture content affected N\textsubscript{2}O production, and N\textsubscript{2}O fluxes via denitrification were higher under soil pre-wet at 20% WFPS than that at 75% WFPS. The effect of WFPS on the relative contributions of nitrification and denitrification to N\textsubscript{2}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 that for O\textsubscript{2} (Hu et al., 2015).

2.2.2 The effect of soil pH on N\textsubscript{2}O emission

A major controller of nitrification and denitrification is pH (Šimek and Cooper, 2002). Many ecological studies have shown that soil pH had a significant effect on the abundance and composition of N-cycling functional genes, and nitrification and denitrification rates (He et al., 2007; Shen et al., 2008; Hu et al., 2013). There was a negative relationship between the ratios of N\textsubscript{2}O / (N\textsubscript{2} + N\textsubscript{2}O) and soil pH (range 5 - 8) (Chapuis-Lardy et al., 2007). Global meta-analysis of field experiments has also revealed that the amounts of N\textsubscript{2}O substantially increase in soils with lower pH values (Shcherbak et al., 2014). In contrast Richardson et al. (2009) indicated that N\textsubscript{2} was the end product of denitrification and there was less N\textsubscript{2}O production in alkaline conditions. By contrast, under the acidic conditions, more N\textsubscript{2}O production was observed (Wrage et al., 2001). These findings are supported by observations that the reductases for nitrate, nitrite and nitric oxide are more active at pH < 7 (Richardson et al. 2009; Bakken et al., 2012). Nitrification is highly sensitive to soil pH and maximum nitrification generally occurs between pH 7 and 8 (Haynes, 1986; Avrahami et al., 2003). Moreover, N\textsubscript{2}O production from nitrifier denitrification increased with decreasing soil pH (pH < 7), with more N\textsubscript{2}O produced under acidic than alkaline conditions (Moth-apo et al., 2013). Kool et al. (2010) proposed that soil pH was a significant indicator of the relative contribution of N\textsubscript{2}O sources,
with nitrifier denitrification being positively correlated with soil pH, and denitrification being negatively correlated with soil pH.

### 2.2.3 The effect of organic matter on N$_2$O emission

Heterotrophic denitrifying organisms are also influenced by the interactions between soil moisture, texture, structure and temperature (Gregorich et al., 1996). The survival of heterotrophic denitrifying organisms depends on the presence of organic matter for its C and energy source. Decomposable C can be high in anaerobic microsites which commonly occur in soils due to insufficient replenishment of air by diffusion (Dalal et al., 2003).

A positive correlation was observed between soil denitrification capacity and organic C content, especially water-soluble C content (Burford and Bremner, 1975). This is consistent with studies that found incorporation of plant materials favoured denitrification (Davidson, 1993). It has been reported that in peat soils the organic C availability and NO$_3^-$ concentration under anaerobic conditions can affect denitrification activity (Maljanen et al., 2003). Different land uses and agricultural management practices can influence soil organic carbon and subsequently affect N$_2$O production (Merino et al., 2004).

### 2.2.4 Other factors effecting N$_2$O emission

Apart from the previously mentioned impacts of soil water content, soil pH and soil organic matter content on N$_2$O emissions, other factors were reported to strongly affect N$_2$O emissions. For example, soil temperature is regarded as an important factor that can influence N$_2$O emissions via nitrification (Lang et al., 2011). Allen et al. (2010) showed that N$_2$O emissions fluctuated during wet and hot months. Soil temperature between 25°C and 35°C is considered optimal for nitrification (Haynes, 1986). Temperatures from 30°C to 65°C are reported as optimal for denitrification (Tiedje, 1994; Malhi et al., 1990). Sommerfeld et al. (1993) and Granli and Bøckman (1994) indicated that although denitrification occurred at temperatures < 0°C, N$_2$O emitted from denitrification usually became significant at >5°C. Boumans and Batjes (2002) suggested, in a modelling study, that a greater loss of N$_2$O occurred from urea than from (NH$_4$)$_2$SO$_4$ fertilizer. This was consistent with the observations in laboratory
incubations of loam and sandy loam soils by Zhu et al. (2013). Bremner and Blackmer (1978) found more N\textsubscript{2}O emissions when NH\textsubscript{4}+ or urea-N was added compared to NO\textsubscript{3}− application under aerobic conditions. Also, they found a positive correlation between the rate of N\textsubscript{2}O emission and N addition rates (Bremner and Blackmer, 1978). N\textsubscript{2}O production from soils can be stimulated by the application of degradable organic materials, and NH\textsubscript{4}+ based fertiliser (Murakamin et al., 1987). Materials rich in degradable organic N can also enhance N\textsubscript{2}O emission (Goodroad and Keeney, 1984). In summary, organic C and N are important regulators of N\textsubscript{2}O production. Furthermore, for the soil types, nitrification-related N\textsubscript{2}O production was found to be significantly higher in grassland and arable soils than in forest soils (Kool et al., 2010).

In conclusion, all of these factors confound the relationships between microbial sources of soil N\textsubscript{2}O production and surface fluxes of N\textsubscript{2}O, and add to the difficulty to predict their shifts based on a single environmental factor. More research is ultimately needed to elucidate how soil variables interact to control N\textsubscript{2}O emissions from complex soil environments.

2.3 Methods to measure N\textsubscript{2}O emission from nitrification

Because multiple pathways involved in N\textsubscript{2}O production and N\textsubscript{2}O consumption occur simultaneously in different micro-environments in the same soil, a great challenge exists in allocating their relative contributions. Nitrification inhibitors and isotope signature techniques are commonly utilised to separate N\textsubscript{2}O-producing and -reducing pathways (Zhang et al., 2009). However, recently attempts have been made to correlate rates of nitrification, denitrification and soil N\textsubscript{2}O fluxes with the abundance, community composition, and expression of the key nitrogen-cycling functional genes like \textit{amoA}, \textit{nirK}, \textit{nirS}, \textit{narG}, and \textit{nosZ} in various ecosystems by using molecular methodologies (Philippot et al., 2002; Balser and Firestone, 2005; Ma et al., 2008; Avrahami and Bohannan, 2009; Dai et al., 2013).

2.3.1 Limiting factors

The favourable conditions for nitrifiers and denitrifiers are different, as outlined previously. Therefore the relative contributions of nitrification and denitrification to N\textsubscript{2}O production can be determined by using different incubation conditions to favour one or other pathway.
However, some studies have revealed that nitrification and denitrification cannot be isolated completely by controlling these factors. For example, Robertson and Kuenen (1990) found denitrification under aerobic conditions which is inconsistent with the common knowledge that oxygen inhibits denitrification reductases. Therefore, to investigate more accurately the contribution of nitrification to N$_2$O production, it is important to confirm whether denitrification is occurring in the presence of the limiting factors e.g. aerobic conditions, and if so, to what extent. To further complicate this process, the reduction of N$_2$O to N$_2$ also occurs in denitrification under high soil moisture content. So using N$_2$O as an indication of denitrification occurrence under these conditions may underestimate denitrification.

2.3.2 Nitrification inhibitors

The application of nitrification inhibitors is one possible and widely adopted method to distinguish between nitrification and denitrification (Inubushi et al., 1996). Nitrification inhibitors specifically retard the oxidation of NH$_3$ to NH$_2$OH (Weiske et al., 2001; Li et al., 2008). Low levels (10–100 Pa) of acetylene (C$_2$H$_2$) can effectively inhibit nitrification, and therefore were considered suitable to eliminate N$_2$O production from both NH$_3$ oxidation and denitrification (Zhu et al., 2013). Thus N$_2$O produced in the low-level C$_2$H$_2$ added treatments was predicted to be only from denitrification. Notably, C$_2$H$_2$ (> 10kpa) is known to block the reduction of N$_2$O to N$_2$ by denitrifying bacteria (Klemedtsson et al., 1994). High levels of C$_2$H$_2$ (> 10kpa) could inhibit both nitrification and the last step of denitrification. Therefore, appropriate care is needed with the C$_2$H$_2$ level used to avoid biased estimation of any particular microbial pathway. Because inhibitors are presumed to be ineffective in inhibiting heterotrophic nitrification (Hynes and Knowles, 1982), heterotrophic nitrification and denitrification cannot be distinguished from one another using nitrification inhibitors. Also there are some limitations in the effectiveness of inhibitors. For example, high soil organic matter content and low soil pH can restrict the effectiveness of nitrapyrin (Sahrawat et al., 1987), whereas C$_2$H$_2$ has been found to be ineffective in acid soils and short experiments (Boer et al., 1993), and high soil temperature and loamy soils have been shown to reduce the efficacy of DMPP (Barth et al., 2008).

Although nitrification inhibitors can discriminative between nitrification and denitrification, as well as autotrophic and heterotrophic nitrification, it remains unclear whether
they can differentiate the relative contributions of AOA and AOB to N₂O (Chen et al. 2008b). The relative sensitivity of AOA and AOB to inhibitors may be different because they are divergent over many biological and generic aspects, such as belonging to different domains in the tree of life, having different gene sequences for **amoA**, and different cell sizes (He et al., 2012). Many studies have been conducted to elucidate specific inhibitors exclusively targeting AOA or AOB, though the results have generally been inconsistent. For example, Zhang et al., (2012) indicated that AOA were more strongly inhibited by dicyandiamide (DCD) compared with AOB in acidic soils, while DCD effectively inhibited AOB rather than AOA in N-rich grassland soils (Di et al., 2009; Dai et al., 2013). C₂H₂ was also shown to be a non-selective nitrification inhibitor, and can impede growth of AOA or AOB depending on which group is functionally dominant in nitrification (Liu et al., 2015; Jia and Conrad, 2009; Offre et al., 2009).

3,4-dimethylpyrazol phosphate (DMPP) is another widely used nitrification inhibitor in field studies and agricultural practices, but the effects of DMPP on soil N₂O emissions are highly variable across soil types. For instance, application of DMPP can reduce the annual N₂O emissions by 38% in a wheat–maize rotation, (Liu et al., 2013), and reduce the cumulative N₂O emissions by 99% in a calcareous fluvo aquic soil (Huang et al., 2014). Although field application of C₂H₂ produced from CaH₂ granules was sometimes reported (Klemetsson and Mosier, 1994), this approach was widely criticized due to field decomposition of C₂H₂, utilization of C₂H₂ for denitrification, C₂H₂ catalyzed oxidation of NO (Murray and Knowles, 2003) and inadequate diffusion in water-saturated and fine-textured soils (Watts and Seitzinger, 2000). Although no nitrification inhibitor exclusive to either AOB or AOA has yet been identified, choosing an appropriate inhibitor may be a promising approach to separate soil N₂O emission sources.

### 2.3.3 Stable isotope (**₁⁵N**) tracer technique

Stable isotope enrichment approaches have been developed to identify N₂O sources following the application of **₁⁵N**-labeled fertilizers in short-term experiments, through the measurement of **₁⁵N** enrichment in N₂O and mineral N pools (Baggs, 2008). Application of **₁⁵N** labelled NH₄⁺ and NO₃⁻ enables the source of fertilizer-derived **₁⁵N**-N₂O to be determined. Generally, denitrification-derived N₂O is quantified following the supply of **₁⁵NO₃⁻**, while nitrification derived N₂O is measured following the supply of **₁⁵NH₄⁺** (Baggs, 2008). The reduction of N₂O to N₂ can also be quantified by determining **₁⁵N** in N₂ after the supply of **₁⁵NO₃⁻** (Stevens and Laughlin, 1998). For example, applications of **¹⁴NH₄**₁⁵NO₃ and **₁⁵NH₄**¹⁴NO₃ have been used to
determine the relative contributions of nitrification and denitrification to N₂O production (Baggs and Blum, 2004). Recently, the method of ¹⁵N pool dilution has been used to separate autotrophic and heterotrophic nitrification (Barraclough and Puri, 1995). After the addition of ¹⁵N labelled NO₃⁻, NO₃⁻ produced by nitrification will dilute the ¹⁵N-enriched NO₃⁻ pool, and this dilution can be used to calculate total nitrate production rate. Although the ¹⁵N isotope technique has many advantages in differentiating nitrification-related N₂O and denitrification-oriented N₂O, it cannot separate N₂O emitted from nitrifier denitrification and nitrification (Wrage et al., 2005), and has proved powerless in presenting critical knowledge regarding the metabolic activity of the functional microorganisms. Combining ¹⁵N enrichment technique with DNA/RNA based molecular techniques could substantiate the ¹⁵N isotope technique application, and may identify the functionally active players involved in NH₃ oxidation (Zhang et al., 2012; Hu et al., 2014) possibly by indirectly linking them with N₂O production.

2.3.4 Biotechnology

Nitrification and denitrification are mediated by microbial communities in soils, so quantifying the functional genes that are involved may be a suitable way to identify the source of N₂O. The diversity, composition and abundance of nitrifier and denitrifier microorganisms in soil are of great importance for directly regulating the amount of N₂O emission.

Pure cultures of microbes have commonly been used for investigating the physiology, genetics and biochemistry of microorganisms. However, since only 0.3% of the soil microbiota can be cultured, these studies result in a significant underestimation of microbial diversity and function (Amann et al., 1995). To overcome this problem, molecular biology based-techniques including polymerase chain reaction (PCR) have been developed to detect nitrifiers and denitrifiers in environmental samples. Quantitative-PCR (qPCR) is an effective technique to detect specific genes and can be used to measure the abundance of functional genes in soil.

2.4 Soil microbial communities involved in N₂O emission

2.4.1 Ammonia-oxidising archaea (AOA) and ammonia-oxidising bacteria (AOB)

Until recently, AOB were believed to be the only microbes active in nitrification, however
ammonia-oxidizing archaea activity in soils has been reported based on in situ expression of archaeal amoA genes (Offre et al., 2009; Treusch et al., 2005; Leininger et al., 2006). More recently, it has been revealed that AOA can also be present in large numbers in terrestrial environments (He et al., 2007; Shen et al., 2008). However, data about the relative importance of AOA to nitrification in soils has been limited, and its significance to nitrification in agricultural soils in particular is in need of further investigation. Recently studies have focused on investigating the importance of AOA and AOB to nitrification in terms of the changes in NO$_3^-$ and NH$_4^+$ concentrations (Di et al., 2009). However few studies have been conducted to determine the contribution of AOA and AOB to N$_2$O leakage from nitrification in different soil types and environmental conditions. Variations in N$_2$O emission rates between AOA and AOB were observed in soil samples incubated under similar conditions (Jiang and Bakken, 1999; Shaw et al., 2006), showing a potential effect of soil microbial communities on N$_2$O emission rates. However, the relationship between populations of AOA and AOB and N$_2$O production by nitrification in natural environments is still unclear and needs further clarification.

Moreover, there are some controversial conclusions on the contributions of AOA and AOB to nitrification (Di et al., 2009; Jia and Conrad, 2009). Jia and Conrad (2009) reported that AOB were more active in nitrification than AOA in agricultural soils even though the AOA were the predominant NH$_3$ oxidizer. Di et al. (2009) showed that there was no relationship between the abundance of AOA and the nitrification rate in N-rich grass soils, with nitrification driven by AOB. The relative importance of AOA and AOB may vary in soils depending on the environmental conditions, although it is generally considered that AOA can grow in conditions which are unfavourable for AOB growth, such as low ammonium and low soil pH conditions (Di et al., 2010a; Verhamme et al., 2011). Studies have shown the community structure of AOA and AOB changes in response to external conditions, including fertilization, soil pH, temperature, ammonium levels, sulphide, phosphate levels and organic C (He et al., 2007; Erguder et al., 2009; Hayden et al., 2010).

2.4.2 The effect of fertilization on AOA and AOB

Application of N-fertilizers can lead to stimulation of bacterial and archaeal amoA gene numbers and N$_2$O emission (Verhamme et al., 2011). Enwall et al. (2005) indicated that both the activity and composition of AOB were influenced by fertilizer management regimes, with
the AOB community favoured by high N conditions, consistent with the observation by Avrahami et al. (2003) and Shen et al. (2008). Taylor et al. (2012) also presented that a larger population of AOB under the N-fertilized treatment compared with the AOB community under unfertilized treatments, and there was an increase in AOA amoA abundance in unfertilized treatments, compared with their AOA counterparts in the N-fertilized treatment. However, conflicting results exist with reports that manure application doubled the abundance of archaeal amoA based on phylogenetic analysis (Chan et al., 2013). Ai et al. (2013) also found that manure application increased AOA abundance while inorganic fertilizer increased AOB population abundance. Application rates and forms of N-fertilizers are also important factors acting on the distribution of AOA and AOB (Verhamme et al., 2011; Strauss et al., 2014). Tian et al. (2014) suggested that N application rates increased bacterial amoA abundance. The results from field fertilization experiments indicated that mineral fertilizers had more significant impact than organic manure on increasing AOB community size and potential nitrification rate (Chu et al., 2008; Fan et al., 2011). This probably because in organic manure the N is largely in organic form and therefore not available to the microbes. Strong evidence offered by Levičnik-Höfferle et al. (2012) showed that organic nitrogen utilized by AOA facilitated the increasing nitrification rate, which was combined with corresponding gene abundance increases. However AOB remained stable after inorganic nitrogen addition in acid forest soils. AOB were believed to be the key players in NH₃ oxidation in agricultural soils under high NH₃ concentrations (Jia and Conrad, 2009; Di et al., 2009, 2010b), while there was no positive relationship between N₂O production by nitrification and the total abundance of AOB in low fertilizer concentration treatments (Avrahami and Bohannan, 2009; Zhang et al., 2012). Considering the contrasting results, further investigation is required to understand the relative importance of decreasing pH fuelled by fertilization, and highly available substrates to bacterial NH₃ oxidation.

2.4.3 The effect of soil pH on AOA and AOB

Numerous studies have suggested that soil pH is of importance in shaping the distribution of AOA and AOB. Under acidic pH conditions, Zhang et al. (2012) and Hu et al. (2014) indicated that nitrification was mainly regulated by AOA, however, Di et al. (2009) presented that nitrification activity was mainly due to AOB in alkaline soils. It is reasonable to hypothesize that in strongly acidic soils, AOA may substantially contribute to N₂O production via the NH₃
oxidation pathway, due to their high affinity for NH$_3$ substrates in NH$_3$-poor acidic soils (He et al., 2012). He et al. (2007) first showed the predominant role of AOA in nitrification in acid soils after long-term application of chemical fertilizers. Similar results were observed by Nicol et al. (2008). There are many controversial results on the response of AOA and AOB to the application of nitrification inhibitors. Zhang et al. (2012) demonstrated that AOA rather than AOB was the target of DCD in an acidic soil in China. Gubry-Rangin et al. (2010) indicated that C$_2$H$_2$ inhibited nitrification through declining archaeal amoA abundance, rather than their bacterial counterpart in two acid agricultural soils (pH 4.5 and 6.0). Yao et al. (2011) also found significant positive correlations between AOA population abundance, community composition and potential nitrification rate, but not for AOB in twenty acid tea orchard soils. Moreover, function-based studies provided the evidence that AOA rather than AOB were capable of autotrophic NH$_3$ oxidization with $^{13}$CO$_2$-DNA-SIP in five acid soils (pH<4.50) (Zhang et al., 2012). However, Di et al. (2009) indicated that nitrification was driven by AOB not AOA in N-rich grass neutral soils. DMPP has been reported to only inhibit AOB, whereas AOA remained unaffected in acid soil under field conditions (Kleineidam et al., 2011). A study comparing the effects of DMPP and DCD on AOB and AOA gene copy numbers following the application of animal urine in six grazed grassland acid soils showed that the AOB population was strongly inhibited by both DMPP and DCD (Di and Cameron, 2011).

Recent studies have indicated that pH is the most crucial factor for niche segregation of AOA and AOB. The *Nitrososphaera* cluster accounted for 80.94% of AOA community diversity (Hu et al., 2013). Another Thaumarchaeal lineage, *Nitrosotalea devanaterra*, only occurred with pH ranging from 4.0 to 5.5, which further demonstrated AOA can survive at low pH (Lehtovirta-Morley et al., 2011). Bacteria in the *Nitrosospira*-related cluster were widely considered as the most abundant bacteria NH$_3$ oxidizers (Hu et al., 2014). Recent research showed that soil pH could directly affect AOB activity, by affecting availability of its substrate, NH$_3$ (Norman et al., 2014).

