The influence of temperature on emissions of nitrous oxide and dinitrogen from soils

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Acronyms and abbreviations

µg	Microgram
^{15}N (%)	Labelling nitrogen (percentage of excess ^{15}N atom)
ANOVA	Analysis of variance
AOA	Ammonia-oxidising archaea
AOB	Ammonium oxidising bacteria
BD	Bulk density
C_2H_2	Acetylene
CT	Constant temperature
DEA	Denitrification enzyme activity
DNRA	Dissimilatory nitrate reduction to ammonium
E_a	Activation energy
FC	Field capacity
FTP	Fluctuating temperature pattern
HSD	Honestly significant difference
mg	Milligram
MPN	Most probable number
N	Nitrogen
N_2	Dinitrogen
N_2O	Nitrous oxide
N_2OR	Nitrous oxide reduction
na	Not applicable
NH_2OH	Hydroxylamine
NH_4^+	Ammonium
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
ns	Not significant
O_2	Oxygen
OC	Organic carbon
P_n	The proportion of N_2O to NO_3^- by nitrification
SE	Standard error
TEA	Terminal electron acceptor
T_{max}	Maximum monthly temperature
T_{min}	Minimum monthly temperature
T_{opt}	The optimum temperature
v/v	Volume per volume
WFPS	Water-filled pore space

Abstract

Nitrification and denitrification are two major soil biological processes that release nitrous oxide (N_2O) from soils. N_2O production and reduction have been well-documented at temperatures below 35 ºC, but are poorly understood at higher temperatures. N_2O production from nitrification was compared at a range of temperatures (10 ºC to 45 ºC) to mimic the typical temperatures encountered in soils from dairy pasture systems in Australia. Temperature was more important than soil type in controlling N_2O from nitrification, which was slow at 10 – 25 ºC and peaked at 35 – 40 ºC, suggesting a higher optimum temperature for N_2O production from nitrification than previous studies reported. Autotrophic nitrification produced N_2O predominantly below 35 ºC, while heterotrophic nitrification, which used NH_4^+ for nitrifying, released N_2O principally between 35 ºC and 40 ºC. Total N_2O emissions measured at different temperatures were influenced by the climatic region from which the soils were sourced. The magnitudes of N_2O emissions in the tropical soil exceeded those in the temperate soil under experimental conditions, although N_2O/NO_3^- from nitrification at different temperatures was independent of the climatic region from which soils were sourced. The N_2O/NO_3^- ratio was positively correlated with increased temperature and was above 1.0% at 35 ºC, regardless of climate.

Temperature interacted with soil moisture and NO_3^- availability to regulate N_2O from denitrification, while the conversion of N_2O to N_2 was affected principally by temperature. The highest denitrification ($N_2O + N_2$) was found at 35 ºC in the soils treated at 75% FC and N contents between 100 – 150 kg N ha^{-1}. Low N_2O/N_2 ratios at 40 – 45 ºC was due to the enhancement of N_2 production at these temperatures, suggesting greater soil NO_3^- loss as N_2 during summer, particularly in soils that are wet at that time.

Interestingly, high NH_4^+ availability was observed at 45 ºC, which was hypothesised to relate to low nitrification rate and high rates of N mineralisation or dissimilatory nitrate reduction to ammonium at this temperature.

This work has improved the knowledge of N cycling processes at high temperatures. Soil moisture or NO_3^- content alone are poor predictors of N_2O and N_2 production, since these elements interacted with temperature to control denitrification. High soil NH_4^+ availability at 45 ºC is a particularly interesting finding with potential to contribute to N losses. The findings confirm that management of soil moisture and NO_3^- availability, and a consideration of crop N demand are likely to reduce N losses as N_2O and N_2.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Last but not least, my family. Special thanks to my wife and son, who have been with me during the journey.

Chapter 1 **Introduction**

Nitrous oxide (N_2O) is a powerful greenhouse gas that contributes to global warming and is one of the most important ozone-depleting substances (Crutzen, 1981; Pierzynski et al., 2005; Ravishankara et al., 2009). Atmospheric N_2O concentrations have increased since pre-industrial times and the agriculture sector currently accounts for 60% of anthropogenic N_2O emissions (Butterbach-Bahl et al., 2013). The global N_2O production from soil increased by 17% from 1990 to 2005, due mainly to excess applications of N fertilisers to agricultural soils (IPCC, 2006). The applied nitrogen to soil can be lost into the atmosphere as NO, N_2O or N_2, which reduces the efficiency of N utilisation and has adverse ecological impacts (Mosier et al., 2004). Thus, the sustainability of agricultural systems should involve the measurement of N losses as nitrogenous gases, particularly N_2O emissions.

Nitrous oxide can be released from both chemical and biological processes in soil. The oxidation of hydroxylamine (Bremner, 1997; Heil et al., 2015) and chemo-denitrification (Van Cleemput, 1998) can release N_2O emissions but the amounts of N_2O production are considered to be much lower than that from biologically meditated pathways. The biological processes, which produce approximately 70% of global N_2O production (Butterbach-Bahl et al., 2013), include nitrification, denitrification and nitrifier-denitrification (Figure 1.1). The rates of these biological processes vary with climates, soil properties and agricultural practices, which result in high temporal and spatial variability of N_2O production (Hénault et al., 2012).

Temperature is a fundamental factor regulating biological N_2O emission from soil because it has both direct and indirect effects on the biological processes responsible for N_2O production. Temperature may influence the growth of microorganisms and kinetic reactions for nitrification and denitrification. The indirect effects of temperature on N_2O

production are mediated via changes in oxygen (O_2), organic carbon (OC), substrate availability (NH_4^+ and NO_3^-) and soil pH. Global warming was estimated to increase by 0.8 ºC in the 20th century and is predicted to continuously increase at an average rate of + 0.2 °C per decade in the 21th century (IPCC, 2001; Hansen et al., 2006). The thermal responses of microorganisms mediating the N cycle may influence soil N losses as N_2O or N_2 from nitrification and denitrification and need to be better understood (Smith, 1997; Castaldi, 2000). Thus, it is vital to investigate the impact of the temperature on biological N_2O and N_2 production from agricultural soils. Temperature may interact with factors such as soil moisture and N availability to control N_2O production and N_2O reduction (corresponding to N_2 production) under field conditions. There are contradictory findings about the relationship between the N_2O/N_2 in relation to temperature, and therefore this needs further investigation, particularly at temperatures that have rarely been explored.

Australian soils typically experience high diurnal temperature fluctuations and seasonal fluctuations vary from – 5 °C to 45 °C (Meteorology, 2015). It has been well-documented that N_2O emissions increase exponentially with rising soil temperature below 30 °C (Goodroad and Keeney, 1984; Maag and Vinther, 1996; Smith, 1997; Avrahami et al., 2003). Only a few studies have examined temperature responses above 30 °C in terms of quantification of N losses as N_2O and N_2 to the atmosphere. Thus, the production of N_2O and N_2 from soils at higher temperatures are poorly understood. In addition, most studies investigating the impact of temperature on biologically produced N_2O emissions have used a daily constant temperature and often neglected the diurnal fluctuations in temperature, which occur naturally under field conditions. Thus, it is important to assess the impact of diurnal temperature regimes on N_2O production.

The different magnitudes of N_2O production among geographic regions and ecosystems have been suggested to relate to the thermal acclimation and/or adaptation of soil

microorganisms. The available data on N_2O production are aggregated from separate experiments conducted under different experimental conditions (Goodroad and Keeney, 1984; Maag and Vinther, 1996; Gödde and Conrad, 1999; Holtan-Hartwig et al., 2002). Therefore, incorporation of thermal responses of N_2O production from further climatic regions, particularly from contrasting regions, will assist in model development. Some models such as DNDC (Li et al., 1992; Li, 2000) and NASA CASA (Potter et al., 1996) have been developed to predict N_2O emissions from different agricultural systems. However, these models have limitations in their applicability across climatic regimes and ecosystems (Chen et al., 2008) due to the high variability of climate and soil conditions, which alter the temperature optima, as described by Stark (1996).

A better understanding of the relationship between temperature and N losses as N_2O and N_2 would contribute to improve the knowledge of N transformations in soil and provide direction for the development of effective management strategies to mitigate N_2O emissions from agricultural soils.