### 2.4.4 Other factors affecting AOA and AOB

Beyond the significant impact of soil pH and fertilizer application, other factors such as climatic scenarios, land-use, soil types, and CO$_2$ concentration have also been reported to strongly affect the abundance and activity of AOA and AOB. For example, land-use exhibited
a larger effect on AOA amoA expression and community structure than on bacterial oxidizers (Shen et al., 2013). Morimoto et al. (2011) indicated that AOB abundance was mainly related to nitrification rates in different land-use soils, while there was a weak relationship between the AOA abundance and nitrification activity. In addition, soil water content was regarded as another key factor affecting the size of microbial and composition of autotrophic nitrifiers (Hu et al., 2014). Soil temperature effects on the abundance and distribution of AOA and AOB were also reported (Taylor et al., 2012). Moreover, community structure of NH₃ oxidizers was tightly correlated with soil properties (Jiang et al., 2011; Hayden et al., 2010), especially soil pH which was shown to be the most important driver (Bru et al., 2011). Ai et al. (2013) demonstrated that the rhizosphere has a marked influence on shifting the AOA community structure, even more significant than long-term fertilization. Examination of the spatial distribution patterns of AOA and AOB at landscape scale suggested there was a spectrum of conditions that impacted the ecological niche differentiation of AOA and AOB (Wessén et al., 2011).

2.5 Summary of current knowledge gaps

Whilst extensive research has been conducted on N₂O emission from soils, there are still knowledge gaps, with currently limited information available on:

1) the source of N₂O, particularly the relative production of N₂O from nitrification and denitrification, with many models estimating the contribution of nitrification using a fixed ratio;

2) the relative importance of heterotrophic and autotrophic nitrification to nitrification, and the microbial community (AOA and AOB) response to the factors affecting nitrification; and

3) the relationship between N₂O emission and abundance and diversity of functional genes associated with N₂O emission under different environmental conditions and soil types.

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Chapter 3

General methodology

The research included sets of microcosm incubation experiments conducted in the laboratory. Soils were collected from different field sites (Table 1). In order to separate the sources of N₂O production from soil and to identify key factors affecting N₂O emissions from nitrification, a combination of chemical and microbial methods e.g. stable isotope ($^{15}$N) labelling, nitrification inhibitor application and molecular biology techniques were used (Figure 1). The combination of methods was used because each applied alone would not have provided an unequivocal answer. The knowledge gained will be used to improve modelling capability and our understanding of N₂O emissions. In this chapter, I will introduce the general materials and methods which were used in the project.

![Diagram showing nitrogen cycle and methods for N₂O source separation](image)

**Figure 1** Methods to separate N₂O sources showing the N species and pathway that can be identified from the use of nitrification inhibitors (NI), stable isotopes ($^{15}$N) and molecular biology techniques (microbial functional genes $amoA$, $narG$, and $nosZ$)

3.1 Soil sampling

3.1.1 Field site description

Soils were collected from eight sites in Australia. The land management and climate conditions at each site are shown as Table 1. The soil characteristics are provided in Table 2.
### Table 1 Field site description

<table>
<thead>
<tr>
<th>Site</th>
<th>Climate</th>
<th>Land Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamilton I, VIC</td>
<td>Temperate</td>
<td>Cropping</td>
</tr>
<tr>
<td>Hamilton II, VIC</td>
<td>Temperate</td>
<td>Cropping</td>
</tr>
<tr>
<td>Bundaberg, QLD</td>
<td>Subtropical</td>
<td>Sugarcane</td>
</tr>
<tr>
<td>Clare, QLD</td>
<td>Subtropical</td>
<td>Sugarcane</td>
</tr>
<tr>
<td>Longworry, VIC</td>
<td>Temperate</td>
<td>Dairy pasture</td>
</tr>
<tr>
<td>Glenormiston, VIC</td>
<td>Temperate</td>
<td>Dairy pasture</td>
</tr>
<tr>
<td>Boneo, VIC</td>
<td>Temperate</td>
<td>Vegetable</td>
</tr>
<tr>
<td>Tamworth, NSW</td>
<td>Temperate</td>
<td>Cropping</td>
</tr>
</tbody>
</table>

### Table 2 Characteristics of soils used in this study

<table>
<thead>
<tr>
<th>Soil names</th>
<th>Texture</th>
<th>pH&lt;sub&gt;H2O&lt;/sub&gt;</th>
<th>Clay</th>
<th>Sand</th>
<th>Silt</th>
<th>NH&lt;sub&gt;4&lt;/sub&gt;-N</th>
<th>NO&lt;sub&gt;3&lt;/sub&gt;-N</th>
<th>BD</th>
<th>CEC</th>
<th>TOC</th>
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<tbody>
<tr>
<td>Hamilton I</td>
<td>Loam</td>
<td>4.5</td>
<td>19</td>
<td>44</td>
<td>38</td>
<td>13</td>
<td>93</td>
<td>0.85</td>
<td>9.42</td>
<td>6.2</td>
</tr>
<tr>
<td>Hamilton II</td>
<td>Loam</td>
<td>5.7</td>
<td>10</td>
<td>61</td>
<td>29</td>
<td>5.1</td>
<td>10</td>
<td>0.9</td>
<td>-----</td>
<td>3.7</td>
</tr>
<tr>
<td>Bundaberg</td>
<td>Sand</td>
<td>6.0</td>
<td>5</td>
<td>90</td>
<td>5</td>
<td>2.6</td>
<td>8.8</td>
<td>1.11</td>
<td>2.6</td>
<td>1.2</td>
</tr>
<tr>
<td>Clare</td>
<td>Clay loam</td>
<td>7.0</td>
<td>53</td>
<td>26</td>
<td>21</td>
<td>4.6</td>
<td>7.6</td>
<td>1.13</td>
<td>27</td>
<td>4.7</td>
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<tr>
<td>Longworry</td>
<td>Clay loam</td>
<td>4.8</td>
<td>4</td>
<td>75</td>
<td>21</td>
<td>16</td>
<td>47</td>
<td>0.7</td>
<td>-----</td>
<td>9.3</td>
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<tr>
<td>Glenormiston</td>
<td>Loam</td>
<td>5.9</td>
<td>10</td>
<td>56</td>
<td>34</td>
<td>11</td>
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<td>0.84</td>
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<tr>
<td>Boneo</td>
<td>Sand</td>
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<td>91</td>
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<td>1.19</td>
<td>6.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Tamworth</td>
<td>Clay loam</td>
<td>8.0</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>6.2</td>
<td>65.3</td>
<td>0.95</td>
<td>-----</td>
<td>1.5</td>
</tr>
</tbody>
</table>

### 3.1.2 Soil sampling

At each site, soil samples were randomly collected from nine locations to a depth of 10 cm. Collected samples were mixed into one composite sample. Collected soils were air-dried, and the remaining roots and leaf pieces were removed. Air-dried soil was ground and sieved (< 2 mm) prior to establishment of the microcosm incubation. A subsample of each soil (10 g of air-dried soil) was taken for gravimetric moisture content measurement after drying (105°C, 48 h) and for analyses of general soil properties (100 g of air-dried soil each).
3.2 Microcosm design

Soil (60 g oven-dry equivalent) was placed into 500 ml incubation vessels and incubated in the dark under 15°C, 25°C and 35°C for three weeks. In order to adjust soil moisture content to the target moisture content (50%, 60%, 70% and 85% water-filled pore space, WFPS) the required amount of distilled water was calculated based on the bulk density (BD) of the soil in the vial. Distilled water (2 ml less than the target amount) was added to soil and the microcosms were pre-incubated for 3 weeks to stabilise the soil microbial communities and prevent effects associated with wetting events. After pre-incubation, 2 ml of each treatment was applied to each incubation vessel to reach the targeted soil moisture content. Five ml of C₂H₂ (1% v/v) was injected into the headspace of the vessel using an air-tight syringe and the vial was kept airtight for 8 hours to ensure sufficient reaction between the C₂H₂ and soil. The soils were aerated by removing the caps for 30 min every 3 days, with water and C₂H₂ replenished every 3 days.

3.3 Treatments

3.3.1 N₂O emission sources

Four treatments were used: ¹⁵NH₄Cl+KNO₃, NH₄Cl+K¹⁵NO₃, ¹⁵NH₄Cl+KNO₃+C₂H₂ (nitrification inhibitor) and control (no fertilizer) to distinguish N₂O production pathways. Through the application of ¹⁵N labelled NH₄⁺, NO₃⁻ and the use of the nitrification inhibitor, C₂H₂, the source of fertilizer-derived ¹⁵N₂O can be determined. The C₂H₂ should inhibit NH₄⁺ oxidation by autotrophic nitrifying bacteria and thereby prevent N₂O production by these microbes. The source of ¹⁵N₂O produced in the ¹⁵NH₄⁺ treatment (without any inhibitor) can be a combination of autotrophic nitrification, heterotrophic nitrification and denitrification. The source of ¹⁵N₂O in the ¹⁵NO₃⁻ treatment is denitrification only. ¹⁵N₂O produced in the ¹⁵NH₄⁺+KNO₃+C₂H₂ treatment should be due to denitrification and heterotrophic nitrification, as the inhibitor will prevent autotrophic nitrification. Therefore, the difference in ¹⁵N₂O between the ¹⁵NO₃⁻ treatment and the ¹⁵NH₄⁺+C₂H₂ treatment is the contribution of heterotrophic nitrification to N₂O emission. The difference in ¹⁵N₂O between the ¹⁵NH₄⁺ treatment and the ¹⁵NH₄⁺+C₂H₂ treatment represents the contribution of autotrophic nitrification.
3.3.2 Microbial community population

Three treatments were used for the assessment of the amoA gene in the soil microbial communities: $^{15}$NH$_4$Cl+KNO$_3$, $^{15}$NH$_4$Cl+KNO$_3$+C$_2$H$_2$, and control (no fertilizer). Subsamples (2 g of soil) were collected from the microcosms described above, before the soil was destructively extracted with 2M KCl. Samples were stored in a -80°C freezer (see section 1.5).

3.4 Gas collection and measurement

Gas samples were collected on days 0, 4, 8, 12, 16 and 20 after fertilizer application. Gas samples (20 ml) for N$_2$O and CO$_2$ analysis were taken from the 500ml vials using gas tight syringes. On each sampling day, gas samples (20 ml) were collected at 0, 8, 16, 24, 48 and 72 hours after vessels closure, using a dynamic chamber technique whereby removed air was replaced with Zero air. Each sample collected was transferred into a pre-evacuated extainer (Exetainer®, Labco Ltd., Lampeter, Ceredigion, UK). Samples were analysed for N$_2$O and CO$_2$ concentrations by a gas chromatograph (Hewlett Packard 6890). Prior to collection of samples, the vials were opened to ensure that N$_2$O and CO$_2$ concentration in the headspace were at ambient levels.

The gas concentration was converted from a volumetric basis (µL L$^{-1}$) to a mass basis (µg cm$^{-2}$) following Equation 1, using the ideal gas law that quantifies the relationship between pressure, volume, mass and temperature of the measured gas concentration.

$$G_m = \frac{G_v \times P \times M_w \times V \times 10^{-6} \times 10^6}{R \times T \times A}$$  \hspace{1cm} (1)

where $G_m$ is the concentration of the trace gas (N$_2$O-N or CO$_2$-C) in µg cm$^{-2}$, $G_v$ is the volume of the trace gas (N$_2$O or CO$_2$) in the headspace during the vessel closure period (µL L$^{-1}$), $P$ is the atmospheric pressure that was assumed to be 1 atm., $M_w$ is molar weight of the component of interest i.e. 28 g mol$^{-1}$ for N or 12 g mol$^{-1}$ for C, $V$ is the vessel headspace volume (cm$^3$), $R$ is the gas law constant 0.08206 L atm. Mol$^{-1}$ °K$^{-1}$, $T$ is the air temperature within the vessel (°K), $A$ is the surface area of the measuring vessel (cm$^2$), $10^{-6}$ converts µmol L$^{-1}$ of trace gas to mol L$^{-1}$, $10^6$ converts g L$^{-1}$ to µg L$^{-1}$. For CO$_2$ the $G_m$ value was multiplied by a factor of $10^{-3}$ to convert CO$_2$-C concentration from µg cm$^{-2}$ to mg cm$^{-2}$.

Gas samples (70 ml) for the analysis of fertilizer-derived $^{15}$N$_2$O were taken on days 0, 4, 8, 12 and 15 using the air tight syringes. Samples were analysed for $^{15}$N enrichment in N$_2$O by...
a mass spectrometer (Hydra 20–20, SerCon, Crewe, UK).

3.5 Soil mineral nitrogen analysis

Following the gas sampling, a subsample (2 g each) of soil was taken from each vessel for microbial analysis and transferred into a 15 ml sterile tube which was then dipped into liquid nitrogen for 5 min and stored in a -80 °C freezer prior to DNA extraction. The remaining 50 g of soil in the incubation vessels was shaken with 250 ml 2M KCl (1:5 ratio soil:KCl) for 1 h at 200 rpm at room temperature, and the extract filtered through a qualitative filter paper (Whatman 42). The extracts (30 ml) were stored at -20°C prior to analysis for mineral N, NH₄⁺ and NO₃⁻, and for ¹⁵N abundance in mineral N (100 ml). Concentrations of NH₄⁺ and NO₃⁻ in the KCl extracts were determined with a segmented-flow analyzer (Skalar, SAN++). The ¹⁵N enrichment of NH₄⁺ and NO₃⁻ was determined by a micro-diffusion method as reported by Saghir et al. (1993), with the modification of an acidified filter paper disc used instead of the petri dish of acid to absorb NH₃, and analysis by the Isotope Ratio Mass Spectrometer (Hydra 20–20, SerCon, Crewe, UK) (see section 3.8).

3.6 Soil DNA extraction

The Power Soil DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) was used for DNA extraction following the manufacturer’s instructions. DNA extractions were quantified by using a Nanodrop spectrophotometer and the quality of extracted DNA was checked on a 1% agarose gel.

3.7 Quantitative polymerase chain reaction (qPCR)

Standards for each gene to be quantified were developed by first confirming the primers amoA F/amoA R (Francis et al., 2005) for archaea and amoA F/Archaeal amoA R (Rotthauwe et al., 1997) for bacteria, amplified a single PCR product of the correct target size (archaeal amoA: 635 bp and bacterial amoA: 491 bp). Endpoint PCR for the bacterial amoA gene was performed in a 20 µl reaction mixture that contained 10 µl of Premix Ex Taq (version 2.0, Takara Bio), 1 µl of the forward primer amoA 1F (Rotthauwe et al., 1997) and 1 µl of the reverse primer amoA 2R (Rotthauwe et al., 1997) and 2 µl of extracted DNA and 6 µl of sterile ultrapure water. For the archaeal amoA PCR the reaction mix contained: 10 µl of Premix Ex Taq (version 2.0, Takara
Bio), 1 µl of the forward primer Arch-amoA (Francis et al., 2005) and 1 µl of the reverse primer Arch-amoAR (Francis et al., 2005) and 2 µl of extracted DNA and 6 µl of sterile ultrapure water. The bacterial amoA amplification was conducted as follows: 2 min of 94°C, 40 cycles consisting of 20 s at 94°C, 30 s at 57°C, and 30 s at 72°C, followed by 72°C for 7 min. The archaeal amoA amplification was conducted as follows: 2 min of 94°C, 40 cycles consisting of 20 s at 94°C, 30 s at 56°C, and 30 s at 72°C, followed 72°C for 7 min. The PCR product was then isolated, purified and cloned into a plasmid. A dilution series was then prepared for the standards to develop a standard curve for determination of gene copy number. The qPCR was optimised for primer concentration, using a primer matrix under different annealing temperatures to identify the best qPCR reaction concentrations and amplification conditions.

The PCR product was isolated using a QIAquick Gel extraction Kit (QIAGEN, Limburg, Netherlands) according to the manufacturer’s instructions and purified, and cloned into a plasmid using a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA). The clone was transformed into E. coli (12 µl/ml) and transformants were selected by plating on Luria Bertani medium containing ampicillin (at 100 µg ml⁻¹). Sixteen clones were selected for sequencing. Plasmids were extracted from the clones by minipreparation with a Qiaprep spin miniprep kit (QIAGEN). The plasmid sequence inserts were amplified for Sanger sequencing using the M13 primer and the BigDye Terminator Cycle Sequencing system (Applied Biosystems, Foster City, CA, USA). Raw sequence files were imported into Sequencher (Gene Codes Corporation, Ann Arbor MI, USA) for trimming and alignments of the plasmid inserts. The sequences of the plasmids for use as QPCR standards were identified and confirmed to be bacterial and archaeal amoA by BLASTn against NCBI Genbank.

Triplicated DNA extracts were analysed by quantitative PCR (qPCR) using iTaq Universal SYBR GREEN Supermix (BioRad Laboratories Inc., US) and a C1000™ Thermal Cycler with 96-Well Reaction Module (Bio-Rad). Each archaeal amoA qPCR reaction was performed in a 20 µl volume containing 10 µl SensiFAST (Bio-Rad Laboratories, USA), 0.5 µM of each primer and 2 µl 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, 45 s at 72°C. Each bacterial amoA qPCR reaction was performed in a 10 µl volume containing 5 µl iTaq Universal SYBR GREEN Supermix (Bio-Rad Laboratories, USA), 0.6 µM of each primer and 2 µl of 10-fold dilution DNA template (1-10 ng). Amplification conditions were the same
as for the archaeal amoA assay. All qPCR assays included a melting-curve protocol for analysis of primer specificity. A known copy number of plasmid DNA for AOA or AOB was used to create a standard curve. For all assays, PCR efficiency was at the range of 90-100% and $r^2$ was 0.96-0.99. Spiking experiments, in which a dilution series is made from a standard template, were prepared in the sample matrix to verify the absence of PCR inhibitors.

3.8 $^{15}$N enrichment in mineral N extracts

The $^{15}$N microdiffusion method followed that of Saghir et al. (1993) with the modification of an acidified filter paper disc used instead of the petri dish of acid to absorb NH$_3$. An aliquot of the 2 M KCl extract containing between 50 and 150 µg N) was placed into a 1 L jar to run the micro-diffusion method. A small disc (5 mm diameter) of a Whatman 41 glass fibre filter wetted by 10 µl of 2.5 M KHSO$_4$ was hung on a stainless steel hook glued to the lid of the jar. Magnesium oxide (MgO) (0.2 g) was added to the extract in the bottom of the jar to make the solution alkaline and cause NH$_4^+$ to be released as NH$_3$. Jars were incubated for 7 days at room temperature (24°C). After 7 days, the lid was removed and the acidified filter disc was detached using tweezers and placed into a desiccator to dry off. New acidified filter paper discs (5 mm diameter) were placed into the same vials. Two drops of 0.3% Brij35 surfactant was added to the solution to moderate H$_2$ release and 0.4 g Devarda’s alloy was weighed out and added to the jar to reduce NO$_3^-$ to NH$_4^+$ and generate NH$_3$. The lid was closed immediately and the diffusion of NH$_3$ proceeded for 7 days. Discs were removed and dried as above. The filter paper discs, once dried were removed and folded into tin cups ready for immediate analysis by the Isotope Ratio Mass Spectrometer (Hydra 20–20, SerCon, Crewe, UK).

3.9 $^{15}$N enrichment in organic N

The 2 M KCl-extracted soil was rinsed with 0.2 M CaCl$_2$ to remove residual mineral N immediately following extraction. Ten g of the 2 M KCl-extracted soil was transferred into a 50 ml centrifuge tube and 45 ml of 0.2 M CaCl$_2$ was added. The sample was then shaken for 30 min at 200 rpm at room temperature and centrifuged at 3500 rpm for 5 min. Supernatant was discarded and the above procedure was repeated. Following this the soil was oven-dried at 60°C for 48 h. And oven-dried soil was then ground by a TissueLyser (TissueLyser II, QIAGEN, USA) and analysed by the Isotope Ratio Mass Spectrometer (Hydra 20–20, SerCon, Crewe, UK).
References
Chapter 4

Nitrification inhibitor selection: DMPP and C$_2$H$_2$, which works best with soils?

This chapter is based on a published article entitled:

The effect of nitrification inhibitors in reducing nitrification and the ammonia oxidizer population in three contrasting soils

Rui Liu · Helen Hayden · Helen Suter · Jizheng He · Deli Chen

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Abstract
Purpose 3,4-Dimethylpyrazole phosphate (DMPP) and acetylene (C2H2) are widely used nitrification inhibitors. These nitrification inhibitors have shown inconsistent efficacy in different soils, demonstrating the importance of determining which soil and microbial factors cause this variability. The aim of the present study was to investigate the efficacy of DMPP and C2H2 to inhibit nitrification and the ammonia oxidizer population in three contrasting soil types from Australia.

Materials and methods Three contrasting soils of different pH ranges (4.6, 7.0, and 8.0) collected from different agriculture systems in Australia were used in a laboratory incubation experiment for 28 days to compare the efficacy of DMPP and C2H2 to inhibit nitrification. We measured mineral nitrogen (N) concentrations during the incubation. In addition, quantitative PCR was applied to quantify the ammonia oxidizer population and to investigate the population change in response to DMPP and C2H2 addition.

Results and discussion Acetylene completely blocked nitrification in the three soils while DMPP was more effective in inhibiting nitrification in the neutral soil (93.5 %) than in the alkaline soil (85.1 %) and acid soil (70.5 %). Ammonia-oxidizing archaea (AOA) were more abundant than ammonia-oxidizing bacteria (AOB) in all three control soils, with the highest AOA abundance found in the acid soil. The addition of DMPP and C2H2 significantly decreased AOA abundance in all soils (P<0.05) and significantly suppressed AOB abundance in the neutral soil and slightly blocked AOB growth in the alkaline soil, though it had no effect on AOB abundance in the acid soil.

Conclusions Our results show C2H2 completely inhibited nitrification and performed better than DMPP in our study. DMPP was more effective in the neutral soil than other two soils. Neither DMPP nor C2H2 was a selective nitrification inhibitor in neutral and alkaline soils in which both AOA and AOB were inhibited. Neither DMPP nor C2H2 had any effect on AOB abundance in the acid soil. Soil pH plays an important role in the effectiveness of DMPP and C2H2 in inhibiting nitrification and ammonia oxidizer population.

Keywords 3,4-Dimethylpyrazole phosphate (DMPP) · Acetylene · AmoA · Archaea · Bacteria · Soil pH

1 Introduction
Nitrification is an important nitrogen transformation process in soils, which converts a relatively immobile form of nitrogen, ammonium (NH4+), into a mobile form (nitrate, NO3-) (Joye and Hollibaugh 1995). Nitrite is subjected to losses by leaching and denitrification when oxygen is limited. Nitrification and denitrification can also lead to the emission of potent greenhouse gas, nitrous oxide (N2O). The application of nitrification inhibitors is one possible way to reduce nitrogen losses and thereby increase nitrogen fertilizer efficiency for ammonium-based fertilizers. Ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) are responsible for the first and rate-limiting step of autotrophic
nitrification. Many studies have found that nitrification inhibitors block nitrification by stopping AOA or AOB growing (Di et al. 2009, 2010; Gubry-Rangin et al. 2010).

3,4-Dimethylpyrazole phosphate (DMPP) is a widely used nitrification inhibitor which in some cases has been very effective in reducing nitrification at low application rates of 0.5–1.0 kg active compound ha⁻¹ (Zerulla et al. 2001; Barth et al. 2008). However, its efficacy at inhibiting nitrification varies markedly with soils (Barth et al. 2001; Shi et al. 2011), temperature, and moisture (Chen et al. 2010). Acetylene (C₂H₂) is another effective inhibitor of nitrification (Bremner and Blackmer 1978). Moreover, C₄H₂ is bacteriostatic (Jaliette et al. 1993) and commonly works on autotrophic nitrification at a low concentration (e.g., 10 Pa) (De Boer and Kovalchuk 2001). Previous studies have shown that C₂H₂ is very effective in inhibiting nitrification in acid soils (Hynes and Knowles 1982; Gubry-Rangin et al. 2010), but the interactions between C₂H₂ and soil properties are rarely reported. Therefore, it is essential to determine what causes the inconsistent efficacy of nitrification inhibitors. Soil pH is believed to be a key factor that affects biological processes in soils (Śmiele and Cooper 2002) by affecting the chemical form, concentration, and availability of substrates (Kenmott et al. 2006) and influencing bacterial diversity and community structure on a global scale (Fierer and Jackson 2006). It has been reported that soil pH was the main factor driving the community changes of AOA and AOB in a series of soil pH gradient plots (He et al. 2007; Nicol et al. 2008; Shen et al. 2008). However, the relationship between soil pH and the effectiveness of nitrification inhibitors has rarely been studied. There are several studies on the responses of AOA and AOB to the addition of nitrification inhibitors (Shen et al. 2008; Di et al. 2009, 2010; Gubry-Rangin et al. 2010; Zhang et al. 2011), though their findings are often contradictory. A comprehensive study is needed to determine the effects of nitrification inhibitors on nitrification and ammonia oxidizer population in different soils.

The objectives of this study were (i) to investigate the efficacy of two types of nitrification inhibitors (DMPP and C₂H₂) to inhibit nitrification in three contrasting soil types of different pH from Australia and (ii) to determine the effects of DMPP and C₂H₂ on ammonia oxidizers in these soils.

2 Materials and methods

Surface soil samples (0–10 cm) of three soil types from different regions and industries and with different pH values were collected from Clare (sugarcane, pH 7.0), Queensland (19.78°S, 147.23°E), Tamworth (pasture, pH 8.0), New South Wales (31.09°S, 150.93°E), and Hamilton (cropping, pH 4.6), Victoria (38.32°S, 142.07°E), air-dried, and ground to pass through a 2-mm sieve prior to analysis. Details of selected soil properties are shown in Table 1. The soils were stored at 4 °C prior to incubation experiments.

Laboratory incubation experiments were conducted in the dark. Sixty grams of air-dried soil from each of the three soil types was placed in capped 500-ml vials at 25 °C and 60 % water-filled pore space (WFPS). Samples were pre-wetted and incubated for 3 weeks under 25 °C and just below 60% WFPS to equilibrate the soil before the application of treatments. Ammonium chloride (NH₄Cl) was applied to all soils at the rate of 100 µg N g⁻¹ soil. The treatments applied were as follows: control (NH₄Cl), DMPP (3.37 ml of 29 % DMPP per g NH₄Cl), and C₂H₂ (1 % of the headspace in the vials). The vials were aerated every 3 days when water content and C₂H₂ were replenished.

Triplicate samples were extracted for NH₄⁺ and NO₃⁻ analyses on days 0, 7, 14, 21, and 28 with 2 M KCl (soil:water ratio 1:5) by shaking for 1 h. The soil extracts were filtered through Whatman number 42 filter papers and analyzed for NH₄⁺ and NO₃⁻ using a segmented flow analyzer (Skalar SAN ++ ).

The amoA gene copy numbers were quantified from triplicate samples on day 28 using real-time polymerase chain reaction (PCR) with two different primer sets to target the AOA (Francis et al. 2005) and AOB (Rothhauwe et al. 1997). Each archaean amoA real-time PCR reaction was performed in a 20-µl volume containing 10 µl SensiFAST (Bio-Rad Laboratories, USA), 0.5 µM of each primer, and 2 µl of 10-fold dilution DNA template (1–10 ng). Amplification conditions were as follows: 95 °C for 3 min, 40 cycles of 3 s at 95 °C, 30 s at 60 °C, and 45 s at 72 °C. Each bacterial amoA real-time PCR reaction was performed in a 10-µl volume containing 5 µl Taq Universal SYBR GREEN Supermix (Bio-Rad Laboratories, USA), 0.6 µM of each primer, and 2 µl 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, PCR efficiency was 90–100 % and r² was 0.96–0.99.

Data were analyzed using SPSS 19, and means were compared using one-way ANOVA between treatments to test the variance with a level of significance of P<0.05.

<table>
<thead>
<tr>
<th>Table 1 Properties of the surface soil (0–10 cm) collected at field sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
</tr>
<tr>
<td>Soil type</td>
</tr>
<tr>
<td>Clay (%)</td>
</tr>
<tr>
<td>Silt (%)</td>
</tr>
<tr>
<td>Sand (%)</td>
</tr>
<tr>
<td>pH&lt;sub&gt;soil&lt;/sub&gt;</td>
</tr>
<tr>
<td>Organic C (%)</td>
</tr>
<tr>
<td>Total N (%)</td>
</tr>
</tbody>
</table>
3 Results

The inhibition of nitrate production by \( \text{C}_2\text{H}_2 \) and DMPP varied with soil type. Nitrate concentrations in the three control soils increased gradually during the incubation, with more \( \text{NO}_3^- \) being produced in the alkaline soil than the other two soils (Fig. 1b, d, f). The addition of \( \text{C}_2\text{H}_2 \) completely blocked the production of \( \text{NO}_3^- \) in all soils (Fig. 1b, d, f). DMPP completely inhibited the production of \( \text{NO}_3^- \) in the neutral soil (Fig. 1b) and markedly slowed its formation in the other two soils (Fig. 1d, f and Table 2).

In the first 7 days, the \( \text{NH}_4^+ \) concentrations in the control soils decreased from 105, 106, and 113 mg N kg\(^{-1}\) soil to 60, 28, and 90 mg N kg\(^{-1}\) soil for the neutral, alkaline, and acid soils, respectively. After that, the rate at which the \( \text{NH}_4^+ \) concentrations decreased was slower, and overall, more \( \text{NH}_4^+ \) was lost from the alkaline soil and less from the acid soil (Fig. 1a, c, e). In the inhibitor treatments, a slight decrease in \( \text{NH}_4^+ \) concentration was observed during the first 7 days, but after that, the concentrations were generally increased. The largest increase occurred in the acid soil treated with \( \text{C}_2\text{H}_2 \) (Fig. 1a, c, and e).

AOA and AOB populations changed with incubation time. After incubation of the soils with \( \text{NH}_4\text{Cl} \) (100 µg N g\(^{-1}\) soil)

### Table 2

<table>
<thead>
<tr>
<th>Soil Type</th>
<th>Nitrification rate (mg N kg(^{-1}) day(^{-1}))</th>
<th>Inhibition* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>DMPP</td>
</tr>
<tr>
<td>Neutral clay</td>
<td>1.7</td>
<td>0.11</td>
</tr>
<tr>
<td>Alkaline clay loam</td>
<td>3.82</td>
<td>0.57</td>
</tr>
<tr>
<td>Acid loam</td>
<td>1.95</td>
<td>0.57</td>
</tr>
</tbody>
</table>

* Inhibition of nitrification = \( (\text{NO}_3^- \text{N produced in control soil}) - (\text{NO}_3^- \text{N produced in inhibitor-treated soil}) / (\text{NO}_3^- \text{N produced in control soil}) \times 100 \)
for 28 days, the AOA amoA gene copy numbers in the control soils ranged from 5.2 to 39×10⁶ g⁻¹ dry soil. The AOA population in the acid soil was greater (39×10⁶ g⁻¹ dry soil) than that in the alkaline soil (18×10⁶ g⁻¹ dry soil) and neutral soil (5.2×10⁶ g⁻¹ dry soil) (P<0.05) (Fig. 2). The AOB population was smaller than that of the AOA in all three soils (Fig. 2). There were more AOB amoA gene copy numbers in the alkaline soil (3.7×10⁶ g⁻¹ dry soil) than in the acid soil (2×10⁶ g⁻¹ dry soil) and neutral soil (0.4×10⁶ g⁻¹ dry soil). In the acid control soil, the ratio of AOA to AOB was highest at 19.5 followed by neutral control soil at 13 and alkaline control soil at 4.9.

The inhibition of AOA and AOB populations by C₃H₅ and DMPP varied with soil type. The addition of DMPP and C₃H₅ significantly reduced the AOA population in all soils, but the effect on the neutral soil was much greater than that on the acid and alkaline soils. Acetylene significantly inhibited AOB population in the neutral soil (P<0.05) and slightly blocked AOB growth in alkaline soil (Fig. 2). However, the AOB population in the acid soil was not affected by the application of the nitrification inhibitors (Fig. 2). The reduction in the ratio of AOA to AOB was greatest (from 19.5 to 2.1) with C₃H₅ in the acid soil and least (from 4.9 to 3.4) with DMPP in the alkaline soil.

4 Discussion

Our study indicated that application of DMPP and C₃H₅ reduced nitrification for all three soil types, but to different extents. Acetylene was much more effective than DMPP in inhibiting nitrification in all three soil types. DMPP performed better in the neutral soil than the other two soils. There was no effect on the rate of NH₄⁺ immobilization after inhibitor addition (Fig. 1), although the inhibitors blocked transformation of NH₄⁺ to NO₃⁻ supporting previous reports by Chalk (1990) and Crawford and Chalk (1993). In our study, soil pH might be a key factor influencing the effectiveness of DMPP and C₃H₅ on nitrification; however, their effectiveness might also have been affected by other soil properties such as soil texture (Burh et al. 2001). A multiple regression including more soil physico-chemical properties is therefore necessary. Although C₃H₅ completely inhibited nitrification, we still measured N₂O emission (data not reported) which we hypothesize must have originated from denitrification of the original NO₃⁻ or heterotrophic nitrification.

In the neutral soil, the two inhibitors significantly suppressed both AOA and AOB and decreased NO₃⁻ content (by 57–85%). In contrast, the effect of these inhibitors on nitrification differed in the other two soil types. This difference may be attributed to soil pH or other properties such as organic matter content (Table 1). It has also been shown that the relative abundance of these organisms is affected by NH₄⁺ concentration (Di et al. 2009; Martens-Habbena et al. 2009; Verhamme et al. 2011), pH (Nicol et al. 2008; Hu et al. 2013), soil type (Girvan et al. 2003; Suzuki et al. 2006), and nutrient content (Di et al. 2009; Erguder et al. 2009). In the alkaline soil, both DMPP and C₃H₅ halved the AOA abundance and decreased AOB gene copy numbers by 27 and 35%, respectively. Our results differ from the study conducted by
Kleineidam et al. (2011) who observed that DMPP only reduced AOB but not AOA abundance in an acid soil 8 weeks after fertilizer application. He et al. (2007) found that AOB and AOA population sizes were the lowest in the N treatment with the lowest soil pH and that soil pH was significantly correlated with the abundance of AOB and AOA. However, this study found no clear relationship between soil pH and AOB abundance. Over the incubation time, we measured soil pH at each sample time and found there was no obvious difference between day 0 (Table 1) and day 28.

In the acid soil, both inhibitors inhibited only AOA but not AOB indicating that nitrification in acid soil was mainly associated with the dynamics of the AOA populations rather than that of AOB. DMPP was less effective in lowering AOA, indicating AOA may be more sensitive to C2H4 than DMPP in acid soil. Office et al. (2009) demonstrated a similar result in which AOA was inhibited by C2H4 in acid soil. It has been reported that soil environmental factors can determine the ecological niche of AOA and AOB (Girvan et al. 2003; Suzuki et al. 2009). Compared to AOB, AOA is better adapted to low NH4 availability (Martens-Habbena et al. 2009) and low pH (Nicol et al. 2008). Studies have shown that AOA were more abundant in unfertilized agricultural soils with low NH4+ content (Office et al. 2009), while more AOB were found in fertilized soils or grazed pastures receiving additional N from animal excretion (Di et al. 2009; Jia and Conrad 2009). Soil heterogeneity and physiological differences between AOA and AOB may explain why they can coexist in the same soil despite competing for NH4+. AOA and AOB have different niches and may therefore respond differently to inhibitors having specific targets.

5 Conclusions

Acetylene and DMPP effectively inhibited nitrification in all three soil types. C2H4 provided better inhibition than DMPP in our study, and DMPP was most effective when applied to neutral soil than alkaline and acid soils. AOA were significantly inhibited by C2H4 and DMPP in all soils; however, AOB were significantly inhibited by both inhibitors in neutral soil, slightly inhibited in alkaline soil, and was not affected in acid soil. Therefore, we propose that AOA might play a more important role than AOB in autotrophic nitrification in alkaline and acid soils in Australia. DMPP and C2H4 were effective in inhibiting both AOA and AOB in neutral soil.

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Chapter 5

The relative significance of heterotrophic nitrification to gross nitrification

This chapter is comprised of two published articles entitled:

Chapter 5.1

Nitrate production is mainly heterotrophic in an acid dairy soil with high organic content in Australia. *Biology and Fertility of Soils* DOI: 10.1007/s00374-015-1026-z, in press.

Chapter 5.2

Nitrate production is mainly heterotrophic in an acid dairy soil with high organic content in Australia

Rui Liu1 · Helen Suter1 · Helen Hayden2 · Ji Zheng He1 · Deli Chen1

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Abstract A laboratory incubation experiment was conducted using the nitrification inhibitor acetylene and 15N isotope techniques in order to determine the relative significance of heterotrophic and autotrophic nitrification in three acid soils from different locations in Australia. The contribution of heterotrophic nitrification to total nitrification was found to vary from 20–88% amongst the three soils. In the less acidic Glenormiston soil (pH 6.2, organic C content 5.6%), nitrification was largely autotrophic with heterotrophic nitrification accounting for only 20%. However, in the more acidic and higher organic C content Longwarry soil (pH 4.8 and organic C content 9.3%), heterotrophic nitrification was the predominant NO3− production pathway accounting for 88% of total nitrification.

Keywords Heterotrophic nitrification · Acetylene · 15N isotope technique · Acid soils

Introduction

It is generally believed that nitrate (NO3−) can be produced through either autotrophic or heterotrophic nitrification pathways in soils (De Boer and Kowalchuk 2001; Zhang et al. 2013a, b) (Fig. 1), and many studies have proposed that heterotrophic nitrifiers can use both inorganic and organic substrates (De Boer and Kowalchuk 2001; Zhang et al. 2014). Recently, nitrification inhibitors and 15N isotope technique have been used widely to distinguish autotrophic and heterotrophic nitrification (Pedersen et al. 1999). Nitrification inhibitors work by acting upon the ammonia monooxygenase enzyme (AMO), which catalyses the limiting step of nitrification, the ammonium oxidation (Mccarty 1999). In contrast, heterotrophic nitrification is unaffected by nitrification inhibitor (Moir et al. 1996). Acetylene is a highly effective a nitrification inhibitor (Killoham 1987). In most of studies only net nitrification and mineralisation are measured which do not necessarily reflect actual scale of processes, particularly where substrate is subject to losses of other pathway (Stark and Hart 1997). The 15N isotope pool dilution (Barrachlough and Puri 1995) was used to calculate the gross rate of autotrophic and heterotrophic nitrification. Therefore, a combination of nitrification inhibitor method and the 15N isotope techniques can be used to distinguish heterotrophic and autotrophic pathways (Kirkham and Bartholomew 1954).

Because heterotrophic nitrifiers can carry out nitrification under harsh conditions, such as in acid soils (De Boer and Kowalchuk 2001), heterotrophic nitrification may be the dominant form of nitrification in acid soils (Wood 1980). However, De Boer and Kowalchuk (2001) and He et al. (2012) stated that active nitrification in acid soils could also be attributed to acid-tolerant autotrophs, and therefore, the importance of heterotrophic and autotrophic nitrification in soils is still under debate (Killoham 1986; De Boer and Kowalchuk 2001). The relative importance of heterotrophic and autotrophic nitrification in acid soils has been evaluated in several studies, although the findings were often contradictory. KuoIwa et al. (2011) showed that heterotrophic nitrification was the minor pathway for gross nitrification in most acid forest topsoils (pH 4.1–5.6) in Japan. De Boer et al. (1992) suggested nitrification was autotrophic after a test of four acid
forest soils (pH 4.0–6.0) using acetylene as the nitrification inhibitor. Klimasch (1987) also found that nitrification in arable acid grassland soil (pH 4.4) was largely autochthonous. Using a combination of $^{15}$N and nitrapyrin-inhibition methods for the measurement of heterotrophic nitrification in acid woodland soil (pH 3.8) in the laboratory, Barral and Puri (1995) showed that heterotrophic nitrification accounted for only 8% of observed nitrification. Islam et al. (2007) indicated that heterotrophic nitrification accounted for 19 and 7% of total nitrification in two acid pasture soils in Australia (pH 4.8–5.3). However, Zhang et al. (2011) demonstrated that the contribution of heterotrophic nitrification was higher than that of autotrophic nitrification in an acid coniferous forest soil (pH 4.3) and was governed by soil pH and organic matter content. In contrast to the other studies of acid forest soils, Zhang et al. (2013b) showed that heterotrophic nitrification was the predominant NO$_3^-$ pathway in an acid coniferous forest soil (pH 4.5) in subtropical China. Zhang et al. (2014) revealed that the contribution of heterotrophic nitrification to total nitrification was 93% when maize straw was used as a N substrate in acidic forest soils (pH 4.7). Therefore, it is necessary to determine the relative roles of heterotrophic and autotrophic nitrification in different soils to develop models for nitrification to provide information to guide soil management to improve N use efficiency.