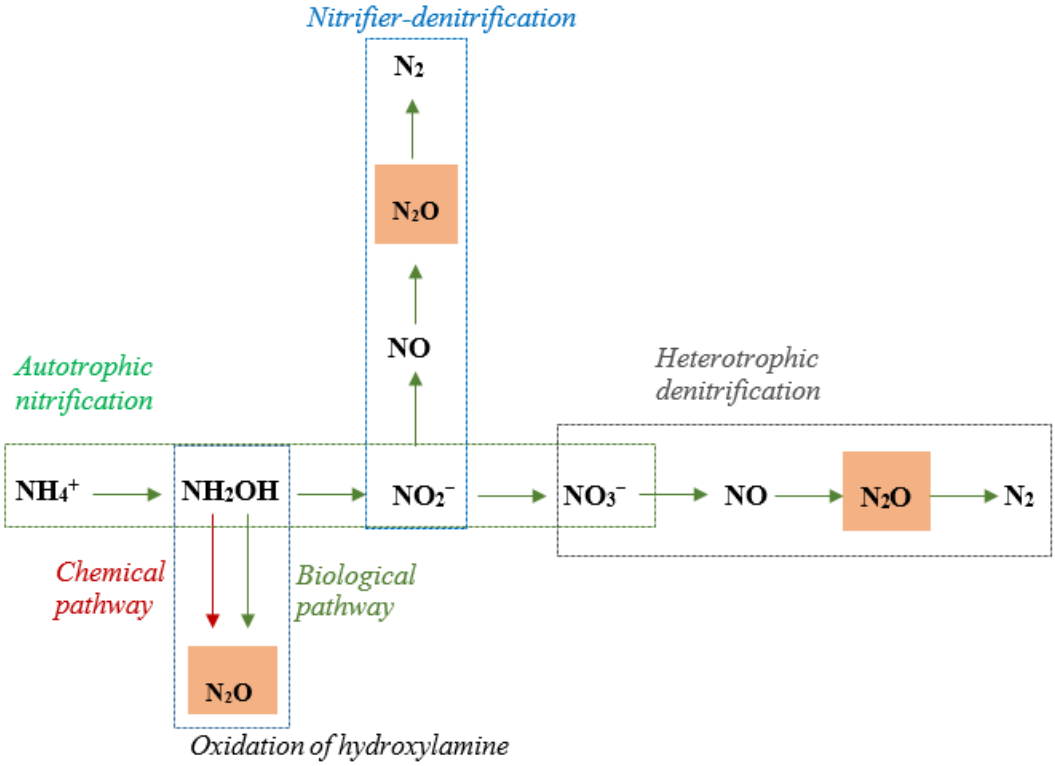

Figure 1.1. The pathways of N_2O from nitrification, denitrification and chemical reactions in soil.

1.1 Aims

The overall aim of the work described in this thesis was to investigate the effects of high temperatures, soil moisture and nitrogen availability on the rates of nitrification, denitrification and N_2O emissions from soils. The specific questions developed as experiments to address this overall question are identified in Figure 1.2.

1.2 Thesis structure

This thesis structure is indicated in Figure 1.2. **Chapter 1** provides the general introduction, research objectives and organisation of the chapters. **Chapter 2** presents a review of the literature and methodologies applied in the experimental work. The influence of daily fluctuation temperature and substrate availability on nitrification and N_2O production are examined in **Chapter 3**. **Chapter 4** and **5** cover two major research components that include: N_2O production from nitrification in response to high temperature (**Chapter 4**) and the temperature responses of nitrification and N_2O emissions in soils from contrasting climatic regions (**Chapter 5**). **Chapter 6** presents the effect of temperature and soil moisture on denitrification. In **Chapter 7**, the effects of temperature and nitrate availability on N_2O production and N_2O reduction during denitrification are examined. **Chapter 8**, the general discussion, synthesizes the results of the experimental work, presents a conceptual model and discusses potential mechanisms in context with the literature and highlights some potential areas for future work.

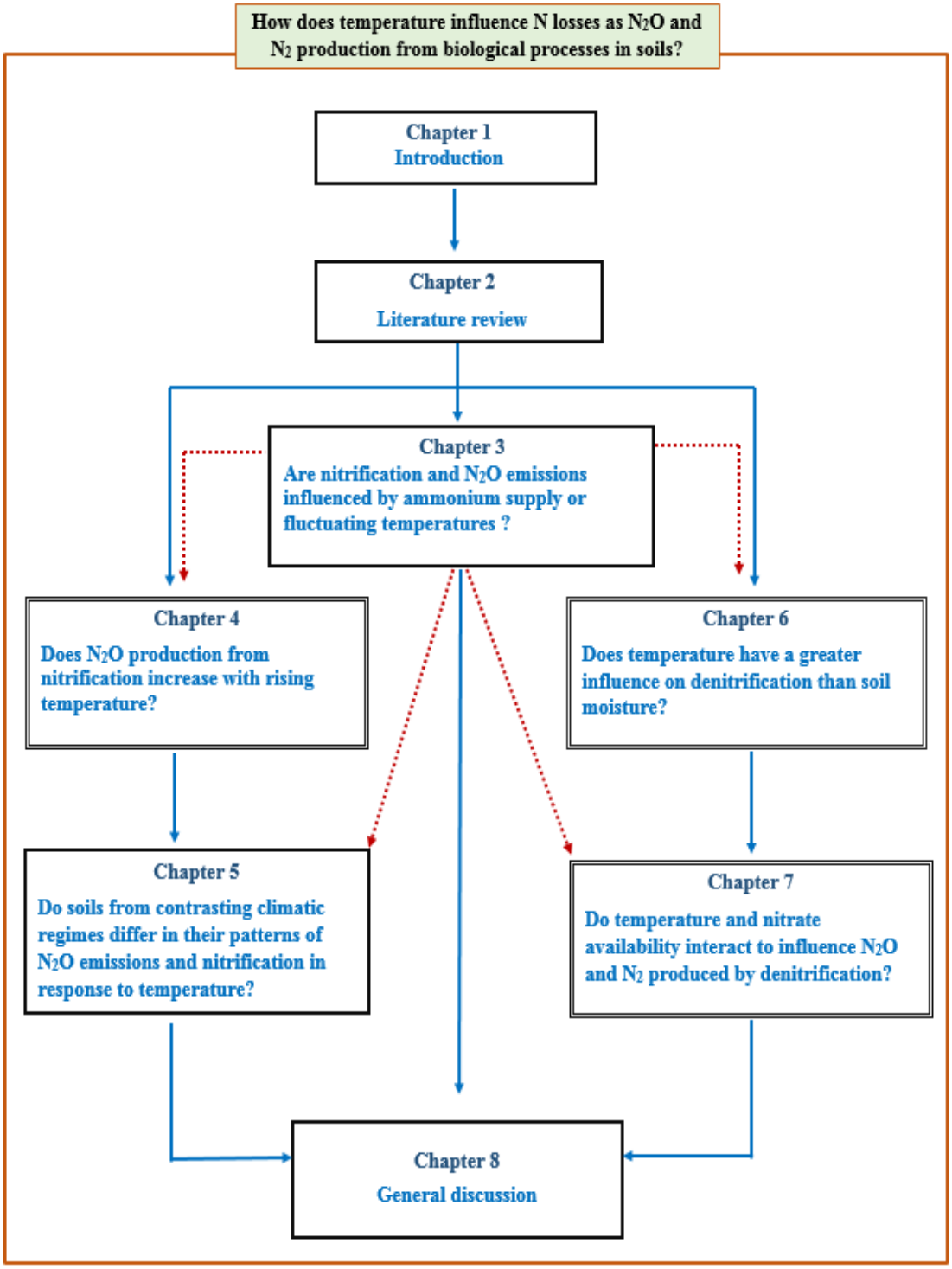

Figure 1.2. Thesis structure indicating the scientific relationships among chapters, indicated as solid blue lines, and some methodological relationships among chapters, indicated as broken red lines. Data from chapters bounding with double line were based on isotopic techniques (^{15}N).

Chapter 2 **Literature review**

2.1 Introduction

This chapter provides a background on the nitrogen (N) cycle and N_2O production in soil as influenced by environmental factors. In particular, the possible effects of temperature on N_2O production from different processes and the advantages and disadvantages of different techniques used to distinguish N_2O emissions from biological processes will be discussed.