The main objective of this study was to determine the importance of heterotrophic nitrification in three acid soils in Australia and to investigate the relative contribution of heterotrophic and autotrophic nitrification to total nitrification. To achieve these objectives, competition for NH$_4^+$-N and NO$_3^-$-N in soils between autotrophic and heterotrophic microorganisms was assessed using C$_2$H$_2$ inhibition and $^{15}$N isotope pool enrichment techniques.

Materials and methods

Surface soil samples (0–10 cm) were collected from three locations with contrasting land-use histories: Longwoory (dairy, pH 4.8), Victoria (33.73° S, 84.43° E), Hamilton (cropping, pH 4.5), Victoria (38.32° S, 142.07° E) and Glenormiston (dairy, pH 6.2), Victoria (38.18° S, 142.97° E). Soil samples were air-dried, ground and sieved (<2 mm), prior to analysis and incubation experiments. The Longwoory soil was sandy loam and contained 4% clay, 21% silt, 75% sand, 9.3% organic C and 0.8% total N. Hamilton soil was clay loam and contained 19% clay, 44% silt, 38% sand, 6.2% organic C and 0.5% total N. Glenormiston soil was clay loam and contained 10% clay, 34% silt, 56% sand, 5.6% organic C, and 0.6% total N.

Laboratory incubation experiments were conducted in the dark at 25 °C. Samples were pre-wetted just below 60% water-filled pore space (WFPS) to equilibrate the soil before the application of treatments (each had three replicates), which were as follows: addition of labelled ammonium ($^{15}$NH$_4$Cl 10 atom% enrichment) plus KNO$_3$ (control) and with C$_2$H$_2$ (1% v/v) and addition of labelled nitrate (K$^{15}$NO$_3$, 10 atom% enrichment) plus NH$_4$Cl. For each soil, a series of 500-ml capped vials were prepared with air-dried soil (equal to 60 g of 105 °C dried soil). The $^{15}$N-spike solution was added to each of the vials to supply a final soil N concentration of 100 μg NH$_4$-N g$^{-1}$ soil and 50 μg NO$_3$-N g$^{-1}$ soil. For C$_2$H$_2$ treatments, C$_2$H$_2$ (1% v/v) was injected into the headspace of the vials using an air-tight syringe, and the vials kept airtight for 8 h to ensure C$_2$H$_2$ was totally incorporated into the soil. The soil was adjusted to 60% WFPS and incubated for 15 days at 25 °C. The soils were aerated by removing the caps for 30 min every 3 days, and water and C$_2$H$_2$ were replenished as well.

The gross rates of nitrification were determined by $^{15}$N dilution (Kirkham and Bartholomew 1954; Barral and Puri 1995). Briefly, the NO$_3$-N pool was labelled with K$^{15}$NO$_3$, and changes in the isotope composition of NO$_3$-N was determined from measurement of at% excess, with dilution of the signal indicating nitrification. In contrast, pool enrichment involves labelling the NH$_4$-N pool and monitoring the $^{15}$N enrichment in the NO$_3$-N pool. Gross nitrification, heterotrophic nitrification (NH$_4^+$ and organic N oxidation) and autotrophic nitrification rates can be calculated as follows:

Gross nitrification rate (N$_{nitr}$) = autotrophic nitrification + heterotrophic nitrification, calculated using the $^{15}$N dilution method reported by Barola and Puri (1995). Autotrophic nitrification rate (N$_{aut}$) analysed by $^{15}$N enrichment method with $^{15}$NH$_4^+$ addition, using $^{15}$NO$_3^-$ isotope enrichment data with and without C$_2$H$_2$ and the equation of Barola and Puri (1995). Heterotrophic NH$_4^+$ nitrification rate (N$_{heter}$) calculated using the $^{15}$NO$_3^-$ isotope enrichment data where the $^{15}$NH$_4^+$ applied with C$_2$H$_2$ Barola and Puri (1995).

Heterotrophic nitrification of organic N (N$_{org}$) = N$_{heter}$ – N$_{Aut}$.

Measurements of NH$_4^+$-N and NO$_3$-N concentrations and their respective $^{15}$N enrichment were carried out after adding a $^{15}$N-spike solution. After 0, 7 and 15 days, soil samples were extracted with 2 M KCl (soil/solution ratio 1:5) for 1 h under shaking, filtered through quantitative filter paper and frozen.
until analysis. Concentrations of NH₄⁺-N and NO₃⁻-N in the KCl extracts were determined with a segmented-flow analyser (Skalar, SAN++)- The ¹⁵N enrichment of NH₄⁺-N and NO₃⁻-N was determined by a micro-diffusion method as reported by Saghiri et al. (1993), with the modification of an acidified filter paper disc used instead of the petri dish of acid to absorb NH₃, and analysis by Isotope Ratio Mass Spectrometry (IRMS, Hydor 20-20, SerCon, Crewe, UK).

Results and discussion

The NO₃⁻ concentration in the presence of C₂H₂ was lower than that in the absence of C₂H₂ for all soils, and NH₄⁺ concentration in the presence of C₂H₂ was higher than that in the absence of C₂H₂ (Fig. 2a, b) indicating that autotrophic nitrification occurred. C₂H₂ was assumed to totally inhibit autotrophic nitrification based on the used concentration (Lu and Jia 2013; Zhang et al. 2014); therefore, any NO₃⁻ produced was considered to have resulted from heterotrophic nitrification. Sahrawat et al. (1987) found that C₂H₂ was a very effective nitrification inhibitor in organic and mineral soils and only blocked autotrophic nitrification (Killion 1987; Zhang et al. 2013b). Our previous study (allowed sufficient time for C₂H₂ to diffuse to soils and sealed incubation vials for 8 h) also demonstrated that C₂H₂ totally blocked autotrophic nitrification in neutral clay, alkaline clay loam and acid loam (Liu et al. 2015). During the incubation, the NO₃⁻-N concentration in the presence of C₂H₂ was stable for Glenormiston and Hamilton soils, suggesting that autotrophic nitrification was the predominant nitrification process. The highest NO₃⁻-N production was observed in the Glenormiston control soil, and NO₃⁻-N concentration remained unchanged in the C₂H₂ treatment indicating that the process responsible for NO₃⁻-N production was autotrophic nitrification. Previous studies have shown that low soil pH may inhibit autotrophic nitrification (Weber and Gainey 1962), and this supports the observed lower NO₃⁻-N production in the Hamilton control soil (pH 4.5) compared to the Glenormiston soil (pH 6.2). This may indicate that autotrophic nitrification in Hamilton soil accounted less for total nitrification than in the Glenormiston soil. In contrast, in the Longworry soil, the NO₃⁻-N production was less than NO₃⁻-N consumption, and showed a tendency to decrease as shown by the NO₃⁻-N concentration decreasing by nearly 50% during the incubation both in the presence and absence of C₂H₂. However, NH₄⁺-N concentration (Fig. 2b) increased by 5% during the incubation up to 200 mg kg⁻¹. Rice and Tiedje (1989) showed that high NH₄⁺-N concentration could inhibit NO₃⁻-N immobilisation. Therefore, we hypothesise that NO₃⁻-N was mostly consumed through denitrification. The Longworry soil had high organic carbon (93 g kg⁻¹) compared to other soils, and thus, it may also contain labile C pools that can be used by the soil microbes (Kramar et al. 2002). Qin et al. (2013) indicated that NO₃⁻-N production was significantly correlated with the abundance of ammonia oxidizing archaea (AOA). Therefore, another possible reason for the decrease in the NO₃⁻-N production in the Longworry soil may be the changes in the composition and

![Fig. 2 a, b Nitrates and ammonium concentrations in the presence and absence of acetylene (C₂H₂) in the three soils during incubation. c, d ¹⁵N atom% excess of the NO₃⁻-N and NH₄⁺-N pool in the labelled ¹⁵NH₄⁺- treatment in the three soils during incubation. e, f ¹⁵N atom% excess of the NO₃⁻-N and NH₄⁺-N pool in the labelled ¹⁵NH₄⁺- treatment in the three soils during incubation. Error bars indicate standard errors of three replicates.](image-url)
population of the microbial community which can be responsible for the observed nitrification.

The $^{15}$N-NO$_3$ enrichment in the Glenormiston and Hamilton soils was significantly lower at day 7 in the presence of $C_2H_2$ than that in the absence of $C_2H_2$ ($P<0.05$) (Fig. 2c), suggesting that the oxidation of NH$_4$-N to NO$_3$-N by autotrophic nitrifiers was inhibited. However, in the Longworry soil, the $^{15}$N-NO$_3$ enrichment was unaffected by $C_2H_2$ (Fig. 2c), suggesting NO$_3$ production was mainly heterotrophic. $^{15}$N-NO$_3$ enrichment significantly increased with incubation time in $C_2H_2$ treatments in Glenormiston and Hamilton soils ($P<0.05$), suggesting that NO$_3$-N

<table>
<thead>
<tr>
<th>Soil</th>
<th>$N_{\text{tot}}$ mg kg$^{-1}$ d$^{-1}$</th>
<th>$N_{\text{NH}_4}$ mg kg$^{-1}$ d$^{-1}$</th>
<th>$N_{\text{NO}_3}$ mg kg$^{-1}$ d$^{-1}$</th>
<th>$N_{\text{NO}_2}$ mg kg$^{-1}$ d$^{-1}$</th>
<th>$N_{\text{BiO}_3}$ mg kg$^{-1}$ d$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Longworry</td>
<td>d7  56.8 (2.55)</td>
<td>9.08 (1.22)</td>
<td>39.62 (1.34)</td>
<td>2.10 (0.41)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>d15 39.8 (1.46)</td>
<td>2.50 (0.31)</td>
<td>34.41 (1.52)</td>
<td>2.90 (0.73)</td>
<td></td>
</tr>
<tr>
<td>Hamilton</td>
<td>d7  15.6 (2.59)</td>
<td>8.46 (0.23)</td>
<td>6.24 (0.99)</td>
<td>0.87 (0.056)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>d15  4.9 (0.87)</td>
<td>3.06 (0.74)</td>
<td>1.6 (0.17)</td>
<td>0.22 (0.045)</td>
<td></td>
</tr>
<tr>
<td>Glenormiston</td>
<td>d7  47.6 (8.49)</td>
<td>37.7 (3.45)</td>
<td>8.04 (0.91)</td>
<td>1.9 (0.04)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>d15 41.3 (5.44)</td>
<td>31.85 (1.49)</td>
<td>8.10 (1.2)</td>
<td>1.31 (0.032)</td>
<td></td>
</tr>
</tbody>
</table>

Values in brackets are standard error (n=3)
produced from inorganic heterotrophic nitrification occurred in the studied soils (Fig. 2c). The increase in $^{15}$N-N$_2$O$_4$ enrichment in the first week of incubation was attributed to the $^{15}$N-NH$_4^+$ solution addition. $^{15}$N-NH$_4^+$ enrichment significantly decreased with incubation time in all treatments ($P<0.05$). A greater difference was shown between C$_3$H$_2$ and non-C$_3$H$_2$ treatments for the Glenormiston and Hamilton soils whilst for the Longwoory soil, the difference in $^{15}$N-NH$_4^+$ enrichment was small between the C$_3$H$_2$ and non-C$_3$H$_2$ treatments (Fig. 2d). suggesting that nitrification in Longwoory soil was predominantly heterotrophic. $^{15}$N abundance in NO$_3^-$ pool with $^{15}$NO$_3^-$ addition significantly decreased with incubation time ($P<0.05$) (Fig. 2e) suggesting receiving unlabelled NO$_3^-$ via nitrification. Gross nitrification rates varied with soil. In the Longwoory and Glenormiston soils, gross nitrification rates were high at 39.2 and 41.3 mg kg$^{-1}$ d$^{-1}$ and in the Hamilton soil were low (4.9 mg kg$^{-1}$ d$^{-1}$) at day 15 (Table 1). The contribution of heterotrophic NH$_4^+$ nitrification to total nitrification was 19.6, 87.8 and 33.3 % in Glenormiston, Longwoory and Hamilton soils, respectively. In the Glenormiston soil, autotrophic nitrification accounted for the biggest proportion of total nitrification and the high NO$_3^-$-N production is most likely to be related to autotrophic nitrification. However, heterotrophic nitrification was predominant in the dairy soil (Longwoory). Heterotrophic nitrification played a greater role in soils with a low pH (4.8 in Longwoory and 4.5 in Hamilton) compared to Glenormiston soil with pH 6.2. In general, heterotrophic nitrification is more widespread in native ecosystems with established microbial community compositions (Wood 1990) than in agricultural ecosystems. The difference in heterotrophic nitrification activity of the Longwoory and Glenormiston soils highlights the fact that soil pH can influence microbial community composition. Moreover, compared to the Longwoory soil, the Glenormiston soil has lower soil organic carbon content.

In conclusion, heterotrophic nitrification was the predominant NO$_3^-$-N production pathway in an acid dairy soil with relative high organic C content (Longwoory), whilst autotrophic nitrification was prevalent in another dairy soil (Glenormiston) with a higher pH and a lower organic C content, and an acidic cropping soil (Hamilton) particularly the presence of labile C and composition of nitrifier. Further studies are needed to determine the key factors affecting the contribution of heterotrophic nitrification to total nitrification, particularly labile C and soil biology analyses to characterise the nitrifier communities in different soils.

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Chapter 5.2

Influence of temperature and moisture on the relative contributions of heterotrophic and autotrophic nitrification to gross nitrification in an acid cropping soil

Rui Liu1 · Helen Suter1 · Jizheng He1 · Helen Hayden2 · Deli Chen1

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Abstract
Purpose Nitrate can be produced through autotrophic and heterotrophic nitrification. Soil temperature and moisture are key factors affecting nitrification; however, how they influence the relative importance of autotrophic and heterotrophic nitrification is still unknown. The aim of this study was to determine the effects of soil temperature and moisture on autotrophic and heterotrophic nitrification in an acid cropping soil.
Materials and methods An acid cropping soil (pH_{H2O} 4.5) with high organic matter content (6.2 %) was collected from a wheat cropping system and used in a laboratory incubation experiment. Nitrogen as 15NH4Cl and K15NO3 was applied to soil samples which were then incubated for 7 days under 15 °C, 25 °C and 35 °C and 50 % and 70 % water-filled pore space (WFPS). 15N dilution and acetylene (C2H2) inhibition techniques were used to distinguish autotrophic and heterotrophic nitrification. We measured nitrate (NO3−) and ammonium (NH4+) concentration and 15NO3− and 15NH4+ enrichment during the incubation.
Results and discussion The result showed that more heterotrophic nitrification was observed under lower temperature (15 °C) conditions. Around 69 % of the NO3− produced was a result of heterotrophic nitrification at 15 °C and 50 % WFPS and 50 % at 15 °C and 70 % WFPS. However, at 25 and 35 °C, nitrification was largely autotrophic regardless of moisture.
Conclusions Our results demonstrate that heterotrophic nitrification can be an important N transformation pathway in the studied soil. The contribution of heterotrophic nitrification decreased with increasing temperature and moisture.

Keywords 15N dilution technique · Acetylene · Autotrophic nitrification · Heterotrophic nitrification · Moisture · Temperature

1 Introduction
Nitrification is the process of oxidation of ammonia (NH3) to nitrate (NO3−) and is a major part of the global nitrogen (N) cycle which determines the balance between reduced and oxidized forms of N. Nitrification can be either autotrophic or heterotrophic. Autotrophic nitrification is driven by autotrophic nitrifying bacteria and archaea which oxidize NH3 to NO2− and then NO3−. Conversely, heterotrophic nitrification is carried out by heterotrophic nitrifying bacteria or fungi, which can oxidize either NH3 or organic N to form NO3−. Heterotrophic nitrifiers utilize organic C for their energy. In acidic soils (pH_{H2O}<5), nitrate production is generally low and nitrate accumulation historically has required liming or other pH-raising measures (Noyes and Conner 1919; Houbilt 1929). In the early twentieth century, it was usually believed that nitrification could not occur in acid soils because the nitrifying bacteria, the autotrophic nitrifiers, are sensitive to soil pH (Weber and Gainea 1962).

It is now generally accepted that nitrification can occur in a wide range of acid soils with different land use including agricultural fields, tea plantations, coniferous and deciduous forests, heathlands, natural grasslands and vegetated soils.
The hypothesis of this study was that (1) under acidic conditions, heterotrophic nitrification is important and might be the dominant for nitrification and (2) temperature and moisture will affect the relative contributions of heterotrophic nitrification in an acid cropping soil in Australia.

2 Materials and methods

Surface soil (0–10 cm) was collected from a cropping site near Hamilton, Victoria, Australia (38.32°S, 142.07°E). The soil had a pH$_{H_2O}$ of 4.5, organic carbon content of 6.2 %, NH$_4^+$-N concentration of 13 mg kg$^{-1}$, NO$_3^-$-N concentration of 43 mg kg$^{-1}$ and total N content of 0.52 %. Collected soil was air-dried and ground to pass through a 2-mm sieve prior to incubation experiments.

The lab incubation experiment consisted of six treatments, which were combinations of two water contents (50 and 70 % water-filled pore space (WFPS)) and three temperature treatments (15 °C, 25 °C and 35 °C). Each treatment was conducted with three sub-treatments with 15N at 10 % atom excess. These were addition of (1) 15NH$_4$Cl+KNO$_3$ (control), (2) NH$_4$Cl+K$_2$SO$_4$ (K$_2$SO$_4$) and (3) 15NH$_4$Cl+KNO$_3$+C$_2$H$_4$ (1 %v/v) (C$_2$H$_4$). The treatments 15NH$_4$Cl+KNO$_3$ and 15NH$_4$Cl+KNO$_3$+C$_2$H$_4$ (1 %v/v) were used to separate the heterogeneous and autotrophic nitrification. The treatment NH$_4$Cl+K$_2$SO$_4$ was used for gross nitrification rate calculation by isotope dilution technique. The calculation equation was $\frac{\Delta N_{15N}}{\Delta t}$ = $N_{15N}(1+\theta \cdot N_{15N})$ (Barnes and Puri 1995), where $N_{15N}$ was the 15N atom excess in NO$_3^-$ pool, $N$ was gross rate of nitrification, $\theta$ was observed rate at which pool size changes and t and 0 refer to time t and 0, respectively. Air-dried soil, equivalent to 60 g of oven-dry soil, was placed into 500-ml vials and pre-incubated at 15, 25 and 35 °C for 3 weeks before the experiment started at just below the target moisture content. Nitrogen as 100 mg NH$_4$N and 50 mg NO$_3$ kg$^{-1}$ soil was applied in solution to the soil surface. MilliQ water was sprayed onto the soil layer to achieve the target water content (50 and 70 % WFPS). Acetylene (C$_2$H$_4$) (5 ml) was injected into the headspace of the vials through a gas-tight fitting, to achieve a 1 %v/v concentration in the C$_2$H$_4$ treatments, and was replenished every 3 days. The vials were capped for 8 h after treatments were applied, and C$_2$H$_4$ was injected to ensure complete diffusion of the C$_2$H$_4$ through the soil. Samples were incubated at 15, 25 and 35 °C for 1 week. Every 3 days, the vials were opened for aeration and water and C$_2$H$_4$ replenishment.