Agricultural soils are the major source of N_2O emissions, contributing to 50 – 60% of global anthropogenic production (Kroeze et al., 1999; EPA, 2010). This source increased from 10 to 12 Tg N_2O-N yr^{-1} between 1900 and 2000 and it is expected to increase up 16 Tg N_2O-N yr^{-1} in 2050 (Bouwman et al., 2013) due mainly to excess application of N fertiliser. The magnitudes of N_2O production often vary with climatic regions (Hénault et al., 2012). Temperature is known as one of key factors that control biological N_2O production. Global warming has been recognised in many regions around the world (IPCC, 2001; Alexander et al., 2006; Holthaus, 2016), suggesting that high temperatures play a significant role in regulating soil microorganisms that mediate the N cycle, potentially influencing N losses as N_2O and N_2. Rising soil temperature would directly influence microbial communities such as nitrifiers and denitrifiers and has indirect effects on N_2O production by changing oxygen (O_2), N pools, OC availability and soil pH. The interactive effects among temperature, soil moisture and N availability likely control biological N_2O and N_2 production under field conditions. However, the interactive effect between temperature and soil moisture or temperature and soil nitrate availability is poorly documented. Therefore, these effects need further investigations to provide a

better understanding of thermal responses of N_2O and N_2 in conjunction with other soil factors.

2.2 The nitrogen cycle and processes related to N_2O

Dinitrogen gas is abundant in the atmosphere but it takes a lot of energy to break the triple bond to produce reactive forms of N that are readily used by plants. Essentially, N enters into soil via decomposition of organic matter (OM), N fixation by legumes and the application of N inorganic fertiliser (Figure 2.1). Symbiotic N fixation was annually estimated at 50 – 70 Tg N in agricultural systems, mainly from soybean (Herridge et al., 2008). Despite this, the major N sources in soil come from the application of N as inorganic fertiliser and OM (IFA, 2016). The increased use of N fertiliser in agricultural systems has caused environmental problems such as contaminated soil water via N leaching and N is lost from the soil via N_2O, N_2, or NO_x (Figure 2.1). For many agricultural systems, the addition of organic sources of N to soil has been considered as an alternative option for inorganic fertiliser application (Quilty and Cattle, 2011) since organic addition can also contribute to improved soil physical, chemical and biological properties (Benbi et al., 1998; Liu et al., 2010). Soil N added as fertiliser N and OM can be transformed into other forms of N by microbial processes (biotic transformation) and/or chemical reactions (abiotic transformations).

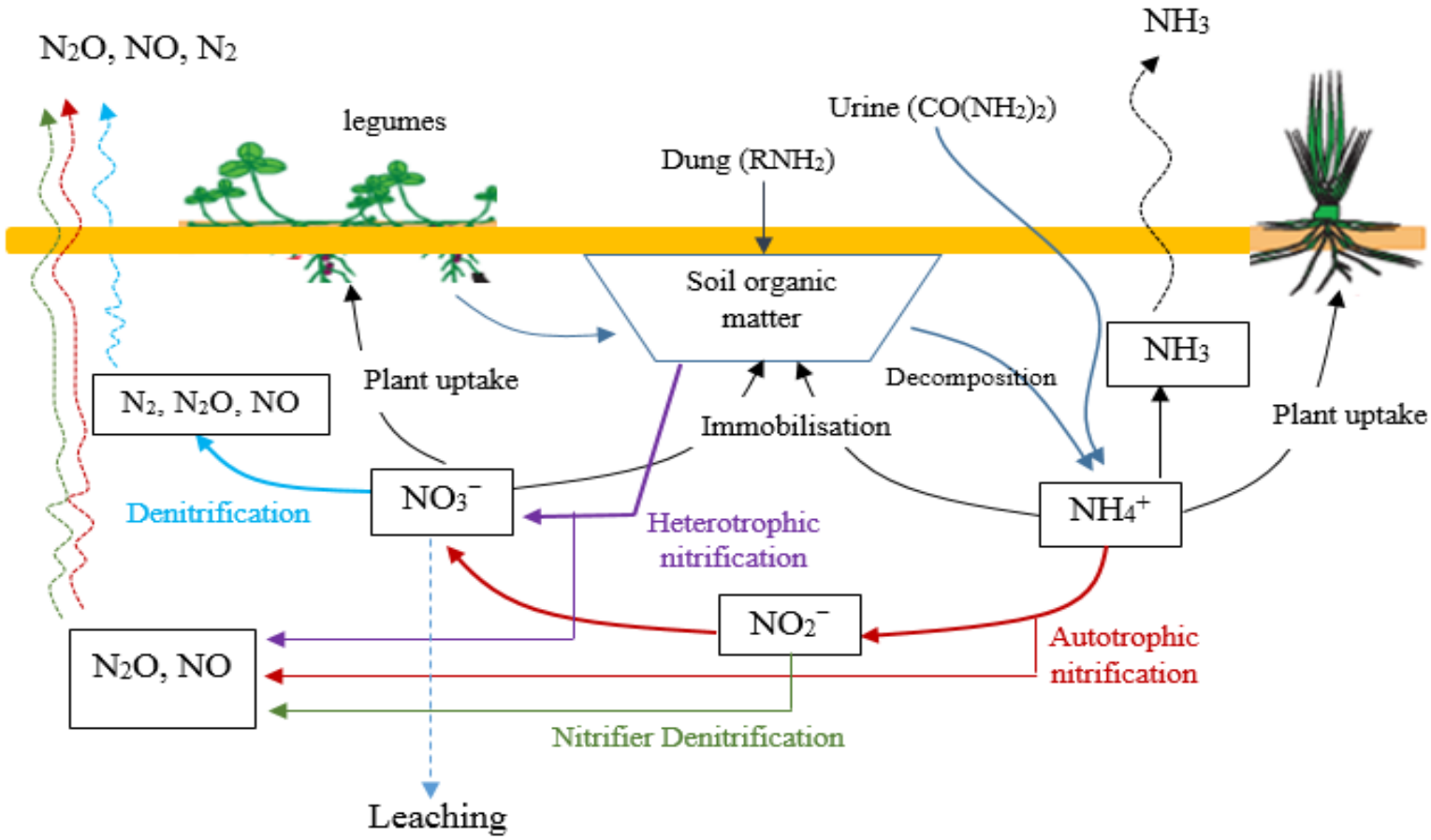

Figure 2.1. The nitrogen cycle in agricultural systems (adapted from Bolan et al. (2004))

2.2.1 Biotic transformations

2.2.1.1 Decomposition, mineralisation and immobilisation

Soil microorganisms can decompose OM to capture energy for their activity through respiration, which results in the release of the nutrients into the soil, such as the breakdown of proteins and amino acids to mineral N. The microbial process by which protein and amino acid are converted into available mineral N forms (NH_4^+ and NO_3^-) is known as N mineralisation (Figure 2.2). There are two distinct microbial reactions in N mineralisation, aminisation and ammonification.

In aminisation, the organic forms of N are first subjected to a microbial process in which heterotrophic microorganisms hydrolyse the macromolecules of organic N compounds such as proteins, into simple N compounds such as amines and amino acids (Dendooven et al., 1996) (Figure 2.2). Ammonification is a biological process in which microorganisms convert amines and amino acids into NH_4^+. For example, urea [$CO(NH_2)_2$] in animal urine and fertilizer releases NH_4^+ and hydroxyl (OH^-) ions via ammonification. This process is known as 'urea hydrolysis' and is carried out by the urease enzyme in the soil. Ammonium ions formed by ammonification or addition of ammonia based fertilisers undergo several processes such as uptake by plants, oxidation to NO_2^- and NO_3^- (nitrification), microbial utilisation (immobilisation), retention onto soil particles (NH_4^+ fixation) and loss to the environment as NH_3 volatilisation.

In the presence of readily available C containing OM, NH_4^+ and NO_3^- ions are assimilated into microbial biomass, which is defined as N immobilisation. High C/N ratios in plant residues added to soils often inhibit decomposition or lead to soil the microbes competing (immobilising) available N from the soil (Pierzynski et al., 2005). Immobilisation often exceeds N mineralisation when the C/N ratios are above 30. The two processes of

immobilisation and decomposition are about equal where the range C/N is around 20 to 30. Mineralisation typically exceeds immobilization when the C/N ratio of the residue is less than 20 (Bolan et al., 2004; Pierzynski et al., 2005).

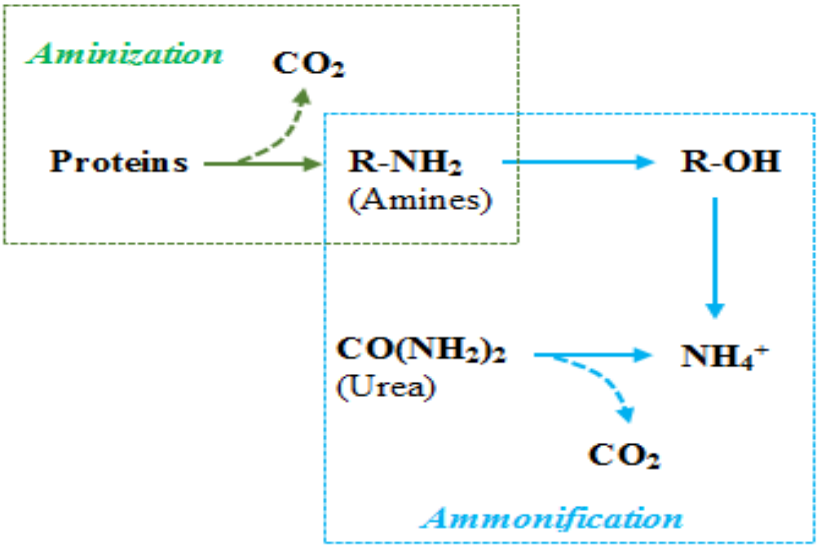

Figure 2.2. Mineralisation of nitrogen in soil including the components of aminisation and ammonification (adapted from Bolan et al. (2004)).

2.2.1.2 Nitrification

When microbial growth is limited by available C and energy, NH_4^+ is oxidised to NO_3^- to provide the energy for biosynthesis and maintenance (Schmidt, 1982). The microbial process of oxidation of NH_4^+ to NO_3^- is defined as nitrification. The majority of microorganisms involved in nitrification are autotrophic nitrifiers, which use NH_4^+ as a substrate. Autotrophic nitrification involves two steps, namely ammonium oxidation and nitrite oxidation (Bremner and Blackmer, 1981). In ammonium oxidation, the oxidation of NH_4^+ to NH_2OH is followed by the reduction of NH_2OH to NO_2^- (Figure 2.3) by ammonia oxidising bacteria (AOB) or ammonia oxidising archaea (AOA). Ammonia oxidising bacteria are highly important for the turnover of inorganic N in agriculture. Most AOB occur in the two genera *Nitrospira* and *Nitrosomonas* (Schmidt, 1982). In nitrite oxidation, NO_2^- is oxidised to NO_3^- by autotrophic nitrite oxidising bacteria, namely *Nitrobacter* (Schmidt, 1982). N_2O can be produced directly from NH_2OH during nitrification or from NO_2^- during nitrifier-denitrification (Wrage et al., 2001).

Heterotrophic nitrifiers, such as *Achromobacter and Corynebacterium* genera, can oxidise NH_4^+ or organic N forms to form NO_3^- and produce N_2O during nitrification (Schmidt, 1982; De Boer and Kowalchuk, 2001). Some studies (Schimel et al., 1984; Islam et al., 2007) have suggested that NH_4^+ is not as important as organic-N compounds for heterotrophic nitrification. It was hypothesised that OM may contain oxidised organic N, which is readily to turns to NO_3^- by heterotrophic nitrifiers (Schimel et al., 1984). However, a recent finding by Liu et al. (2015) found that the majority of NH_4^+ added to soil is nitrified by heterotrophic nitrifiers, resulting in the enhanced NO_3^- from nitrification.

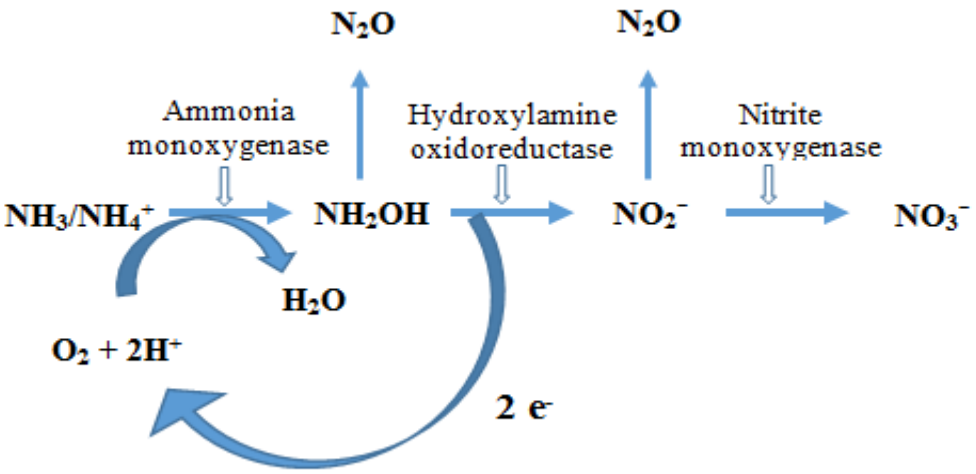

Figure 2.3. Nitrification: outline of the pathway and enzymes involved (adapted from Wrage et al. (2001)).

2.2.1.3 Denitrification

When O_2 becomes limiting in soil microsites, some microorganisms have the capacity to reduce nitrogen oxides and use them as electron acceptors for cell metabolism (Tiedje, 1988). The microbial process by which nitrogen oxides, principally NO_3^- and NO_2^-, are reduced to dinitrogen gases is referred as the respiratory denitrification (Tiedje, 1988). Denitrification may ultimately produce N_2 through a series of gaseous N oxide products such as NO and N_2O (Figure 2.4).

Denitrification is carried out by a diverse group of microorganisms, including bacteria, archaea, and fungi (Tiedje, 1988). The reduction of nitrogen oxides during denitrification is catalysed by a range of enzymes, namely nitrate reductase, nitrite reductase, nitric-oxide reductase and nitrous oxide reductase (Figure 2.4). There are more than 60 genera of bacteria, archaea, fungi and yeast involved in denitrification (Philippot, 2002; Watanabe et al., 2009) but most soil denitrifiers fall under four major genera: *Pseudomonas*, *Bacillus*, *Alcaligenes*, and *Flavobacterium* (Tiedje, 1988). Heterotrophic bacteria such as *Flavobacterium* are very active under low O_2 levels and use NO_3^- as a terminal electron acceptor (Lin et al., 2009) while others such as *Thiosphaera pantotraopha* can denitrify in both aerobic and anaerobic conditions (Wang et al., 2009).

2.2.1.4 Nitrifier-denitrification

Nitrifier-denitrification is another pathway of nitrification in which the oxidation of NH_3 to NO_2^- is followed by the reduction of NO_3^- to N_2O and N_2 by only one group of microorganisms, namely autotrophic NH_3-oxidisers (Wrage et al., 2001). These organisms are very responsive once O_2 becomes limiting under acidic conditions (Poth and Focht, 1985).

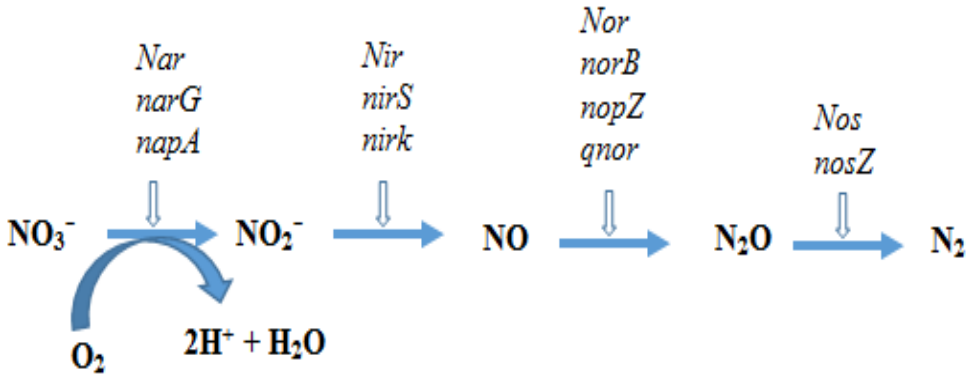

Figure 2.4. Denitrification: outline of the pathways and enzymes involved (adapted from Bolan et al. (2004)).

2.2.2 Abiotic transformations

2.2.2.1 Chemo-denitrification

Chemo-denitrification is a non-biological reaction which can occur under low soil pH and in the presence of Fe^{2+} (Van Cleemput, 1998). Nitrite (NO_2^-) produced during denitrification can rapidly react with Fe^{2+} in soil to release N_2O. The magnitudes of N_2O production from this pathway are closely related to the Fe^{2+} concentration in soil because it acts as a catalyst (Jones et al., 2013). The isotopic compositions of N_2O from chemo-denitrification differ to N_2O production emitted from denitrification (Jones et al., 2013).

2.2.2.2 Hydroxylamine oxidation

Hydroxylamine (NH_2OH), which is produced from the oxidation of NH_4^+ in nitrification, can chemically react with several transition metals in soil to form N_2O (Bremner, 1997). The amount of NH_2OH has been found to be positively correlated with AOB during nitrification, thus the oxidation of NH_2OH potentially plays an important role in N_2O production from soil, in the presence of transition metals and rising temperature (Heil et al., 2015).