Measurements of mineral N (NH$_4^+$/N, NO$_3^-$/N) concentrations and their respective 15N abundances were carried out. On days 0 and 7, the soil in the sampled vials (three replicates for each treatment) was extracted with 2 M KCl (soil: KCl = 1: 5) by shaking for 1 h and extracts were filtered through quantitative filter paper (Whatman 42). Samples were kept at -20 °C
prior to analysis by segmented flow analyser (Skalar, SAN++)). The δ 15N enrichment of NH4+-N and NO3--N was determined after micro-diffusion, as reported by Saghiri et al. (1993) with the modification that an acidified filter paper disc was used instead of a Petri dish of acid to absorb NH3 and analysed on an Isotope Ratio Mass Spectrometry (IRMS, Hydra 20–20, SerCon, Crewe, UK).

3 Results and discussion

During the incubation, NO3--concentration increased and NH4+-concentration reduced for all non-C3H2 treatments, albeit to varying degree, indicating that nitrification occurred (Fig. 1). At day 0, NO3--concentration in the 35 °C 70 % WFPS were significantly lower than that of other treatments. This suggests that at 35 °C and 70 % WFPS, denitrification most likely occurred during the pre-incubation phase due to availability of liable C in the air-dried soil (Cabrera and Chiang 1994). The gross rate of nitrification was 3.8, 43.2 and 59.5 μg NO3--N g⁻¹ soil day⁻¹ for 15, 25 and 35 °C 50 % WFPS, respectively, and was 5.0, 59.9 and 45.4 μg NO3-N g⁻¹ soil day⁻¹ for 15, 25 and 35 °C 70 % WFPS, respectively (Table 1). For the C3H2 treatments, NO3--concentration was lower than that in the absence of C3H2 (Fig. 1) over the 7-day period. Moreover, NH4+-concentration in the C3H2 treatment was higher than that of the non-C3H2 treatment (Fig. 1). Previous research showed that C3H2 can completely block autotrophic nitrification in this acidic soil (Liu et al. 2015). Therefore, we conclude that there is autotrophic nitrification occurring in this soil. Szukics et al. (2010) indicated that denitrifier community structure was most affected by water content and that abundance rapidly increased under wetter conditions until NO3-- became limiting. Nitrate concentration did not increase in the C3H2 treatment at 25 and 35 °C 50 % WFPS, indicating autotrophic nitrification was the main NO3--production process. However, the increase of NO3--concentration at 15 °C 50 % WFPS in the presence of C3H2 indicates that heterotrophic nitrification occurred (Fig. 1). In contrast, NO3--concentration in the presence of C3H2 remained stable over 7 days at 15 °C 70 % WFPS, suggesting effective inhibition and hence predominance of autotrophic nitrification. Therefore, it appears that heterotrophic nitrifiers prefer drier and lower temperature conditions where aerobic conditions dominate. The decreasing heterotrophic nitrification rates with increasing soil moisture and
Table 1 The gross nitrification and the mean contribution of heterotrophic nitrification and autotrophic nitrification to total nitrification for the different temperatures and moistures in the whole incubation

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<th>50 % WFPS</th>
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<td>15 °C</td>
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<td>Gross nitrification (µg NO₃-N g⁻¹ soil day⁻¹)</td>
<td>3.8 (1.1)</td>
<td>43.2 (7.9)</td>
<td>59.5 (6.4)</td>
<td>5.0 (1.3)</td>
<td>59.9 (5.2)</td>
<td>45.4 (1.4)</td>
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<td>Heterotrophic nitrification (%)</td>
<td>69.2</td>
<td>7.6</td>
<td>6.8</td>
<td>49.9</td>
<td>6.8</td>
<td>4.2</td>
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<td>Autotrophic nitrification (%)</td>
<td>30.8</td>
<td>92.4</td>
<td>93.2</td>
<td>50.1</td>
<td>93.2</td>
<td>95.8</td>
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Values in brackets are standard deviation (n=3)
*Percent heterotrophic nitrification was calculated as heterotrophic nitrification rate/gross nitrification rate × 100 %

Fig. 2 ¹⁵N atom% excess of the NO₃⁻-N and NH₄⁺-N pools in the labelled ¹⁵NH₄⁺ treatment during the incubation at 50 and 70 %WFPS. Error bars indicate standard errors of three replicates.
temperature was likely associated with the appearance or extension of anaerobic microsites. Higher soil moisture content can restrict O$_2$ diffusion into the soil (Tiedje et al. 1984), but heterotrophic-nitrifying bacterium actively convert NH$_3$ to nitrogen gas under more aerobic conditions (Robertson et al. 1988). Further research is needed to investigate why heterotrophic nitrification is favoured by lower temperatures and drier conditions.

Enrichment of $^{15}$N in the NO$_3^-$ pool significantly ($p<0.05$) increased while $^3$N abundance in NH$_4^+$ pool significantly ($p<0.05$) decreased within 7 days of incubation regardless of whether C$_2$H$_4$ was added or not (Fig. 2). This indicated that NO$_3^-$ produced from nitrification of the $^{15}$N-NH$_4^+$ occurred and suggested that heterotrophic nitrifiers can use inorganic N substrate. It has been controversial for a long time about the substrate for heterotrophic nitrifiers. It was generally accepted that heterotrophic nitrifiers can only utilize organic N (Kilham 1986); however, recently, many studies have revealed that heterotrophic nitrifiers can oxidize either NH$_3$ or organic N to form NO$_3^-$ (Islam et al. 2007; Zhang et al. 2014) which was consistent with our results. The $^{15}$N-NO$_3^-$ enrichment in the 25 and 35 °C and 50 and 70 % WFPS treatments in the presence C$_2$H$_4$ was significantly lower than that in the non-C$_2$H$_4$ treatments ($p<0.05$) indicating that the oxidation of $^{15}$N-NH$_4^+$ was inhibited which confirmed autotrophic rather than heterotrophic nitrification. However, for the 15 °C and 50 and 70 % WFPS treatments, there was no obvious difference between $^{15}$N-NO$_3^-$ and $^{15}$N-NH$_4^+$ in the presence or absence of C$_2$H$_4$ (Fig. 2) indicating that nitrification was not inhibited and therefore was heterotrophic.

The ratio of production of $^{15}$N-NO$_3^-$ in the presence of C$_2$H$_4$ to the production of $^{15}$N-NO$_3^-$ in the absence of C$_2$H$_4$ was defined as the relative contribution of heterotrophic nitrification to total nitrification, based on our knowledge that autotrophic nitrification is completed inhibited by C$_2$H$_4$ in this soil (Liu et al. 2015). Although net apparent nitrification decreased at higher temperature and wetter conditions (decreasing the nitrate concentrations), the overall gross nitrification rate increased with increasing temperature and moisture, with the exception of 50 % WFPS and 35 °C. Soil temperature had a significant influence ($p<0.05$) on gross nitrification rate and the relative contribution of heterotrophic and autotrophic nitrification, more so than soil moisture. Heterotrophic nitrification accounted for 69 % of the NO$_3^-$ produced at 15 °C and 50 % WFPS and 50 % at 15 °C and 70 % WFPS (Table 1). Conversely, autotrophic nitrification was the predominant form of nitrification (>90 %) at 25 and 35 °C and 50 and 70 % WFPS. This is consistent with the optimum temperature of 25–35 °C for autotrophic nitrification in pure culture (Focht and Verstraete 1977). It appears that at 25 and 35 °C, autotrophic nitrification is the predominant form of nitrification. Heterotrophic microorganisms may carry out nitrification in environments which are unfavourable (low temperature and low moisture) for autotrophic nitrifying bacteria (Cai et al. 2010).

4 Conclusions

In conclusion, the importance of heterotrophic nitrification varied with temperature and moisture indicating that both are important factors in controlling the significance of heterotrophic nitrification. Heterotrophic nitrification was the predominant nitrification process at 15 °C and 50 % WFPS but contributed less than 10 % under higher temperature and moisture conditions. Conversely, the significance of autotrophic nitrification increased with increasing temperature and moisture, comprising more than 90 % of total nitrification at 25 and 35 °C and 50 and 70 % WFPS. Combining the gross nitrification rate and the high contribution of heterotrophic nitrification indicates that heterotrophic nitrification is important in the studied soil.

Acknowledgments The authors would like to acknowledge the financial support of Insects Pivot Limited and the Australian Government Department of Agriculture through the Grains Research and Development Corporation.

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Chapter 6

Effect of temperature and moisture on the contribution of nitrification to N$_2$O emission and ammonia oxidizer growth

This chapter is based on a manuscript which is ready to submit entitled:

The effect of temperature and moisture on the contribution of N$_2$O sources and ammonia oxidizer growth in a cropping soil. Ready to submit to *Soil Biology and Biochemistry*.
Chapter 6

The effect of temperature and moisture on the contribution of N\textsubscript{2}O sources and ammonia oxidizer growth in a cropping soil

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ABSTRACT

The continuous increase of the greenhouse gas nitrous oxide (N\textsubscript{2}O) in the atmosphere due to increasing anthropogenic nitrogen input in agriculture has become a global concern. In recent years, identification of the microbial sources responsible for soil N\textsubscript{2}O production has substantially advanced with the development of isotope enrichment and inhibition technologies and the discoveries of specific functional genes. However, little information is available to effectively quantify the N\textsubscript{2}O sources. Therefore it is necessary to improve the understanding of N\textsubscript{2}O formation and quantify the contribution of different pathways. To investigate this a \textsuperscript{15}N-tracing incubation experiment was conducted under controlled laboratory conditions (25°C, 35°C and 50%, 70% and 85% water filled pore space (WFPS)). Nitrification was found to be the main contributor of N\textsubscript{2}O production, having the greatest effect at the WFPS conditions of 50% and 70% WFPS. Nitrification contributed to 87%, 80% and 53% of total N\textsubscript{2}O production at 50%, 70% and 85% WFPS respectively at 25°C, and to 86%, 74% and 33% of total N\textsubscript{2}O production at 50%, 70% and 85% WFPS respectively at 35°C. The proportion of nitrified N as
N$_2$O increased with increasing temperature and moisture, except at 85% WFPS. Ammonia oxidizing archaea (AOA) were the dominant ammonia oxidizers, but the ammonia oxidizing bacteria (AOB) population was significantly related to N$_2$O emitted from nitrification ($p < 0.05$). Soil moisture had a more significant ($p < 0.05$) influence on AOA and AOB abundance compared to soil temperature. Increasing moisture content significantly ($p < 0.05$) decreased AOA and AOB abundance. These findings can be used to develop better models for simulating N$_2$O from nitrification to inform soil management practices for improved N use efficiency.

**Keywords** Nitrification, AOA, AOB, bacteria, archaea, climate change

1. **Introduction**

Nitrous oxide (N$_2$O) is a trace greenhouse gas which is of environmental concern, as it contributes to global warming and stratospheric ozone depletion. Soils are a vital and the largest source of N$_2$O, accounting for an estimated 65% of the anthropogenic atmospheric loading of this gas (IPCC, 2007). N$_2$O is generated through several biological processes in soils such as nitrification, denitrification, dissimilatory nitrate reduction to ammonium (DNRA) and nitrifier denitrification (Hu et al., 2015; Wrage et al., 2005) though the principle N$_2$O sources are nitrification and denitrification.

Nitrification is the process of oxidation of ammonia to nitrate by nitrifiers, and is a principal part of the global N cycle, which determines the balance between reduced and oxidized forms of N. Ammonia oxidization, the first step of nitrification which is catalyzed by the *amoA* gene encoding the ammonia monooxygenase (AMO) enzyme, is known to be 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). Ammonia oxidization is the rate-limiting step of the nitrification process and thus is critical for nitrification-derived N$_2$O emissions. Denitrification is the process where nitrate (NO$_3^-$) is reduced to N$_2$ under anaerobic conditions by denitrifiers, and N$_2$O is a regular intermediate. Although the N$_2$O
production pathways are quite well known, the mechanism and relative contribution of each pathway are poorly understood (Hu et al., 2015). Furthermore, there are many factors regulating N₂O emissions from soils, such like agricultural practice, climatic conditions and soil properties (Livesley et al., 2008; Mørkved et al., 2007), adding great uncertainty in predicting N₂O production and consumption pathways in complex soil environments.

Soil water content and temperature are the predominant factors regulating N₂O production and the relative contribution of nitrification and denitrification to N₂O emission from soils. Zheng et al. (2000) and Huang et al. (2004) reported that N₂O emitted from N fertilizer amended fields was affected mainly by soil moisture conditions. Increasing N₂O emissions with temperature from 10°C to 15°C indicated that N₂O production was sensitive to soil temperature (Lang et al., 2011). Allen et al. (2010) showed that N₂O emissions were higher during wet and hot months than in cool and dry months. Higher temperatures were favourable for nitrification within the range (25°C to 35°C) (Haynes, 1986). Generally, the rate of N₂O production is low below 40% water-filled pore space (WFPS), though it increases rapidly from around 50% to 65% WFPS in the absence of other limiting factors, such as NH₄⁺ supply (Bateman and Baggs, 2005; Mathieu et al., 2006, Dalal et al., 2003). Nitrification has usually been assumed to be the principle source of N₂O at low soil water contents, while denitrification is the predominant contributor of N₂O production at high water contents (Mathieu et al., 2006). It has been reported that nitrification could contribute around 80% of total N₂O emissions depending on soil temperature and moisture (Godde and Conrad, 1999). It is usually considered that maximum N₂O production from denitrification and/or nitrification will occur when soil moisture content is within the range of 55-85% WFPS (Granli and Bøeckman, 1994). Higher soil water content stimulates the reduction of N₂O to N₂ in denitrification, thus the ratio of N₂/N₂O increases with soil moisture content above 75% WFPS (Davidson, 1992; Weier et al., 1993) (Figure 1). The effect of a single factor on nitrification and denitrification-sourced N₂O
emissions has been documented well. However, it is difficult to predict the interaction of multiple environment factors affecting N\textsubscript{2}O production and consumption. Limited information is available on the interaction of temperature and moisture regulating N\textsubscript{2}O pathways.

Figure 1 A generalized relationship between water-filled pore space (WFPS) of soils and the relative N\textsubscript{2}O (▲) and N\textsubscript{2} (■) emissions from nitrification and denitrification (Dalal et al., 2003).

Several studies to date have shown a link between N\textsubscript{2}O flux and community composition and abundance of ammonia oxidizers (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). However, there are few studies on changes in the abundance of ammonia oxidizers under variable environmental conditions linked with N\textsubscript{2}O emission. Soil moisture has been found to indirectly affect N\textsubscript{2}O emission by nitrification through changes in the abundances of ammonia oxidizers (Avrahami and Bohannan, 2009). Di et al. (2010) demonstrated that N\textsubscript{2}O flux was associated with the dynamics of the AOB population in nitrogen-rich grassland soils. However Andert et al. (2011) did not find a relationship between AOA and AOB abundance and N\textsubscript{2}O emission.
Hence, further research is needed to reveal the potential impact of soil moisture and temperature on ammonia oxidizers, and the respective contributions of AOA and AOB to N₂O fluxes.

Therefore, the main objectives of this study were to: 1) investigate how soil moisture and temperature affect the contribution of nitrification and denitrification to N₂O emissions and the abundance of AOA and AOB, and 2) whether there is a relationship between N₂O emission and AOA and AOB abundance.

2. Materials and methods

2.1 Soil sampling

Surface soil (0-10 cm) was collected from the Department of Economic Development, Jobs, Transport and Resources (DEDJTR) research farm at Hamilton, which is located 9 km south of Hamilton, Victoria, Australia (38.32°S, 142.07°E). The soil is classified as a Chromosol, which is an acidic gravelly loam. The study site is in a high rainfall zone (688 mm per annum) and subjected to two different land management practices, cropping and pasture, and field studies have shown that high N₂O emissions occurred when the soil was converted from pasture to cropping. We collected soils from the cropping system after harvest (crop was harvested in 01. 2012) and wheat-stubble covered the soil surface. The soil contained 0.52% total N, 5.2% organic C, 19% clay (< 2 µm), 44% silt (2~60 µm) and 18% sand (60~2000 µm). The CEC was 9.42 meq/100g and the soil pH (H₂O) was 4.5. The original NH₄⁺ and NO₃⁻ were 13 and 93 mg/kg soil, respectively.

2.2 Incubation design

The fresh soils were air-dried, and remaining roots and leaf pieces were removed with tweezers. Air-dried soil was ground and sieved (< 2 mm) prior to establishment of the
microcosm incubation. Three subsamples (10 g of air-dried soil each) were taken for gravimetric moisture content measurement after drying (105°C, 48 h) and for chemical analyses (100 g of air-dried soil each). Oven-dry equivalent 60 g of soils were placed into 500 ml incubation vials. Distilled water (2 ml less than the target amount: 50%, 70% and 85% WFPS) was added to soil and the microcosms were pre-incubated under 25°C and 35°C for 3 weeks to stabilise the soil microbial communities and prevent effects associated with wetting events. After pre-incubation, 2 ml of each treatment was applied to each incubation vessel to reach the targeted soil moisture contents. The treatments applied contained 100 mg N kg⁻¹ as NH₄⁺ and 50 mg N kg⁻¹ as NO₃⁻, added to soils as 1) ¹⁵NH₄Cl+KNO₃ (at 10 atom% ¹⁵N excess) and 2) NH₄Cl+K¹⁵NO₃ (at 10 atom% ¹⁵N excess). Each treatment had three replicates for each soil sampling date and four replicates for each gas sampling time. The soils were incubated for 3 weeks at 25°C, 35°C and 50%, 70% and 85% WFPS. The soil samples were aerated by removing the caps for 30 min every 3 days and water was replenished every 3 days.

2.3 Gas sampling and analysis

Gas samples (20 ml) for N₂O and CO₂ analysis were taken from the headspace of the 500 ml vials on days 0, 4, 7, 12, 15 and 21 after fertilizer application using gas tight syringes. Gas samples (60 ml) for the analysis of fertilizer-derived ¹⁵N₂O were taken on days 0, 4, 7 and 15 using the air tight syringes. Before gas collection, the vials were opened, evacuated and flushed with ambient air to make sure that N₂O and CO₂ concentrations in the headspace were equal to those in the ambient air at the onset of the incubation. Then, the vials were sealed with special gas collection caps and gas samples were taken from the 500 ml vials using gas tight syringes. On each sampling day, gas samples (20 ml) were collected at 0, 8, 24, 48 and 72 hours after vials closure, with each sample transferred into a pre-evacuated exetainer (Exetainer®, Labco Ltd., Lampeter, Ceredigion, UK). The 20 ml gas samples were analyzed for N₂O and CO₂ by
gas chromatography (GC). The 60 ml gas samples were measured by Isotope Ratio Mass Spectrometry (Sercon Hydra).

2.4 Soil Sampling and analysis

Soils were destructively sampled for soil mineral N measurements on days 0, 7, 15 and 21 and for isotope measurements on days 0, 7, 15 directly after gas sampling. Two grams (2 g) of soil was also collected during the destructive sampling for use in DNA extraction. Fifty grams (50 g) of soil in the incubation vessels was shaken with 250 ml 2M KCl (1:5 ratio soil:KCl) for 1 h at 200 rpm at room temperature, and the extract filtered through a qualitative filter paper (Whatman 42). The extracts (30 ml) were stored at -20 °C prior to analysis for mineral N (NH$_4^+$ and NO$_3^-$) and for $^{15}$N abundance in mineral N. Concentrations of NH$_4^+$-N and NO$_3^-$-N in the KCl extracts were determined with a segmented-flow analyzer (Skalar, SAN++). The $^{15}$N enrichment of NH$_4^+$ and NO$_3^-$ was determined by a micro-diffusion method as reported by Saghir et al. (1993), with the modification of an acidified filter paper disc used instead of the petri dish of acid to absorb NH$_3$, and analysis by the Infrared Mass Spectrometer (Hydra 20–20, SerCon, Crewe, UK).