2.3 Factors influencing N_2O production

Multiple factors are involved in the regulation of biological N_2O production from soil, which are presented in Figure 2.5. These will be described in more detail below.

2.3.1 Temperature

Soil temperature is one key factor controlling nitrification, denitrification and N_2O production in soil (Schmidt, 1982; Tiedje et al., 1983). Temperature has both direct and indirect effects on nitrification, denitrification and N_2O emissions. Temperature can influence the growth of microorganisms and the enzymatic reactions, thus regulating N_2O production from soil. For example, denitrification rate was significantly higher in *P.mandelii* cells grown at 30 °C than those at 10 °C or 20 ºC, due to the enhancement of cell numbers and enzyme rates of reaction at 30 ºC (Saleh-Lakha et al., 2009). Reducing temperature (< 10 ºC) may provide a challenge to the denitrifying bacteria, as total denitrification rate and associated N_2O production decrease (Holtan-Hartwig et al., 2002). Shifting temperature between 14 °C and 3 °C (12h – 12h) in a fluctuating temperature pattern (FTP) slowed the growth of nitrifiers, reducing the production of NO_3^- under FTP compared with that under a constant temperature (CT) of 10 ºC (Campbell and Biederbeck, 1972). The indirect effects of temperature on biological N_2O production have been demonstrated by reducing O_2, OC availability via microbial respiration rate (Smith, 1997; Öquist et al., 2004; Butterbach-Bahl and Dannenmann, 2011) or changing mineral N (NH_4^+ and NO_3^-) contents and soil pH via nitrification (Bolan and Hedley, 2003). These changes would have significant effects on biological N_2O production.

2.3.2 Oxygen and soil moisture

Oxygen (O_2) availability in soil microsites is influenced by temperature, via microbial respiration, soil water content and O_2 consumption by plant roots and soil microorganisms

(Figure 2.5). The availability of O_2 in the soil microsites determines the activity of nitrifiers and heterotrophic denitrifiers. As nitrification is an aerobic process, the majority of N_2O production in soil is produced by nitrification under aerobic conditions, which typically equates to less than 60% water-filled pore space (WFPS). An increase in anaerobic zones above 60% WFPS tends to increase the rates of denitrification (Davidson et al., 1991; de Klein and Van Logtestijn, 1996; Hefting et al., 2003) although it is acknowledged that the values of WFPS may differ according to soil type and may not accurately reflect the porosity volume (Farquharson and Baldock, 2008).

The depletion of O_2 in the soil microsites tends to influence the product ratios from nitrification and denitrification. The ratio of N_2O to NO_3^- production from nitrification increases with a decrease in O_2 concentration, particularly at partial pressures below 3 kPa O_2 (Khalil et al., 2004; Zhu et al., 2013). Decreased O_2 availability often results in increased N_2O/N_2 ratio during denitrification (Davidson, 1992; Weier et al., 1993; Rudaz et al., 1999). Under anaerobic conditions, NO_3^- is increasingly used as a terminal electron acceptor (TEA) relative to N_2O production (Cho et al., 1997a; Strong and Fillery, 2002), thus increasing N_2O/N_2 ratio. Inhibition of the reduction of N_2O to N_2 during denitrification contributes to high N_2O/N_2 ratio. Reduction of N_2O in soils has been attributed exclusively to denitrifiers possessing *nos* operons with *nosZ* encoding N_2OR, the enzyme system catalysing N_2O to N_2 reduction (Kolb and Horn, 2012; Zheng and Doskey, 2015). N_2OR is likely to be inhibited as O_2 content drops below 0.02 atm (~ 2 kPa), where N_2O production still occurrs (Morley et al., 2008; Zhu et al., 2013), resulting in high N_2O/N_2 ratio. Therefore, O_2 availability in soil microsites has a large impact on the activity of nitrification, denitrification and the partitioning of gaseous N losses from these processes.

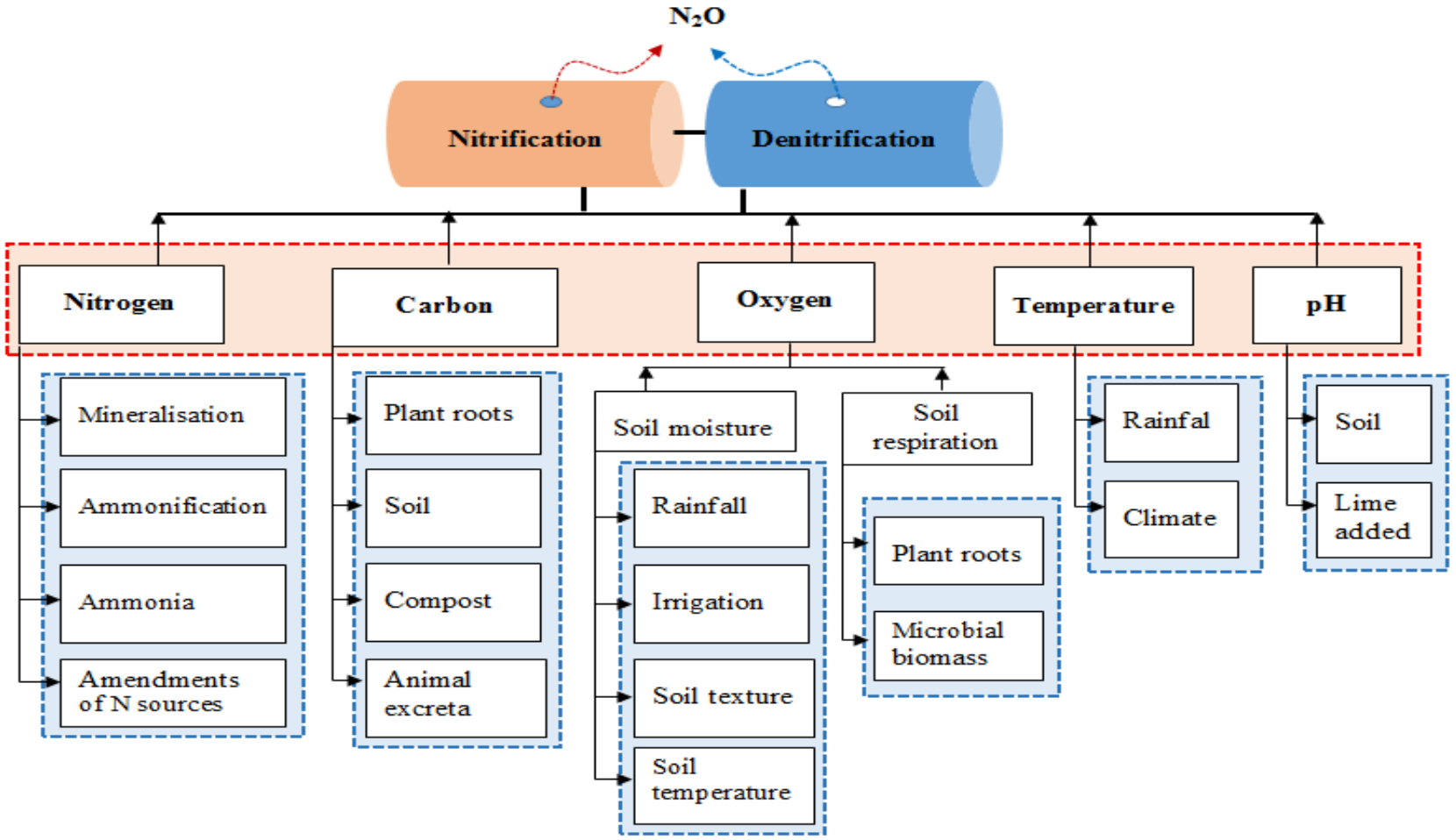

Figure 2.5. Factors affecting nitrification, denitrification and N_2O production in soils (red shaded: proximal factors; blue shaded: distal factors). Adapted from Saggar et al. (2013)

2.3.3 Substrate availability

Soil mineral nitrogen (N) is the substrate for nitrification and denitrification and therefore influences N_2O production from soil. Soil ammonium, NH_4^+, is a substrate for the initial step of nitrification process and is formed through mineralisation of organic N. The limitation of soil NH_4^+ availability has been identified to slow the rates of nitrification and associated N_2O production in few studies (Gödde and Conrad, 1999; Avrahami et al., 2003; Blagodatskaya et al., 2014a). The addition of NH_4^+ to soil increased the cumulative NO_3^- production from nitrification in a study by (Jones and Hedlin, 1970) but the rate of nitrification decreased at NH_4^+ supply above 200 µg N g^{-1} soil (Malhi and McGill, 1982). The production of N_2O from nitrification was less affected by NH_4^+ amendments from 50 to 150 µg N g^{-1} soil (Vermoesen et al., 1996). Some recent studies have demonstrated that heterotrophic nitrifiers can use NH_4^+ as a main substrate to produce a significant NO_3^- production (Liu et al., 2015) and N_2O production (Bateman and Baggs, 2005) below 25 °C. Despite this, N_2O production from heterotrophic nitrification has been observed under few controlled conditions and temperature regimes (Bateman and Baggs, 2005; Islam et al., 2007). Therefore, it is important to examine the thermal response of N_2O production from heterotrophic nitrification.