2.5 Soil DNA extraction

The Power Soil DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) was used for DNA extraction, following the manufacturer’s instructions, on soil samples collected on days 0, 7, and 15. DNA extracted was quantified by using a Nanodrop spectrophotometer and the quality of extracted DNA was checked on the 1% agarose gel. The amoA gene copy numbers were quantified from triplicate samples using quantitate polymerase chain reaction (qPCR) with two different primer sets to target the AOA (Francis et al., 2005) and AOB (Rotthauwe et al., 1997). Each archaeal amoA qPCR reaction was performed in a 20 μl volume
containing 10 µl SensiFAST (Bio-Rad Laboratories, USA), 0.5 µM of each primer and 2 µl 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, 45 s at 72°C. Each bacterial amoA qPCR reaction was performed in a 10 µl volume containing 5 µl iTaq Universal SYBR GREEN Supermix (Bio-Rad Laboratories, USA), 0.6 µM of each primer and 2 µl 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 r² was 0.96-0.99.

2.6 Calculations

N₂O fluxes were calculated according to the following equation:

\[ F = \rho \times \frac{V \times \frac{\Delta c}{\Delta t} \times \frac{273}{273 + T}}{A} \]

where \( F \) is the gas flux in µg N₂O-N cm² d⁻¹, \( \rho \) for the density of N₂O under the standard state (g ml⁻¹), \( V \) is the volume of the head space (ml), \( A \) is the area of the vial (cm²), \( \frac{\Delta c}{\Delta t} \) is the change in gas concentration per unit of time in ppm d⁻¹, and \( T \) is the air temperature within the vessel (°K).

The gross nitrification rate was determined by \(^{15}\)N dilution (Kirkham and Bartholomew 1954; Barraclough and Puri 1995).

The relative contribution of denitrification (Cd) and nitrification (Cn) to the N₂O production was calculated using the method of Stevens et al. (1997) and following the equation:

\[ Cd = \frac{(a_{N2O} - a_{NH4})}{(a_{NO3} - a_{NH4})} \] (1-1)
\[ Cn = 1 - Cd \] (1-2)

where \( a_{N2O} \) is the \(^{15}\)N atom% enrichment of N₂O, \( a_{NO3} \) is the \(^{15}\)N atom% enrichment in NO₃⁻ pool, and \( a_{NH4} \) is the \(^{15}\)N atom% enrichment in NH₄⁺ pool. Based on Stevens et al. (1997), the
relative contribution of nitrification and denitrification to N₂O emission was calculated from the ¹⁵N-NO₃ treatment.

N₂O production from nitrification (N₂Oₙ) was calculated as:

\[ N₂Oₙ = Cn \times N₂O_T \]  \hspace{1cm} (2-1)

N₂O production from denitrification (N₂O₅) was calculated as:

\[ N₂O₅ = Cd \times N₂O_T \]  \hspace{1cm} (2-2)

The proportion of nitrified N emitted as N₂O (Pₙ₂o) was calculated as:

\[ Pₙ₂o = N₂Oₙ / n \]  \hspace{1cm} (3)

Where n is gross nitrification rate.

2.7 Statistical analyses

Data were analysed using SPSS 19 and means were compared using one-way ANOVA between treatments to test the variance with a level of significance of \( p < 0.05 \).

3. Results

3.1 N₂O emission under different environmental conditions

Analysis of the gas samples collected on each sampling date showed that there was no significant difference (\( p > 0.05 \)) in N₂O produced from the ¹⁵NH₄⁺ and ¹⁵NO₃⁻ treatments throughout incubation period under all conditions (Figure 2). N₂O production rates increased over the first 7 days and then decreased until they became stable at 25°C and 50% WFPS. N₂O production reached a peak flux at day 4 at 25°C and 70% and 85% WFPS, and at day 3 at 35°C for all moisture treatments (Figure 2).
Figure 2 N$_2$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) and 35°C 50% WFPS (d),
35°C 70% WFPS (e), 35°C 85% WFPS (f). Error bars are the standard deviation.
The greatest N\textsubscript{2}O production peak was detected at 25°C and 85% WFPS, amounting to 48.92 mg kg\textsuperscript{-1} d\textsuperscript{-1}. Within the incubation time, under the same temperature significantly (p < 0.05) more N\textsubscript{2}O was emitted when the soil moisture content was 70% or 85% WFPS than when the soil moisture content was 50% WFPS. Except for the 85% WFPS treatments, the N\textsubscript{2}O production rates were significantly higher (p < 0.05) when the soil temperature was 35°C compared to 25°C (Figure 2).

3.2 Sources of N\textsubscript{2}O under different soil moisture and temperature

The enrichments of the N\textsubscript{2}O, NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3}\textsuperscript{-} pools under different environmental conditions for each treatment are shown in Figures 3 and 4. At both temperatures and all three WFPS conditions, the \textsuperscript{15}N enrichment in the N\textsubscript{2}O pool over the 21 days incubation was always between the \textsuperscript{15}N enrichment of the NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3}\textsuperscript{-} pools for both treatments, and did not exactly match the enrichment of either the NH\textsubscript{4}\textsuperscript{+} or NO\textsubscript{3}\textsuperscript{-} pools (Figure 3, 4). This suggests that N\textsubscript{2}O was produced by both the nitrification and denitrification pathways. In the \textsuperscript{15}NH\textsubscript{4}Cl +KNO\textsubscript{3} treatment, the enrichment of the \textsuperscript{15}NO\textsubscript{3}\textsuperscript{-} pool increased during the incubation due to \textsuperscript{15}N entering the NO\textsubscript{3}\textsuperscript{-} pool from nitrification (Figure 3). In the NH\textsubscript{4}Cl+K\textsuperscript{15}NO\textsubscript{3} treatment, the enrichment of the \textsuperscript{15}NH\textsubscript{4}\textsuperscript{+} pool was close to natural abundance throughout the incubation period and \textsuperscript{15}N abundance in the NO\textsubscript{3}\textsuperscript{-} pool decreased due to dilution by \textsuperscript{14}NO\textsubscript{3}\textsuperscript{-} production by nitrification (Figure 4).
Figure 3: $^{15}$N enrichment of $\text{N}_2\text{O}$, $\text{NH}_4^+$ and $\text{NO}_3^-$ in the $^{15}\text{N}$ labelled $\text{NH}_4^+$ treatment during the incubation. Fig.3 a, b and c are 25°C 50%, 70% and 85% WFPS, respectively. Fig.3 d, e and f are 35°C 50%, 70% and 85% WFPS, respectively. Error bars are the standard deviation.
The contribution of nitrification (Cn) to N\textsubscript{2}O production decreased with increasing temperature and moisture, while the contribution of denitrification (Cd) to N\textsubscript{2}O emission reacted conversely, increasing with increased temperature and moisture (Table 1). On average, nitrification was responsible for 87.24%, 79.77% and 52.85% of the N\textsubscript{2}O production under 25°C 50%, 70%, 85% WFPS respectively and for 86.34%, 73.89% and 33.14% of total N\textsubscript{2}O production under 35°C 50%, 70%, 85% WFPS respectively. Compared to nitrification, denitrification was less important in N\textsubscript{2}O emission when soil moisture content was at 50% and
70% WFPS. At 25°C and 85% WFPS, the ratio of nitrification to denitrification to N\textsubscript{2}O production was almost 1:1. At 35°C and 85% WFPS, the contribution of denitrification to N\textsubscript{2}O emission was highest at an average of 66.87% throughout the incubation period. The nitrification-derived N\textsubscript{2}O (N\textsubscript{2}O\textsubscript{n}) was significantly influenced by soil moisture and soil temperature ($p < 0.05$) (Table 1). N\textsubscript{2}O from nitrification (N\textsubscript{2}O\textsubscript{n}) increased as the soil moisture content increased from 50% to 85% WFPS, with the greatest production detected at 25°C 85% WFPS in the first week. The highest WFPS (85% WFPS) conditions had higher N\textsubscript{2}O production at 35°C than at 25°C, however, the Cn was lower relative to that at 25°C suggesting there was a interaction of temperature and moisture on the contribution of nitrification to N\textsubscript{2}O emission (Table 1).

**Table 1** Gross nitrification rate and the relative contribution of nitrification to N\textsubscript{2}O production under the different incubation conditions

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Moisture</th>
<th>Time</th>
<th>Gross nitrification rate mg N kg(^{-1}) d(^{-1})</th>
<th>Relative contribution %</th>
<th>N\textsubscript{2}O\textsubscript{n} (\text{mg N}_2\text{O-N cm}^{-2}) d(^{-1})</th>
<th>N\textsubscript{2}O\textsubscript{o} (\text{mg N}_2\text{O-N kg}^{-1}) d(^{-1})</th>
<th>$P_{N2O}$ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>50% WFPS</td>
<td>d0-d7</td>
<td>1.97 (0.32)</td>
<td>10.68</td>
<td>89.32</td>
<td>0.0002</td>
<td>0.0017</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d7-d15</td>
<td>5.26 (1.57)</td>
<td>(1.22)</td>
<td>(1.98)</td>
<td>(2.3E5)</td>
<td>(2.3E5)</td>
</tr>
<tr>
<td>70% WFPS</td>
<td>d0-d7</td>
<td>8.32 (0.69)</td>
<td>13.08 (3.92)</td>
<td>14.85 (1.68)</td>
<td>86.92 (1.168)</td>
<td>0.048 (0.009)</td>
<td>0.317 (0.009)</td>
</tr>
<tr>
<td></td>
<td>d7-d15</td>
<td>2.37 (0.24)</td>
<td>27.42 (0.46)</td>
<td>72.58 (0.004)</td>
<td>0.055 (0.004)</td>
<td>0.147 (0.004)</td>
<td>1.06 (0.012)</td>
</tr>
<tr>
<td>85% WFPS</td>
<td>d0-d7</td>
<td>3.84 (0.78)</td>
<td>(4.050) (6.98)</td>
<td>29.09 (6.98)</td>
<td>70.91 (1.53)</td>
<td>10.979 (1.53)</td>
<td>26.76 (5.22)</td>
</tr>
<tr>
<td></td>
<td>d7-d15</td>
<td>6.65 (1.51)</td>
<td>(2.90) (5.49)</td>
<td>(1.89) (4.64)</td>
<td>65.21 (1.902)</td>
<td>34.79 (1.036)</td>
<td>1.902 (6.14)</td>
</tr>
<tr>
<td>35°C</td>
<td>50% WFPS</td>
<td>d0-d7</td>
<td>5.39 (0.16)</td>
<td>11.14</td>
<td>88.86</td>
<td>0.0024</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td></td>
<td>d7-d15</td>
<td>3.43 (0.41)</td>
<td>(2.11)</td>
<td>(3.46)</td>
<td>(0.0005)</td>
<td>(0.005)</td>
</tr>
<tr>
<td>70% WFPS</td>
<td>d0-d7</td>
<td>7.05 (0.33)</td>
<td>(1.24) (2.37)</td>
<td>16.13 (2.37)</td>
<td>83.87 (0.001)</td>
<td>0.017 (0.0009)</td>
<td>0.0086 (0.01)</td>
</tr>
<tr>
<td></td>
<td>d7-d15</td>
<td>2.35 (0.76)</td>
<td>(2.58) (8.99)</td>
<td>18.02 (1.72)</td>
<td>81.98 (0.024)</td>
<td>0.172 (0.039)</td>
<td>0.785 (1.93)</td>
</tr>
<tr>
<td>85% WFPS</td>
<td>d0-d7</td>
<td>11.17 (2.24)</td>
<td>(1.93) (4.51)</td>
<td>50.10 (3.152)</td>
<td>49.90 (3.152)</td>
<td>3.152 (3.139)</td>
<td>3.152 (23.43)</td>
</tr>
<tr>
<td></td>
<td>d7-d15</td>
<td>0.97 (0.36)</td>
<td>(4.20) (6.78)</td>
<td>83.63 (4.64)</td>
<td>16.37 (0.264)</td>
<td>0.624 (0.109)</td>
<td>0.122 (0.19)</td>
</tr>
</tbody>
</table>
3.3 Ammonia oxidizers population dynamic

Both AOA and AOB populations were assessed by quantifying the *amoA* genes over incubation period after application of the treatments (Figure 5). At day 0, at the same soil moisture content, AOA *amoA* copies were higher at 35°C compared to 25°C, however, AOB *amoA* copies were higher at 25°C than at 35°C. At the same soil temperature, both AOA and AOB abundance were highest under the highest WFPS (85% WFPS). AOA and AOB abundances increased in response to N fertilization. For AOA the copy numbers declined as WFPS increased and there was a significant effect at 85% WFPS (*p < 0.05*) at both temperatures. For the AOB there was a greater increase in copy numbers after fertilizer was added compared to AOA.

**Figure 5** AOA and AOB *amoA* gene copy numbers in 15NH4 treatment at 25°C and 35°C during the incubation period.
There was modest effect of temperature on AOA and AOB populations as a similar pattern of change in AOA and AOB amoA copies occurred at 25°C and 35°C. Over the incubation period AOA copy numbers rose from day 0 to 7 and then started to decline to day 15, indicating a different response to the fertiliser than seen for the AOB, where copy numbers rose from day 0-7 and stayed higher at day 15 (Figure 5). The contribution of nitrification to N₂O (Cn) was significantly related with AOB population \((p < 0.01)\) though AOA were the predominant ammonia oxidizers (Figure 5) in this acidic cropping soil.

4. Discussion

4.1 N₂O production sources

Nitrification was the main source of N₂O production over the incubation period, even at 85% WFPS in the first incubation week, which agreed with previous studies (Stevens et al., 1997; Rutting et al., 2010). One possible reason for our observation of nitrification as the main contributor to N₂O production was that the experiment was done under aerobic conditions with the incubation vials aerated every three days and the soils were not water-saturated. Even at high soil moisture content, because the height of soil layer was only 2 cm, O₂ could still diffuse into the soil. Correspondingly, the gross nitrification rate increased with moisture and temperature. Although our results were consistent with previous results (Garrido et al., 2002; Lan et al., 2013), the nitrification contribution may have been overestimated as a result of aeration during the incubation. The relative contribution of nitrification (Cn) to N₂O decreased as moisture increased, consistent with previous reports (Mathieu et al., 2006). However, many field and laboratory studies have indicated that the contribution of nitrification to total N₂O production increased with increased soil moisture (Schuster and Conrad, 1992; Muller et al., 2008). The dynamic was different when the soil was completely anaerobic (Klemedtsson et al., 1988).
Increased soil temperature and moisture resulted in increased N\textsubscript{2}O emissions. At 50% WFPS, peak N\textsubscript{2}O flux occurred at 7 days after the treatment addition, while at 70% and 85% WFPS the peak N\textsubscript{2}O flux occurred at days 4 and 3 respectively, regardless of soil temperature. This may be due to the effect of soil moisture content on microbial nitrifier growth. The initial populations of archaeal and bacterial nitrifiers, as measured by copy number of the \textit{amoA} gene, were greater in the higher WFPS conditions, while at day 7 the copy numbers were greatest for both AOA and AOB in the 50% WFPS samples. Avrahami and Bohannan (2009) suggested that when low or moderate amounts of fertilizer are applied into soils, the rate of N\textsubscript{2}O emission from nitrification (N\textsubscript{2}O\textsubscript{n}) decreases quickly with increased soil moisture and temperature. However, if the fertilizer application was high, the rate changed conversely. In our study, we applied 100 mg NH\textsubscript{4}-N kg\textsuperscript{-1} soil and 50 mg NO\textsubscript{3}-N kg\textsuperscript{-1}, and nitrification-sourced N\textsubscript{2}O production increased with increasing moisture and temperature, except at 85% WFPS. When soil moisture content was 85% WFPS, N\textsubscript{2}O\textsubscript{n} was higher at 25°C than at 35°C. This was probably because N\textsubscript{2} was emitted rather than N\textsubscript{2}O in the 35°C 85% WFPS treatment because complete denitrification occurred. This can be supported by the total N\textsubscript{2}O production being reduced by around 92% 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 N\textsubscript{2}O and N\textsubscript{2} production occurred.

In our study, the proportions of nitrified N as N\textsubscript{2}O (P\textsubscript{N2O}) were 0.07% to 0.7% at 35°C at the three moisture contents. Our results were higher than those reported in previous studies in the agricultural soils (Magg and Vinther, 1996; Tortoso and Hutchinson, 1990; Garrido et al., 2002). Most previous studies found the proportion of N\textsubscript{2}O emission from nitrification varied from 0.02% and 0.2% under aerobic conditions in agricultural soils (Tortoso and Hutchinson, 1990; Zhu et al., 2011). However, Khalil et al. (2004) and Mathieu et al. (2006) demonstrated a higher ratio of 0.16% to 2.32%. The P\textsubscript{N2O} in our study varied significantly ($p < 0.05$) with
temperature and moisture. Higher temperature favoured a higher N\textsubscript{2}O/\textsubscript{NO}\textsubscript{3} (P\textsubscript{N2O}), except at 85% WFPS, which was consistent with the finding by Goodroad and Keeney (1984). While, Lan et al. (2013) found that the proportion of nitrified N emitted as N\textsubscript{2}O tended to decrease with increasing soil moisture content in an alkaline clay soil, and there was no significant difference among different moistures from 40- 90% WHC in the natural loam soil. Therefore, soil temperature and moisture are also soil properties which can affect P\textsubscript{N2O} value. However, the models such as DNDC (Li et al., 2000) and CENTURY (Mathieu et al., 2006) only use the fixed factor to estimate the contribution of nitrification to total N\textsubscript{2}O production. We have identified that soil water, temperature, and their interaction significantly affect N\textsubscript{2}O emissions, the contribution of nitrification to N\textsubscript{2}O emission, and AOA and AOB abundance, and thus models need to be adapted to include this variability. Further work needs to be done to validate these results in field soils.

4.2 AOA and AOB

AOA were the predominant ammonia oxidizers (Figure 5) in our acidic field soil. Schleper et al. (2005) found a similar result. AOA and AOB carry the functional gene \textit{amoA}, and \textit{amoA} can be detected and quantified to accurately assess abundance and composition of AOA and AOB. The activity of nitrification enzymes, including AMO, can vary depending on environmental changes (Niklaus et al., 2001; Zak et al., 2000). Several studies have reported significant increases in AOA and AOB abundance in response to N fertilization (Okano et al., 2004; Mendum et al., 1999; Hermansson and Lindgren, 2001) which is consistent with our observations. Horz et al. (2004) indicated that AOB abundance was most strongly correlated with soil moisture, and soil temperature only had a modest effect on the abundance of AOB which was consistent with our results. In our study, the results demonstrated increasing moisture content significantly ($p < 0.05$) decreased AOA and AOB abundance. It is plausible
that increased moisture caused a reduction in oxygen ($O_2$) availability. However, previous studies showed that soil moisture content changed AOB abundance either positively or negatively. Hastings et al. (2000) found that elevation of soil moisture increased abundance by reducing water stress. While, Belser (1979) also indicated that larger increases in soil moisture can decrease abundance due to decreased diffusion of $O_2$ into the soil. Another possibility is that elevated CO$_2$ due to increasing moisture and temperature altered the competition for resource substrates between AOA, AOB and heterotrophic microbes (Horz et al., 2004) therefore both AOA and AOB abundance decreased. Moreover, Hungate et al. (2000) found that soil heterotroph abundance and activity were increased as CO$_2$ concentration increased.

The contribution of nitrification to N$_2$O ($C_n$) was significantly related with AOB population ($p < 0.01$). Di et al. (2010) also found that AOB population had quantitative relationship with N$_2$O production. However, previous work did not show a significant relationship between potential nitrification activity and community structure of AOB after 7 weeks (Avrahani and Bohannan, 2009).

5. Conclusion

Soil temperature and moisture had a negative influence on the contribution of nitrification to N$_2$O emission. Nitrification occurred even at 85% WFPS in this study. Compared to soil temperature, soil moisture had a significant influence on AOA and AOB population. In our study, a significant relationship was found between the AOB population and N$_2$O emitted from nitrification.