Nitrate, is the substrate used by bacteria during denitrification. An increased NO_3^- concentration in soil resulting from either N fertiliser application or nitrification may increase the rate of denitrification and the N_2O/N_2 ratio (Smith et al., 1998; Gillam et al., 2008) when conditions such as O_2 and C contents are optimised for the activity of denitrifying bacteria. NO_3^- may be used as a TEA since O_2 availability becomes limiting in the soil microsites (Cho et al., 1997a; Strong and Fillery, 2002), which promotes the production of N_2O from denitrification. Increased NO_3^- in soil often results in high N_2O/N_2 ratios (Gillam et al., 2008) due mainly to a preference for NO_3^- as a TEA, relative

to N_2O production (Cho et al., 1997a) and/or the inhibition of N_2O reductase by high NO_3^- availability (Blackmer and Bremner, 1978). The activity of N_2O reductase is likely to be inhibited once soil NO_3^- concentration is over 200 μg N g^{-1} soil, which results in a high N_2O/N_2 ratio. (Blackmer and Bremner, 1978; Scholefield et al., 1997; Stevens and Laughlin, 1998). Thus, change in soil NO_3^- availability is likely to regulate the total N denitrified and the denitrification product ratios below 30 °C.

2.3.4 Carbon availability

Organic carbon (OC) impacts on the biological pathways of N_2O emission through provision of an energy source to stimulate microbial activity. An increase in C availability in soil microsites would enhance denitrification and associated N_2O production (Tiedje, 1988; Weier et al., 1993) because C supplies energy and electron for the activity of soil denitrifiers (Burford and Bremner, 1975; Azam et al., 2002; Gillam et al., 2008). The consumption of carbon by microbial respiration indirectly affects nitrification and denitrification by reducing O_2 in the soil microsites. A decrease in O_2 concentration determines the relative contribution of nitrification and denitrification to overall N_2O production.

Increased carbon availability typically increases denitrification product ratios, such as $N_2O/(N_2O+N_2)$, as a result of the influence of C on the diffusion of NO_3^- into denitrifying microsites (Swerts et al., 1996; Weymann et al., 2010). The addition of C increases the demand for TEA and enhances the use of NO_3^- as a preferred TEA relative to N_2O once O_2 becomes limiting in the soil microsite. Thus, the $N_2O/(N_2O+N_2)$ ratio generally increase with greater OC and NO_3^- availability (Casella et al., 1984; Henry et al., 2008).

2.3.5 Soil pH

Soil pH can regulate the rates of nitrification and denitrification because pH directly affects the activity of soil microorganisms and indirectly influences biological processes by modifying substrate availability.

The nitrification process is thought to be very sensitive to changes in soil pH (Goodroad and Keeney, 1984; Strong et al., 1997; Islam et al., 2006). The rate of nitrification often increases with increasing soil pH, reaching a maximum NO_3^- production at around 5.0 – 6.5, regarded as the pH optima for the activity of nitrifiers (Islam et al., 2006). The production of NO_3^- from nitrification, however, has been recently measured in many acid soils (Šimek and Cooper, 2002; Islam et al., 2007; Liu et al., 2010) and has been identified from the activity of autotrophic and heterotrophic nitrification. The activity of nitrifiers under acid conditions has been considered to occur in either acid-sensitive or acid-tolerant autotrophic and heterotrophic nitrifiers (De Boer and Kowalchuk, 2001). One possibility for acid sensitive nitrifiers to be active under low pH (< 5) is through intracellular hydrolysis of urea, releasing NH_3 as a substrate for nitrification to proceed (De Boer et al., 1991). Acid-tolerant nitrification has been argued to be important in the lower organic layers in N-saturated forest and heathland soils (De Boer et al., 1992). The yield of N_2O production to nitrified N was much higher in very acidic conditions (pH 4.1 – 4.2) than in soil pH above 5 (Mørkved et al., 2007). Thus, a decrease in soil pH influences the ratio of N_2O to NO_3^- production from nitrification.

Denitrification can occur over a pH range from 3.5 to 8.5 although the mechanisms of pH controlling denitrification are still poorly understood. Denitrification tends to reach a maximum rate at pH 6 to 8 and decline at lower pH (Parkin et al., 1985; Šimek and Hopkins, 1999; Saleh-Lakha et al., 2009). A decrease in soil pH affected both in the cell

density and gene expression of *Pseudomonas mandelii* strain PD30 in cultures negatively, resulting in low denitrification rate and N_2O at pH 5, relative to higher pH (Saleh-Lakha et al., 2009). In another study, denitrifying enzyme activity decreased threefold as soil pH decreased from pH 6.02 to 4.08 (Parkin et al., 1985). The result from a study by (Šimek and Hopkins, 1999) found no significant relationship between soil pH and denitrifying enzyme activity, suggesting that the optimum pH for denitrification can only be identified when other factors (NO_3^-, O_2, OC) are optimised. Despite this, denitrification product ratios are affected by changes in soil pH. The N_2O/N_2 ratio increased with decreased soil pH in some studies (Nägele and Conrad, 1990; Struwe and Kjøller, 1994; Liu et al., 2010) partially due to enhanced N_2O formation in acid soil (Parkin et al., 1985). The production of N_2O in acidic conditions may be related to the amounts of NO_2^-, which is released at greater amounts from abiotic transformations of NO_3^- (Koskinen and Keeney, 1982). The production of N_2 in acidic soils is often low due to inhibition of the activity of *Nos*Z, the enzyme responsible for the reduction of N_2O to N_2, and can result in a high N_2O/N_2 ratio (Liu et al., 2010). The influence of soil pH on the N_2O/N_2 ratio was modelled by Rochester (2003), who showed that the N_2O/N_2 ratio can decrease exponentially with increasing soil pH from 5 to 8, although the impact of soil pH on the total amount of N denitrified, and lost from the system, was not considered.

2.3.6 Availability of trace metals

The availability of metal ions such as iron, copper and molybdenum can affect biological N_2O production. Denitrification enzymes require these ions as co-factors for their activities (Ferguson, 1998). Molybdate ion (Mo^{6+}), for example, acts as a component of the Mo-cofactor for *Nar* and ferric ion (Fe^{3+}) is essential for the cytochrome subunits of both *Nar* and *Nir*, the denitrifying enzymes in the reduction of NO_3^- to NO_2^- and NO_2^- to NO, respectively (Ferguson, 1998). Thus, lack of these metals in soil can potentially

reduce N_2O production from denitrification due to incomplete assembly of *Nar* and *Nir*. This could explain the results observed in several studies (Labbé et al., 2003; Zhou et al., 2007; Pintathong et al., 2009) that the additions of Mo^{6+} and Fe^{3+} to soil increase the cumulative NO_2^- and N_2O production. Copper (Cu) is an obligate component of *Nos*Z in the denitrification process. Thus insufficient Cu in soil can result in the failure of the full complement of the Cu-cofactors for *NosZ*, which inhibits the reduction of N_2O to N_2 during denitrification (Richardson et al., 2009). Therefore, the lack of Cu in soil would slow the rate of N_2 production from denitrification while the limitation of Fe^{3+} and Mo^{6+} would prevent the biological formation of NO_2^-, NO and sequential N_2O production.

2.4 Thermal response of N_2O production

2.4.1 N_2O production from nitrification

The response of nitrification rate (NO_3^- production) and associated N_2O emission to temperature have been studied in a variety of soils and climates. Below 35 °C, it is generally known that the rates of nitrification increase exponentially with increasing temperature. The thermal response of nitrification could be described by using Q_{10}, the relative difference in rate over a 10 °C increase in temperature in some studies. For example, Q_{10} of nitrification was estimated to be 2 within the temperature range 5-35 °C (Focht and Verstraete, 1977) while other studies (e.g. Malhi and McGill (1982); Maag and Vinther (1996)) found that Q_{10} is significantly higher at low temperatures (< 20 °C) than at higher temperatures. However, the use of Q_{10} to describe the response of nitrification to temperature does not allow for a plateau or decline in activity with increasing temperature (Dessureault-Rompré et al., 2010) while an optimum temperature (T_{opt}) for nitrification has been recognised in other studies (Campbell and Biederbeck, 1972; Stark, 1996). T_{opt} for nitrification may differ among geographic regions. For

example, T_{opt} for nitrification was found at 20 °C in a cool climatic soil from Alberta, Canada (Malhi and McGill, 1982) but at 35 °C in a tropical soil from Northern Territory of Australia (Myers, 1975) (Figure 2.6).

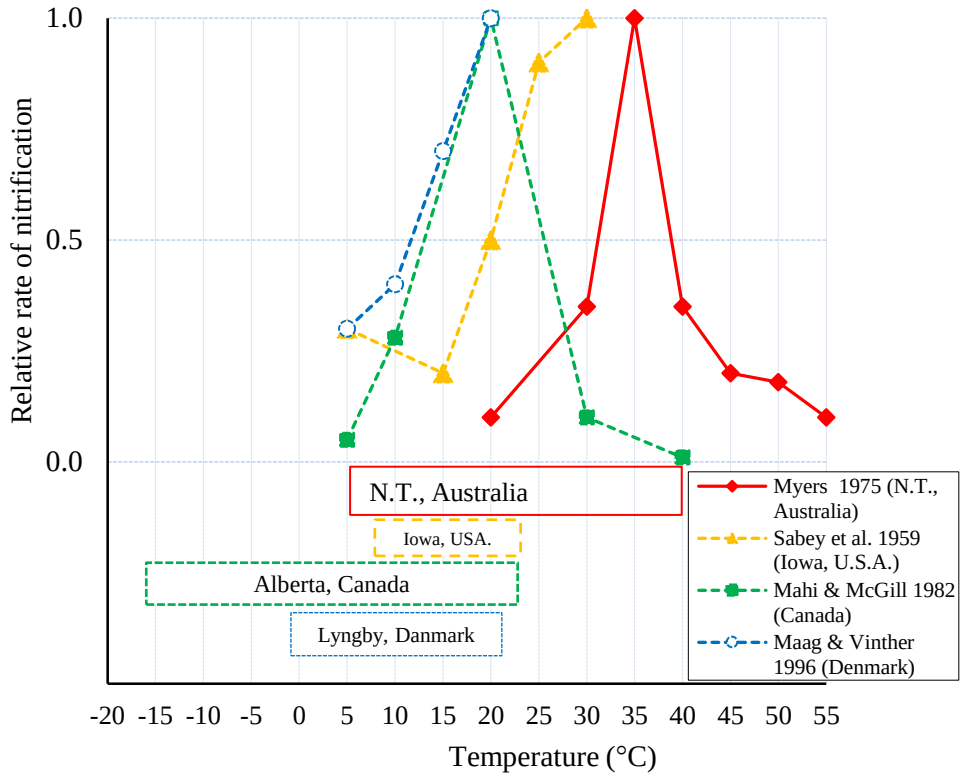

Figure 2.6. The effect of soil temperature on relative nitrification rates from different climatic regions: relative nitrification rates (lines) and the range of mean of monthly air temperatures for each site (bars). Data from Myers (1975), Sabey et al. (1959), Malhi and McGill (1982), Maag and Vinther (1996).

Temperature optima for N_2O production from nitrification may vary with climatic regions. T_{opt} for N_2O production from soil is generally expected to be lower in cool climates than in warm climates, as predicted by the DAYCENT model (Figure 2.7). The variation of T_{opt} may relate to the ability of microorganisms to acclimate and/or adapt to the historic range of soil temperatures (Powlson et al., 1988). The difference in T_{opt} for N_2O production from nitrification among climatic regions challenges the application of general ecosystem models predicting N_2O production from soils across climatic regions (Farquharson and Baldock, 2008). Some complex models such as DNDC (Li et al., 1992; Li, 2000) or NASA CASA (Potter et al., 1996) have been developed to improve the simulation of N_2O production across regions, but their limitations have been recognised by Chen et al. (2008) due to the variability of climate, soil properties and agricultural practices. Thus, the thermal responses of nitrification and N_2O production are required from local regions such as southern Australia to test the accuracy of these models.

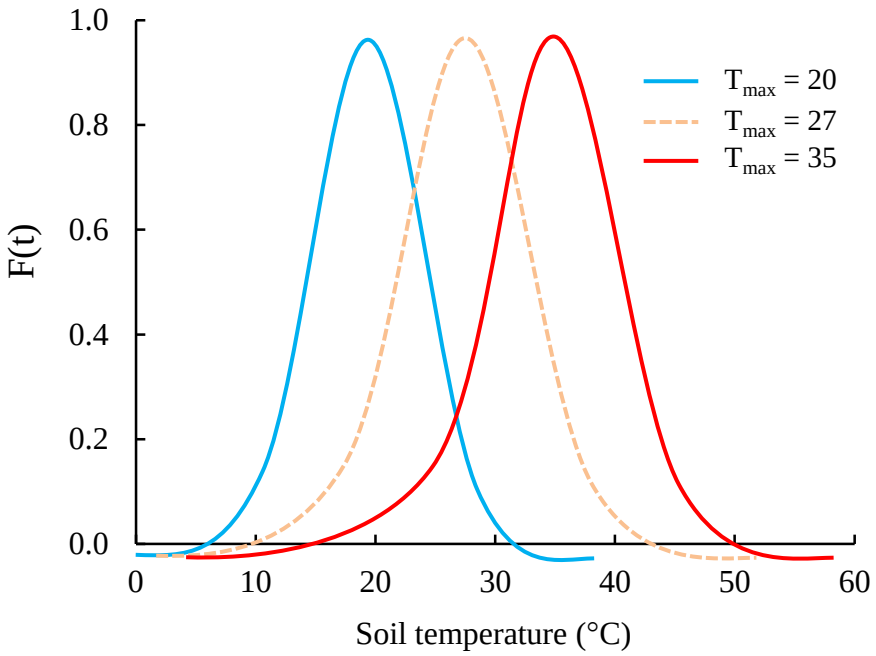

Figure 2.7. The effect of temperature on N_2O and NO_x from soil based on the DAYCENT model (Parton et al., 2001).

The proportion of N_2O to NO_3^- (P_n) released by from nitrification presents the yield of N losses as N_2O emission, during the nitrification process. P_n has been well-documented with the changes in soil moisture and oxygen availability in numerous studies (Table 2.1). P_n increased with decreasing O_2 pressure and has been frequently found to be less than 1.0%, although a few exceptions exist (Table 2.1). For example, the estimated P_n was 1.09% at 1.5 kPa O_2 (Khalil et al., 2004) and 2.9% at 3 kPa O_2 (Zhu et al., 2013). Despite this, there is little information about the response of P_n to temperatures above 30 ºC. P_n was observed to decrease with rising temperature between 5 °C to 25 °C (Maag and Vinther, 1996) while others (Goodroad and Keeney (1984), Ingwersen et al. (1999)) found an increase in P_n with rising temperature, up to 30 °C. Thus, there is some uncertainty in the responses P_n to high temperatures.

The thermal responses of N_2O specifically from autotrophic and heterotrophic nitrification are poorly understood. Autotrophic nitrification was originally thought to be more important than heterotrophic nitrification as the source of N_2O production, as heterotrophic nitrifiers produced a very small fraction of N_2O in agricultural soils such as sodosols (Tortoso and Hutchinson, 1990; Islam et al., 2007). However, recent studies have reported a larger contribution of heterotrophic nitrification to NO_3^- production from nitrification in both forest and pasture soils. Heterotrophic nitrification contributed up to 60% of total NO_3^- production during nitrification in cool (at 15 °C) and dry conditions (< 50% WFPS) (Liu et al., 2015). Heterotrophic nitrification accounted for 20% of total N_2O emissions in soil incubated at 22 °C and 50% WFPS (Bateman and Baggs, 2005). There is no study examining the production of N_2O from heterotrophic nitrification above 30 °C. Thus, the impact of high temperatures on N_2O production from autotrophic nitrification and heterotrophic nitrification should be considered.

Table 2.1. The proportion of nitrified N as N_2O at specified temperatures in laboratory and field studies.