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Chapter 7

Effect of land-use on the contribution of nitrification to N\textsubscript{2}O emission and ammonia oxidizer growth

This chapter is based on a manuscript which is ready to submit entitled:

Impact of different land-use on soil nitrous oxide production and ammonia oxidizing microorganisms in Australian soils. Ready to submit to *Environmental Research.*
Chapter 7

Impact of different land-use on soil nitrous oxide production and ammonia oxidizing microorganisms in Australian soils

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Abstract

Soil ecosystems represent the largest sources of N\textsubscript{2}O emissions, caused primarily by the excessive use of nitrogen fertilizers in agricultural practices. N\textsubscript{2}O emissions in soil are subject to spatial-temporal variability, so it is hard to predict N\textsubscript{2}O emission at regional and national levels based on measurements from a given location without continuous monitoring. Therefore, it is a necessary to understand how soil land-use management affects N\textsubscript{2}O production in order to scale up the estimation of N\textsubscript{2}O fluxes. Furthermore, the potential role of AOA and AOB in nitrification derived N\textsubscript{2}O emissions is still unclear. To improve our understanding of N\textsubscript{2}O emission, a \textsuperscript{15}N-tracing incubation experiment was conducted on soils from different land uses under controlled laboratory conditions. Our results showed that nitrification was the main contributor to N\textsubscript{2}O production in soils from sugarcane, dairy pasture and cropping systems, while denitrification played a major role in N\textsubscript{2}O production in the vegetable cropping soil. Nitrification contributed to 96.7% of the N\textsubscript{2}O emissions in sugarcane soil followed by 71.3%
in the cropping and 70.9% in the dairy pasture system. The proportion of nitrified N as N$_2$O ($P_{N2O}$ value) varied across different soils, with the highest $P_{N2O}$ value (0.26 ‰) found in the cropping soil, which was around 10 times higher than that in other three systems. AOA were the dominant ammonia oxidizers, and were significantly correlated to N$_2$O emitted from nitrification in the sugarcane soil ($p < 0.05$), but AOB was significantly correlated with N$_2$O emitted from nitrification in the cropping soil ($p < 0.01$).

**Keywords** Nitrification, AOA, AOB, bacteria, archaea, land-use management

1. **Introduction**

   How to combat the influences of global environmental changes due to increasing atmospheric greenhouse gas (GHG) emissions (Trivedi et al., 2013) is one of the major challenges of the 21st century. Nitrous oxide (N$_2$O) is a potent GHG with a 300-fold higher global warming potential than CO$_2$, and significantly contributes to global warming and stratospheric ozone depletion (IPCC, 2007; Ravishankara et al., 2009). Ammonium-based fertilizers have been extensively used in agricultural practices to meet the food demand for the increasing human population, resulting in conspicuous increase in atmospheric N$_2$O concentrations (Galloway et al., 2008; Davidson, 2009). Globally, natural and anthropogenic N$_2$O sources are primarily dominated by emissions from soil ecosystems, with occupying approximately 65% of the total N$_2$O emissions (IPCC, 2007). In Australia, agriculture is the second largest greenhouse gas emitter, accounting for 16% of total greenhouse gas emissions, of which N$_2$O emitted from agricultural soils contributes 19% (Australian Greenhouse Office, 2007). The emission of N$_2$O is the result of multiple biological pathways including nitrification
and denitrification (Davidson et al., 1986; Stevens et al., 1997; Hu et al., 2015), which could occur simultaneously in the soil environment (Kuenen and Robertson, 1994).

The relative importance of nitrification and denitrification to N\textsubscript{2}O production can be measured by \textsuperscript{15}N isotope techniques. Differently labelled substrates of \textsuperscript{15}NH\textsubscript{4}\textsuperscript{+} and \textsuperscript{15}NO\textsubscript{3}\textsuperscript{-} were applied into soil and the \textsuperscript{15}N abundance in N\textsubscript{2}O, NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{3}\textsuperscript{-} pools was measured at different sampling times (Stevens et al., 1997). Most studies to date on soil N\textsubscript{2}O emissions have focused either on the estimation of agricultural N\textsubscript{2}O sources (Reay et al., 2012) or on the environmental factors affecting N\textsubscript{2}O fluxes (Cantarel et al., 2011), but limited information is available on the effect of land-use management on N\textsubscript{2}O production and the underlying microbial mechanisms in soil ecosystems. Hadi et al. (2000) showed that N\textsubscript{2}O emission was strongly influenced by land-use management. Moreover, the relative contribution of nitrification and denitrification to N\textsubscript{2}O differ dramatically across different land-use types (Roelandt et al., 2007; Kaiser and Ruser, 2000), therefore, to reduce N\textsubscript{2}O emission and compare N\textsubscript{2}O production from different agricultural systems, the formation and emission rates of N\textsubscript{2}O as affected by land-use must be understood.

Ammonia oxidizers are responsible for the first step of nitrification (oxidation of NH\textsubscript{3} to NO\textsubscript{2}\textsuperscript{-}) and include two groups, ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) (Di et al., 2009, 2010; Gubry-Rangin et al., 2010). The ammonia monooxygenase (AMO), encoded by the \textit{amoA} gene, is the key enzyme to catalyse ammonia oxidization. AOA and AOB populations can be measured by quantifying archaeal and bacterial \textit{amoA} genes. Increasing evidence suggests that AOA and AOB have the genetic potential to produce N\textsubscript{2}O (Di et al., 2010), but their contributions to soil N\textsubscript{2}O emissions in natural environments and the relevant pathways remains unclear.

AOA and AOB abundances varied across different ecosystems and soils (Prosser and Nicol, 2008; Erguder et al., 2009). Land use management is a very important factor affecting
microbial communities in soils. Morimoto et al. (2011) reported that land use affected AOA and AOB abundance and nitrification activity, and there was a strong relationship between the AOB abundance and nitrification activity. Research on different types of flooded paddy soils in China revealed that soil type was a key driver in determining the abundance and community structure of AOB and AOA, irrespective of whether rice was planted (Chen et al., 2010). Research across different soils in Australia has also revealed that changes in soil variables due to different land-use can strongly influence the abundance of AOB amoA genes (Hayden et al., 2010).

Therefore, this study aims to determine the effect of land use on N₂O sources, and to provide comprehensive insights into the microbial community, which enables informed land management to address N₂O emissions in agriculture.

2. Materials and methods

2.1 Soil collection and physicochemical measurement

Soil samples used in this study were taken from four different sites across Australia including Bundaberg (QLD, 24°57’S, 152°20’E), Boneo (VIC, 38°24’S, 144°53’E), Longworry (VIC, 38°08’S, 145°43’E) and Hamilton (VIC, 38°19’S, 142°42’E). The four sampling sites are characterized by different land-use practices as detailed in Table 1. Top soil (0-10 cm) was collected from these four agricultural systems. Soil moisture contents were determined by oven-drying three subsamples (10 g of air-dried soil) at 105°C for 48 h.

2.2 Soil microcosm incubation

The fresh soils were air-dried, and remaining roots and leaf pieces were removed with tweezers. Air-dried soil was ground and sieved (< 2 mm) prior to establishment of the microcosm incubation. Oven-dry equivalent 60 g of soils were placed into 500 ml incubation vials. Distilled water (2 ml less than the target amount 50% WFPS) was added to soil and the
microcosms were pre-incubated under 25°C for 3 weeks to stabilise the soil microbial communities and prevent effects associated with wetting events. After pre-incubation, 2 ml of each treatment was applied to each incubation vessel to reach the targeted soil moisture content. Two sets of treatments were employed with 100 mg N kg\(^{-1}\) as NH\(_4\)\(^+\) and 50 mg N kg\(^{-1}\) as NO\(_3\)\(^-\) added into soils: 1) \(^{15}\)NH\(_4\)Cl + KNO\(_3\) (at 10 atom% \(^{15}\)N excess) and 2) NH\(_4\)Cl + K\(^{15}\)NO\(_3\) (at 10 atom% \(^{15}\)N excess). Each treatment was repeated in three subsets for each soil sampling date and in four replicates for each gas sampling date. Soil moisture content was adjusted to 50% WFPS and samples were incubated for three weeks at 25°C. Aerobic conditions and soil moisture contents in the vials were maintained every three days by opening microcosms for air refreshing and water replenishing.

2.3 Gas Sampling and analysis

Gas samples (20 ml) for N\(_2\)O and CO\(_2\) analysis were taken from the headspace of 500 ml vials on days 0, 4, 7, 12, and 15 after fertilizer application using gas tight syringes. Gas samples (60 ml) for the analysis of fertilizer-derived \(^{15}\)N\(_2\)O were taken on days 0, 4, 7 and 15. Before gas collection, all vials were opened and flushed with ambient air to ensure that N\(_2\)O and CO\(_2\) concentrations in the headspace were equal to those in the ambient air at the onset of gas sampling. The vials were sealed with gas collection caps, and gas samples were taken from the 500 ml vials using gas tight syringes. On each sampling day, gas samples (20 ml) were collected at 0, 8, 24, 48 and 72 h after vials closure, with each sample transferred into a pre-evacuated extetainer (Exetainer®, Labco Ltd., Lampeter, Ceredigion, UK). The 20 ml gas samples were analyzed for concentrations of N\(_2\)O and CO\(_2\) by gas chromatography (GC, Agilent 7890). The 60 ml gas samples were measured by Isotope Ratio Mass Spectrometry (IRMS, Hydra 20–20, SerCon, Crewe, UK).

2.4 Soil Sampling and analysis

Soils were destructively sampled for mineral N measurements and isotope measurements...
on days 0, 7 and 15 immediately after gas sampling. Two grams (2 g) of subsamples were collected for soil DNA extraction, and 50 g of soil in the incubation vessels was shaken with 250 ml 2M KCl (1:5 ratio soil:KCl) for 1 h at 200 rpm at room temperature, and the extract filtered through a qualitative filter paper (Whatman 42). The extracts (30 ml) were stored at -20°C prior to analysis for mineral N, NH₄⁺ and NO₃⁻ on a segmented-flow analyser (Skalar SAN++). The ¹⁵N enrichment of NH₄⁺ and NO₃⁻ was determined by a micro-diffusion method as reported 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 NH₃ and analysis by the Isotope Ratio Mass Spectrometer (Hydra 20–20, SerCon, Crewe, UK).

2.5 Soil DNA extraction and quantitative PCR

The Power Soil DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) was used for DNA extraction on days 0, 7, and 15 following the manufacturer’s instructions. The quantity and quality of the extracted DNA were assessed using a Nanodrop spectrophotometer checked on the 1% agarose gel. The AOA and AOB amoA gene copy numbers were quantified from triplicate samples using quantitative polymerase chain reaction (qPCR) with the primer sets amoA F1/amoA R (Francis et al., 2005) and Archaeal amoA F/Archaeal amoA R (Rotthauwe et al., 1997), respectively. Each AOA qPCR reaction was performed in a 20 µl volume containing 10 µl SensiFAST (Bio-Rad Laboratories, USA), 0.5 µM of each primer and 2 µl 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, 45 s at 72°C. Each AOB PCR reaction was performed in a 10 µl volume containing 5 µl iTaq Universal SYBR GREEN Supermix (Bio-Rad Laboratories, USA), 0.6 µM of each primer and 2 µl of 10-fold dilution DNA template (1-10 ng). Amplification conditions were the same as the AOA qPCR assays. 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 r² was 0.96-0.99.
2.6 Calculations

$N_2O$ fluxes were calculated according to the following equation:

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

where $F$ is the gas flux in $\mu g \ N_2O-N \ cm^2 \ d^{-1}$, $\rho$ represents the density of $N_2O$ under the standard state (g ml$^{-1}$), $V$ is the volume of the head space (ml), $A$ is the area of the vial (cm$^2$), $\Delta c / \Delta t$ is the change in gas concentration per unit of time in ppm d$^{-1}$, and $T$ is the air temperature within the vessel ($^oK$).

The gross nitrification rate was determined by $^{15}N$ dilution (Kirkham and Bartholomew, 1954; Barraclough and Puri, 1995).

The relative contribution by denitrification (Cd) and nitrification (Cn) to $N_2O$ production was calculated using the method by Stevens et al. (1997) following the equation:

$$Cd = \frac{a_{N_2O} - a_{NH4}}{a_{NO3} - a_{NH4}}; Cn = 1 - Cd$$

where $a_{N_2O}$ is the $^{15}N$ atom% enrichment of $N_2O$, $a_{NO3}$ is the $^{15}N$ atom% enrichment in $NO_3^-$ pool, and $a_{NH4}$ is the $^{15}N$ atom% enrichment in $NH_4^+$ pool. Based on Stevens et al. (1997), the relative contribution of nitrification and denitrification to $N_2O$ emission was calculated from the $^{15}N$-NO$_3^-$ treatment.

$N_2O$ production from nitrification ($N_2O_n$) was calculated as: $N_2O_n = Cn \times N_2O_T$

$N_2O$ production from denitrification ($N_2O_d$) was calculated as: $N_2O_d = Cd \times N_2O_T$

The proportion of nitrified N emitted as $N_2O$ ($P_{N2O}$) was calculated as: $P_{N2O} = N_2O_n / n$

Where $n$ is gross nitrification rate.

2.7 Statistical analyses

Data were analysed using SPSS 19 and means were compared using one-way analysis of variance (ANOVA) between treatments to test the variance with a level of significance of $p < 0.05$. 
3. Results

3.1 Soil properties

In this study, the soil physical and chemical properties varied with different land-use (Table 1). All soils except the vegetable soil were acidic (pH $\leq 6$), and inorganic N was dominated by NO$_3^-$ . The dairy pasture soil had the highest total C content (9.3%), and the vegetable soil the lowest (0.8%). Total N in the dairy pasture soil was ten times more than that in the sugarcane and vegetation soils. Mineral N was highest in the dairy pasture soil (Table 1).

Table 1 Field site description and selected characteristics of soils used in this study

<table>
<thead>
<tr>
<th>Site names</th>
<th>Climate</th>
<th>Land-use</th>
<th>Texture</th>
<th>Clay</th>
<th>Sand %</th>
<th>Silt %</th>
<th>pH (H$_2$O)</th>
<th>NH$_4^+$ N mg N/kg soil</th>
<th>NO$_3^-$ N %</th>
<th>OC %</th>
<th>TC %</th>
<th>TN %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bundaberg, QLD</td>
<td>Subtropical</td>
<td>Sugarcane</td>
<td>Sand</td>
<td>5</td>
<td>90</td>
<td>5</td>
<td>6.0</td>
<td>2.6</td>
<td>8.8</td>
<td>0.99</td>
<td>1.18</td>
<td>0.06</td>
</tr>
<tr>
<td>Boneo, VIC</td>
<td>Temperate</td>
<td>Vegetable</td>
<td>Sand</td>
<td>1</td>
<td>91</td>
<td>8</td>
<td>7.8</td>
<td>1.1</td>
<td>19</td>
<td>0.6</td>
<td>0.8</td>
<td>0.08</td>
</tr>
<tr>
<td>Longworry, QLD</td>
<td>Temperate</td>
<td>Dairy pasture</td>
<td>Clay loam</td>
<td>4</td>
<td>75</td>
<td>21</td>
<td>4.8</td>
<td>16</td>
<td>47</td>
<td>2.4</td>
<td>9.3</td>
<td>0.88</td>
</tr>
<tr>
<td>Hamilton, VIC</td>
<td>Temperate</td>
<td>Cropping</td>
<td>loam</td>
<td>10</td>
<td>61</td>
<td>29</td>
<td>5.7</td>
<td>5.1</td>
<td>10</td>
<td>3.1</td>
<td>3.7</td>
<td>0.13</td>
</tr>
</tbody>
</table>

3.2 N$_2$O production rates

Different land-use resulted in a range of N$_2$O emissions. N$_2$O flux decreased throughout the incubation period in the cropping and dairy pasture soils, however in the sugarcane and vegetable soils N$_2$O production became stable after 7 days (Figure 1). Generally, soil with higher total N content had higher N$_2$O production rates. The highest N$_2$O production rate was recorded in the cropping soil (average 0.67 µg N$_2$O-N kg$^{-1}$ soil d$^{-1}$), which was significantly higher than in the sugarcane soil (0.05 µg N$_2$O-N kg$^{-1}$ soil d$^{-1}$), vegetable soil (0.10 µg N$_2$O-N kg$^{-1}$ soil d$^{-1}$), and dairy pasture soil (0.17 µg N$_2$O-N kg$^{-1}$ soil d$^{-1}$).
**Figure 1** Soil N$_2$O production rates from different land-use. Error bars are the standard deviation.

3.3 N$_2$O sources in different soils

The enrichment of N$_2$O, NH$_4^+$, and NO$_3^-$ pool in the different soils for each treatment is shown in Figure 2. In the $^{15}$NH$_4^+$ treatment, the $^{15}$N enrichment in the N$_2$O pool over the course of incubation was always between the $^{15}$N enrichment of the NH$_4^+$ and NO$_3^-$ pools suggesting that N$_2$O was produced by both nitrification and denitrification (Figures 2a, c, e and g). The denitrification pathway was determined to be responsible for only 3.3% of N$_2$O production in the sugarcane soil (Table 2), which was reflected by the $^{15}$N enrichment of N$_2$O from the $^{15}$NO$_3^-$ treatment (Figure 2b). In the sugarcane soil the $^{15}$N enrichment of N$_2$O from the $^{15}$NO$_3^-$ treatment was close to natural abundance (Figure 2b), and N$_2$O was determined to be mainly produced from nitrification (96.7%). However, in the vegetable soil, the $^{15}$N enrichment of the
$\text{N}_2\text{O}$ pool (Figure 2d) was close to the $^{15}$N abundance of the $^{15}\text{NO}_3^-$ at day 7, therefore indicating that denitrification was the predominant pathway of $\text{N}_2\text{O}$ emission and was determined to be responsible for 76.3% of $\text{N}_2\text{O}$ production (Table 2).

**Figure 2** $^{15}$N enrichment of $\text{N}_2\text{O}$, $\text{NH}_4^+$ and $\text{NO}_3^-$ in the $^{15}$N labelled $\text{NH}_4^+$ and $\text{NO}_3^-$ treatments during the incubation. Figure 2 a, c and e represents the $^{15}$N labelled $\text{NH}_4^+$ treatment in the sugarcane, vegetable, dairy pasture and cropping soil, respectively. Figure 2 b, d and e represents the $^{15}$N labelled $\text{NO}_3^-$ treatment in the sugarcane, vegetable, dairy pasture and cropping soil, respectively. Error bars indicated the standard deviation.
The nitrification-derived \( \text{N}_2\text{O} \) peak from the cropping soil was 334.47 \( \mu \text{g N}_2\text{O-N cm}^{-2} \text{d}^{-1} \) (Table 2), which was higher than that in the sugarcane soil (23.40 \( \mu \text{g N}_2\text{O-N cm}^{-2} \text{d}^{-1} \)) although the \( \text{Cn} \) of the sugarcane soil was higher than that of the cropping soil. In the acidic soils, the \( \text{Cn} \) was higher than that of denitrification (Cd) (Table 2), and followed the order sugarcane soil > cropping soil > dairy pasture soil. There was a negative relationship between denitrification capacity and soil pH in the three acidic soils (Table 2).

3.4 The ratios of \( \text{N}_2\text{O} \) derived from nitrification

The gross nitrification rate was calculated by \(^{15}\text{N} \) dilution technique (Barraclough and Puri 1995). The results showed that the gross nitrification rate was 1.70, 5.42, 3.84, and 9.88 mg N kg\(^{-1} \) d\(^{-1} \), for the sugarcane, vegetable, dairy pasture and cropping soils, respectively (Table 2). The nitrification rate in the cropping soil was significantly \( (p < 0.05) \) higher than that in the other soils.

Table 2 Gross nitrification rate and the ratio of \( \text{N}_2\text{O} \) production to nitrification in the studied soils.