Reference	Soil type[a]	Method[b]	Experimental conditions	Temperature (°C)	P_n (%)
Bateman and Baggs (2005)	Silt loam	^{15}N C_2H_2 (0.01% v/v)	35% WFPS	21	0.53
			50%WFPS		0.18
			60% WFPS		0.17
Carter (2007)	Loamy sand	^{15}N	Field study (N.D.)	10	0.02
					0.29
Cheng et al. (2004)	Molisol	Direct measure	60% WFPS	25	0.06-0.23
	Alphiol				0.08-0.36
	Aridisol				0.42
	Ultisol				0.14-0.15
Galbally et al. (2010)	Brown sodosol	^{15}N	Field study	10-25	0.01-0.05
Garrido et al. (2002)	Redoxic luvisol	C_2H_2 (0.1-10 kPa)	- 0.01 MPa	20	0.08
	Pachic calsisol				0.031/0.04
	Hypercalcareous rendosol				1.00
Goodroad and Keeney (1984)	Planosoil	Direct measure	0.2 / 0.3 m^3/m^3	10	0.09/0.08
				20	0.08/0.12
				30	0.09/1.15
Ingwersen et al. (1999)	Acid forest	BaPS	70% w/w	5	0.01
				10	0.026
				15	0.036
				20	0.05
				25	0.054
Khalil et al. (2004)	Orthic luvisol	^{15}N	20.4 kPa O_2	20	0.16
			4.3 kPa O_2		0.42
			1.5 kPa O_2		1.09
			0.76 kPa O_2		1.48
Maag and Vinther (1996)	Sandy loam	C_2H_2 (10 Pa)	55 / 70% FC	5	0.49/0.55
				10	0.37/0.42
				15	0.27/0.33
				20	0.18/0.27

Reference	**Soil type**[a]	**Method**[b]	**Experimental conditions**	**Temperature** (°C)	**P_n (%)**
Martikainen et al. (1993)	Forest soil	C_2H_2 (2.5 kPa)	22% w/w	20	0.03
Mathieu et al. (2006)	Gleyic luvisol	^{15}N	75% FC	20	0.13
			150% FC		2.32
Mørkved et al. (2006)	Molic gleysol	^{15}N	-10 kPa	5	0.27-1.0
Mørkved et al. (2007)	Sapric histosol	^{15}N	18.4 w/w	20	1.4
					7.6[c]
Tortoso and Hutchinson (1990)	Agricultural soil	Direct measure	-0.1 MPa	25	0.02
Zhu et al. (2013)	Sandy loam to clay loam	^{15}N + ^{18}O + C_2H_2 (0.01% v/v)	50% WHC	22	
			21 kPa $_{O2}$		0.08-0.11
			3 kPa $_{O2}$		0.52-2.9
			0.5 kPa O_2		6.9-8.3

[a] Terminology for soil type is not standardised: usage varies from author to author

[b] Method used for quantifying N_2O by nitrification: indirect measure, authors assume that nitrification was the only source of N_2O, and they simply report measured N_2O fluxes to observe nitrification rates, C_2H_2 refers to the use of acetylene as nitrification inhibitor; ^{15}N refers to the use of ^{15}N isotopic tracers; BaPS refers to the barometric process separation method.

[c] Data observed at pH 4.1

2.4.2 N_2O from denitrification

Denitrification can occur at a wide range of soil temperatures ranging from sub-zero to 75 °C (Knowles, 1982). Denitrification enzyme activity (DEA) follows Michaelis–Menten kinetics with a typical Q_{10} value of approximately 2 in soils (Stanford et al., 1975). Rates of denitrification in controlled experiments vary markedly among experiments and with temperature regimes (Table 2.2). The production of N_2O from denitrification exponentially increased with rising temperature (Keeney et al., 1979; Maag and Vinther, 1996; Holtan-Hartwig et al., 2002; Schaufler et al., 2010), with optimal measured denitrification rates close to 30 °C. The thermal response of N_2O production from denitrification was often greater at low temperatures than at high temperatures, with, for example, Q_{10} estimated to be 3.7 between 5 °C to 12 °C and at 2.3 between 12 °C to 18 °C (Dobbie and Smith, 2001).

Another important consideration of denitrification is the proportion of N lost as N_2O emission, relative to N_2, often expressed as the N_2O/N_2. This ratio is not only controlled by N_2O production but also depends on the biological reduction of N_2O to N_2 during denitrification. There has been some conjecture as to how the N_2O/N_2 ratio responds to rising temperatures. It has been suggested that more N_2O is produced at low temperatures, thus the N_2O/N_2 product ratio is increased with reduced temperature (< 15 °C) (Keeney et al., 1979; Avalakki et al., 1995; Maag and Vinther, 1999). Other studies (Focht, 1974; Rudaz et al., 1999) found that temperature had no effect on denitrification products. The production of N_2 in these studies were mainly estimated by the inhibition of N_2O reductase using the C_2H_2 technique (Table 2.2). Incomplete inhibition of N_2O reductase by C_2H_2 could affect the accuracy of the partitioning of gaseous N losses as N_2O and N_2. Thus, the use of the isotopic tracer (^{15}N) technique enables a more precise measurement

of N_2 production to evaluate of N_2O/N_2 responses to temperature and other important factors such as soil moisture and NO_3^- availability.

Table 2.2. Denitrification and associated N_2O production at specified temperatures in laboratory and field studies.

Reference	Soil type	Method	Experimental conditions	N applied (kgN ha^{-1})	Temperature (°C)	Denitrification (kgN ha^{-1} d^{-1}) Mean (min-max)
Senbayram et al. (2012)	Sandy loam	C_2H_2 (10% v/v)	65% WHC	120-KN	15	0.84 (0.72-0.96)
Carter (2007)	Loamy sand	^{15}N-labeled N	35%, 45% WFPS	529-U		0.140
Clough et al. (2004)	Silt loam	^{15}N-labeled N	54, 80 %WFPS	500-U	23	0.046 (0.023-0.1)
de Klein and Van Logtestijn (1996)	Sand, loam	C_2H_2 (9% v/v)	60-95% WFP		10, 20	1.413 (0.03-3.6)
Eckard et al. (2003)	Loam, sandy loam	C_2H_2	20-35% v/v air filled porosity	200-AN	10-22	0.04 (0.018-0.061)
Ellis et al. (1998)	Silty clay loam	C_2H_2 (10% v/v)	22-31% WC v/v	45 to 60-CS, AN	25	0.117 (0.06-0.151)
Hixson et al. (1990)	Loam sand Sandy loam	C_2H_2	10-45% v/v WC		3-22	0.015 (0.007-0.024)
Horwath et al. (1998)	a	C_2H_2 (5-10% v/v)	38-55% v/v WC	240-U+KN	13-16	0.142 (0.011-0.280)
Jarvis et al. (2001)	Coarse sandy loam	C_2H_2 (10% v/v)	a	280-NK	a	0.0645 (0.02-0.37
Kaiser et al. (1996)	Sandy loam	C_2H_2 (10% v/v)	0.07-0.31 g^{-1} g^{-1}	215-AN	11-18	0.059
Luo et al. (2000)	Silt loam	C_2H_2 (10% v/v)	Field study	a	8-16	0.002 (0.003-0.247
Marshall et al. (1999)	Sandy loam, loam, silt loam	C_2H_2	a	103 to 254-poultry	a	0.016 (0.002-0.042)
Misselbrook et al. (1996)	Sandy loam			44 to 72-CS 223 to 278-PS		0.059 (0.002-0.101)

Reference	Soil type	Method	Experimental conditions	N applied (kgN ha^{-1})	Temperature (°C)	Denitrification (kgN ha^{-1} d^{-1}) Mean (min-max)
Patra et al. (2005)	sandy	C_2H_2 (10% v/v)	100% WHC	Grazing intensity	26	1.4 (1.2-1.8)
Rudaz et al. (1999)	Sandy loam	C_2H_2 (12 kPa)	60-87% WFPS	85-AN	5-23	0.078 (0.065-0.091)
Ruz-Jerez and White (1994)	Fine sandy loam	C_2H_2	Field study	500-U	4-6	0.014 (0.005-0.031)
Ryden (1983)	Loam over clay			250, 500-AN		0.038 (0.004-0.080)
Šimek et al. (2004)	Sandy loam	C_2H_2 (10% v/v)	47-64% WFPS	Clover, grass	25	0.141 (0.024-0.464)
Sullivan et al. (2005)	Sandy loam	C_2H_2 (10% v/v)	8-18 % WC v/v	112-PS, AN	a	0.0003
Van Cleemput et al. (1994)	Sandy loam	C_2H_2 (10% v/v)		295-AN	nd	0.240
Van der Salm et al. (2007)	Heavy clay	C_2H_2 (5% v/v)	10-60% WFPS	33 to 264-CS, KN	20	0.276
Velthof et al. (1996)	Sand	Direct measurement	Wet conditions		12-16	0.152 (0.006-0.382)
Vermoesen et al. (1996)	Sandy loam	Direct measurement	100% FC	