<table>
<thead>
<tr>
<th>Land-use</th>
<th>Gross nitrification rate mg N kg(^{-1} ) d(^{-1} )</th>
<th>Relative contribution %</th>
<th>( \text{N}_2\text{O}_4 ) ( \mu \text{g N}_2\text{O-N cm}^{-2} \text{d}^{-1} )</th>
<th>( \text{N}_2\text{O}_4 ) ( \mu \text{g N}_2\text{O-N cm}^{-2} \text{d}^{-1} )</th>
<th>( \text{P}_{\text{N2O}} ) %o</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugarcane</td>
<td>1.70 (0.50)</td>
<td>3.30 (0.45)</td>
<td>96.67 (6.8)</td>
<td>0.80 (0.03)</td>
<td>23.40 (0.34)</td>
</tr>
<tr>
<td>Vegetable</td>
<td>5.42 (0.43)</td>
<td>76.36 (9.2)</td>
<td>23.64 (3.91)</td>
<td>53.65 (7.03)</td>
<td>16.63 (3.30)</td>
</tr>
<tr>
<td>Dairy Pasture</td>
<td>3.84 (0.78)</td>
<td>29.09 (4.1)</td>
<td>70.90 (4.97)</td>
<td>20.24 (1.22)</td>
<td>49.85 (8.34)</td>
</tr>
<tr>
<td>Cropping</td>
<td>9.88 (2.30)</td>
<td>28.74 (8.6)</td>
<td>71.26 (1.82)</td>
<td>134.34 (4.06)</td>
<td>334.47 (6.63)</td>
</tr>
</tbody>
</table>

The proportion of nitrification-derived \( \text{N}_2\text{O} \) (\( \text{P}_{\text{N2O}} \)) over 7 days varied with soils (Table 2). The cropping soil had the highest \( \text{P}_{\text{N2O}} \) value (0.26%) which was significantly \( (p < 0.05) \)
higher than that in other soils. The gross nitrification rate of the other soils followed the order vegetable > dairy pasture > sugarcane, whilst $P_{N2O}$ followed the opposite order being dairy pasture > sugarcane > vegetable.

3.5 Dynamics of ammonia oxidizers

The abundance of AOB population was always lower than that of AOA in all soils (Figure 3). The highest AOA $amoA$ copy number ($1.5 \times 10^7$ g$^{-1}$ dry soil) was found in the vegetable soil at day 0, while the highest AOB $amoA$ copy number ($9.1 \times 10^5$ g$^{-1}$ dry soil) was observed in the cropping soil. Following application of the treatments, both AOA and AOB $amoA$ numbers significantly increased ($p < 0.05$). The cropping soil had the largest AOB population ($2.9 \times 10^7$ g$^{-1}$ dry soil) throughout the incubation period, whilst there were much higher AOA $amoA$ gene copy numbers in the sugarcane soil ($2.5 \times 10^8$ g$^{-1}$ dry soil) than in the vegetable soil ($8.6 \times 10^7$ g$^{-1}$ dry soil), the dairy pasture soil ($1.7 \times 10^8$ g$^{-1}$ dry soil) and the cropping soil ($1.0 \times 10^8$ g$^{-1}$ dry soil) (Figure 3). In the sugarcane soil the ratio of AOA to AOB was the highest and averaged at 61.4, followed by dairy pasture soil at 24.5, vegetable soil at 23.2 and cropping soil at 5.4. The contribution of nitrification to $N_2O$ (Cn) in the cropping soil was significantly related to AOB population ($p < 0.01$) while AOA was mainly responsible for nitrification in the sugarcane soil ($p < 0.05$).
Figure 3 Changes in AOA and AOB amoA gene copy numbers in the ^15^NH_4 treatment during the incubation period. Error bars indicated the standard deviation.

4. Discussion

This study investigated N_2O sources and changes in ammonia oxidizers in four agricultural soils collected from different land uses. The results suggested that land-use may significantly affect the relative contribution of nitrification and denitrification to N_2O emissions and AOA and AOB populations. Different land-use resulted in a range of N_2O emissions. Verchot et al. (1999) demonstrated that there were lower N_2O fluxes from pasture compared
with forests. Studies from the fertilised cropping systems in the humid and subhumid tropics have indicated that N₂O fluxes can be as much as 10 times that of the natural systems depending on the rates and timing of application of fertilizers (Davidson et al., 1996; Veldkamp and Keller, 1997). This was likely attributed to the different soil properties determined by different land-use. In fact, many previous studies have showed that land-use and management practices could significantly affect soil physical, chemical and biological parameters (Lauber et al., 2008; Bissett et al., 2011; Osborne et al., 2011). Firestone and Davidson (1989) indicated that the relative significance of nitrification and denitrification to N₂O was variable mainly due to varying C and N availability. In this study, the different soils were largely characterized by the differences in soil N, C and pH (Table 1). Soil pH can directly affect denitrification enzymes (Simek and Cooper, 2002). This may be a possible explanation for the higher contribution of denitrification to N₂O (76%) in vegetable soil (pH 7.8) compared with the other three acidic soils. Xu and Cai (2007) found there was a negative relationship between denitrification capacity and soil pH in humid subtropical soils which was consistent with our results. However, the contribution of nitrification to N₂O decreased with decreasing soil pH in the acidic soils (Table 2). This is probably because soil pH has an overriding effect on autotrophic nitrification and low soil pH can impede autotrophic nitrifier activities (Weber and Gainey, 1962). Some studies, in subtropical China, have revealed that acidic soils reduced nitrification capacity (Zhao et al., 2007). Weier et al. (1993) demonstrated that total N loss due to denitrification decreased as soil texture became finer and there was sufficient C content. Therefore, in sandy soils (sugarcane and vegetable), the contribution of denitrification to N₂O emission was lower in the sugarcane soil than in the vegetable soil because the C content of the sugarcane soil was higher than that of vegetable soil. N₂O emissions were greatest in the cropping soil because high NO₃⁻ concentration can inhibit the reduction of N₂O (Weier et al., 1993). The results of this study showed the Pₙ₂₀ values were lower than the observations of Zhang et al. (2011). In
this study, the highest ratio (0.26‰) occurred in the cropping soil with the highest organic C content indicating that soil organic C content may have affected N$_2$O production ratios from nitrification. Morkved et al. (2007) found that the ratio of N$_2$O production from nitrification in soils with low pH and high organic C content was higher than the soils with high pH and low soil organic C content.

The different soils had different amoA genes copy numbers (Figure 3). Copy numbers of the AOA and AOB amoA genes were found to be higher in the sugarcane and cropping soils respectively, than in the other soils, suggesting that amoA genes abundances were strongly influenced by land-use. Previous studies found similar results when comparing amoA genes between different agricultural land-use soils (Hayden et al., 2010; Bissett et al., 2014). The size of AOA and AOB populations significantly increased after the addition of the treatments possibly because of increasing NO$_3^-$ concentration.

The important role of AOA in nitrification and their potential for N$_2$O production has been highlighted previously in different ecosystems (Konneke et al., 2005; Francis et al., 2005). Here, it was observed that nitrification-sourced N$_2$O emissions (Cn) in the cropping soil was significantly related to AOB population ($p < 0.01$) while AOA was mainly responsible for nitrification in the sugarcane soil ($p < 0.05$). The sugarcane soil had the lowest substrate concentration, and the highest substrate concentration level was found in the cropping soil. It has been suggested that AOA may be favoured by low fertility environments (Mahmood and Prosser 2006; Schauss et al., 2009; Di et al., 2009), while the AOB community can adapt to the high nutrient availability conditions (Di et al., 2009). Therefore, in the cropping soil with the high substrate, AOA was only playing a limited role in N$_2$O production, and AOB played the predominant role in N$_2$O emission. Di et al. (2010) also found that AOB population had a significant relationship with N$_2$O production in the N-rich grassland soil. We measured amoA gene abundance in different soils using soil DNA, giving insights into genetic potential,
however, measurements of active community based on soil RNA are definitively needed in future study.

5. Conclusions

Land-use and soil properties may be important factors to influencing the contribution of different pathways to N₂O emissions, and the size of the AOA and AOB communities. AOB were found to be the major contributor to N₂O emissions in the cropping soil, while AOA were the predominately responsible for nitrification-sourced N₂O in sugarcane soil. In the vegetable and dairy pasture soils, both AOA and AOB contributed to nitrification and N₂O emissions.

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Chapter 8

General Discussion and Conclusions

8.1 Major findings

The production and consumption of soil N\textsubscript{2}O involves a series of co-occurring processes primarily modulated by microorganisms. A large body of work has been devoted to partitioning the sources of N\textsubscript{2}O by using nitrification inhibitors and \textsuperscript{15}N isotope techniques (Toyoda et al., 2005; Zhu et al., 2013). However, allocating the relative contribution of the different N\textsubscript{2}O sources still remained a great challenge because these techniques alone cannot provide information about the underlying microbial mechanisms (Hu et al., 2015). In this research, a multidisciplinary approach was used, combining use of a nitrification inhibitor, \textsuperscript{15}N isotope techniques and molecular approaches, to differentiate biological N\textsubscript{2}O pathways in laboratory incubation experiments. The significance of nitrification-sourced N\textsubscript{2}O, the relative significance of heterotrophic and autotrophic nitrification, the contribution of AOA and AOB to nitrification and N\textsubscript{2}O formation, and the importance of soil properties to control N\textsubscript{2}O emissions were comprehensively analysed for a range of Australian agricultural soils. The major results are as follows:

1. pH influenced the relative contribution of AOA and AOB to nitrification, and N\textsubscript{2}O emissions. Nitrification activity in an acid soil was correlated with AOA abundance, while in alkaline and neutral soils, both AOA and AOB contributed to nitrification, albeit to varying degrees (Chapter 4).

2. pH, soil organic C content, soil temperature and moisture significantly influenced the relative contributions of heterotrophic and autotrophic nitrification to total nitrification (Chapter 5). Heterotrophic nitrification was favoured by high organic C availability and low soil pH. Unexpectedly we found that heterotrophic nitrification was more dominant at lower soil temperature (15\textdegree C) and in drier (50% WFPS) soil.

3. Nitrification contributed to N\textsubscript{2}O emission under high water content, denitrification
contributed under low water content, and the relative contributions differed between soils.

Elevated soil temperature (35°C) and moisture (85% WFPS) increased the proportion of nitrified N that was emitted as N₂O (\( \text{P}_{\text{N}_2\text{O}} \) value) even though it reduced the contribution of nitrification to N₂O production (Chapter 6). The contribution of nitrification to N₂O production under drier conditions varied with soil type. At 25°C and 50% WFPS, 76% of total N₂O was produced by denitrification in an alkaline vegetable soil (pH 7.8), while nitrification was the main contributor of N₂O emissions in acidic sugarcane, dairy pasture and cropping soils (pH < 6) (Chapter 7). Compared to the cropping, vegetable and dairy pasture soils, more nitrification sourced N₂O was emitted from the sugarcane soil (\( \text{P}_{\text{N}_2\text{O}} 0.03\% \)) (Chapter 7 Table 2).

4. Soil moisture and temperature, and land-use influenced the role of AOA and AOB in nitrification.

The abundance of AOA and AOB and the changes in abundance influenced their functional capacity (nitrification activity). Elevated soil moisture (85% WFPS) reduced both AOA and AOB populations, while soil temperature only had a modest effect on the abundance of AOA and AOB (Chapter 6). Nitrification and nitrification-sourced N₂O was closely related to the AOB population regardless of soil temperature and moisture in the cropping soil. In the sugarcane system, N₂O-from nitrification was strongly related (\( p < 0.05 \)) to AOA abundance, while AOB was the driver for nitrification and nitrification-derived N₂O in the cropping land (Chapter 7).

8.2 General discussion

The use of nitrification inhibitors together with nitrogen fertilizers have shown great potential in improving fertilizer efficiency and to reduce N₂O emissions and nitrate leaching in agroecosystems (Di et al. 2011; Liu et al., 2013). Appropriate use of nitrification inhibitors could also serve as a powerful tool to differentiate biological N₂O pathways in laboratory experiments. However, previous studies have presented inconsistent results on the efficacy of nitrification inhibitors (Chen et al., 2008a; Islam et al., 2007; Weiske et al., 2001). This may be because of the variable importance of heterotrophic nitrification, which is unaffected by nitrification inhibitors, in different soil environments. Therefore, if we can determine where heterotrophic nitrification is the dominant process, we can predict that the efficacy of nitrification inhibitors would be low. In order to develop a greater understanding of N₂O
emissions from soils, to provide appropriate targeted management strategies, it is necessary for us to be able to identify the pathways occurring in different soils. Previous studies have stated that heterotrophic nitrification is the dominant nitrification pathway in acid forest soils (Well et al., 2008; Zhang et al., 2011), and have focused on the contributions of heterotrophic nitrification to N\textsubscript{2}O emissions and nitrification for forestry production systems (Kuroiwa et al., 2011; Zhang et al., 2014). However, limited information is available on heterotrophic nitrification in agricultural systems. Therefore, Chapter 5.1 compared two contrasting dairy soils and one cropping soil and investigated the effects of soil variables and environmental conditions on the contribution of heterotrophic nitrification to nitrification and N\textsubscript{2}O emission.

According to this study (Chapter 5), heterotrophic nitrification is favoured by high organic C content and low soil pH. This may be because soils with high organic C content often also contain labile C pools which can be used by heterotrophs (Kramer et al., 2002). In Chapter 5.2, the effects of environmental conditions were studied and showed that heterotrophic microorganisms may carry out nitrification in environments which are unfavourable (low temperature and low moisture) for autotrophic nitrifying bacteria (Cai et al. 2010). This information suggests that a nitrification inhibitor will not be effective under these conditions.

Another possible explanation for the variable efficacy observed for nitrification inhibitors may be closely associated with the relative roles of AOA and AOB in nitrification. To fully understand N\textsubscript{2}O emissions from soils to develop management strategies, it is necessary for us to be able to identify which of the microbes is dominant in different soils. AOA and AOB are divergent in many biochemical and genetic features, such as membrane structures, cell size, AMO gene structures and pathways of ammonia oxidation (He et al., 2012). These differences are thought to affect their relative sensitivity to inhibitors, and so the relative importance of AOA and AOB to nitrification can be identified through their different responses to inhibitors. I found (Chapter 4) that AOA were more strongly inhibited by inhibitors in acid soil, which was consistent with previous studies (Gubry-Rangin et al., 2010; Offre et al., 2009), suggesting AOA were responsible for nitrification in acid soil. This is consistent with the hypothesis that AOA may dominate ammonia oxidation in strongly acidic soils due to their high affinity for NH\textsubscript{3} substrate (He et al., 2012). However, in neutral and alkaline soils, the inhibitors are non-selective, and they can impede growth of AOA and/or AOB depending on which group is more functionally dominant in nitrification (Liu et al., 2015). Based on this knowledge, I propose that further study is undertaken using exclusive nitrification inhibitors,

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which can completely inhibit either AOA or AOB, but have little effect on the other, and to develop tools to be used to mitigate N$_2$O effectively.

Due to spatial-temporal variability experienced with field N$_2$O measurements, process based agro-ecosystem models are useful for predicting N$_2$O emissions under different situations. In order to better predict and mitigate N$_2$O emissions, it is essential to identify the key environmental factors which govern the dominant microbial N$_2$O sources. However, to date, the quantification of these complex N$_2$O production pathways and identification of the role of microbial communities involved in N$_2$O emission has been difficult, particularly due to the lack of data for modeling N$_2$O emission from nitrification (a fixed ratio is used). The work in my thesis (Chapters 6 and 7) has revealed that N$_2$O-from nitrification was more likely produced under 25°C and 50% WFPS in an acid cropping soil compared to 35°C 50%, 70%, 85% WFPS and 25°C 70%, 85% WFPS. However, under the same conditions denitrification was the main contributor of N$_2$O production in an acid sugarcane soil. This may be because of the differing importance of AOA and AOB to N$_2$O-from nitrification. Di et al. (2010) showed that N$_2$O production was significantly correlated with AOB population. However, the results of the study in my thesis (Chapters 6 and 7) indicated that AOA was the key player in nitrification and N$_2$O production in sugarcane soil, while AOB was responsible for nitrification and nitrification-derived N$_2$O in the cropping soil. This difference may be due to the different soil C and N concentrations in the different land-use soils (Mahmood and Prosser 2006; Schauss et al., 2009). In the sugarcane system, the high C can facilitate AOA growth, while in the cropping soil with the mineral fertilizers addition AOB tends to be the key player (Chu et al., 2008; Fan et al., 2011). This information can assist to improve the accuracy of simulation of N$_2$O emissions from agricultural systems. My thesis has provided knowledge on the proportion of N$_2$O leakage during nitrification, the relative contribution of nitrification and denitrification to N$_2$O emission, and how soil and environmental variables influence (quantitatively) the proportion (amount of N$_2$O) and partitioning between pathways (N$_2$O source) in a range of Australian agricultural soils. This knowledge will inform advancement of current process based simulation models and improve our understanding of nitrification and N$_2$O emissions in agricultural systems.

8.3 Implications for future study

To enable identification of soil variables modulating the relative occurrence of all N$_2$O
production pathways (e.g. nitrification, denitrification, nitrifier denitrification, DNRA), long-term studies are needed. The research reported in my thesis was conducted under controlled laboratory conditions and the results generated may differ somewhat to those observed under field conditions (as reflected in the spatial-temporal variability experienced with N$_2$O field emissions). Therefore, $^{15}$N field experiment and measurement of N$_2$O production and microbial communities in situ are needed to confirm our laboratory outcomes.

From the results obtained in this study, I found a relationship between amoA gene abundance and nitrification-sourced N$_2$O in soils. However, it should be noted that there are other critical nitrogen-cycling biomarkers (including napA, nirK, nirS, narG, nosZ and nifH) involved in N$_2$O production and consumption pathways. Studies on the napA, nirK, nirS, narG, nosZ and nifH genes are very limited, and in order to better understand the underlying mechanisms leading to soil N$_2$O production more work is needed to assess the structure and abundance of these functional genes, and to characterize the environmental factors which influence their activity. Their abundance, structure, expression and metabolic activity could serve as potential indicators for denitrification-derived N$_2$O fluxes in soils (Morales et al., 2010). For example, it is hypothesised that there is a high capacity for N$_2$O production from denitrification and thus high levels of N$_2$O emissions, or vice versa, if soils have high ratios of ($nirK + nirS)/nosZ$ gene copies. This will assist in the development of more accurate methods for predicting N$_2$O emission. If we can assess the structure and abundance of all the genes involved in N$_2$O production and consumption, and the environmental conditions and soil characteristics determining their abundance and activity it will assist in the development of more accurate methods for predicting N$_2$O emission.

Other nitrogen transformation processes influence the emission of N$_2$O from soil, such as nitrate leaching, ammonification, ammonia volatilization, plant nitrogen uptake (Chen et al., 2008b) and oxidation of volatilized NH$_3$ to produce N$_2$O on plant leaves (Bowatte et al., 2014). Therefore, the spatial and temporal variability of N$_2$O emissions is high because N$_2$O production and consumption processes within the soil matrix are shaped by numerous factors (Chapuis-Lardy et al., 2007). The variability of field measurements of N$_2$O emissions is very high (Butterbach-Bahl et al., 2001; Nemeth et al., 2014; Turner et al., 2008), and transient N$_2$O fluxes can be several orders of magnitude higher or lower than under steady state conditions (Elberling et al., 2010; Schreiber et al., 2012). Therefore, more research over a wider variety
of soil types is needed to develop a more confident simulation of future N$_2$O emissions from terrestrial ecosystems.

8.4 Conclusion

In conclusion, the combined methodology of isotope probing, nitrification inhibitors and molecular techniques used in my research is a powerful tool that can be used to resolve the relative importance of nitrification and denitrification to N$_2$O productions, and to identify the relation between N$_2$O and AOA and AOB populations. The results from laboratory incubation experiments revealed the effects of soil variables and environmental condition on N$_2$O production pathways (nitrification and denitrification) and nitrification-related processes (heterotrophic and autotrophic nitrification) and nitrifiers (AOA and AOB). These results are valuable to develop a more confident simulation of future N$_2$O emissions from terrestrial ecosystems and for the development of more targeted mitigation strategies for use in agricultural practices.

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Appendix

Plate 1 Hamilton#1 cropping site (03.2012)

Plate 2 Boneo vegetable site (2013)
Plate 3 Longwoory dairy pasture site (2013)

Plate 4 The incubation experiment conduction----- a series of 500 ml capped vials were prepared with air dried soil (equal to 60 g of 105°C dried soil)
Plate 5 Gas collection vial

Plate 6 1 L microdiffusion jars for measuring $^{15}$N enrichment in NO$_3^-$ and NH$_4^+$ pools
Plate 7 The modified hook for an acidified filter paper disc

Plate 8 The Power Soil DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA)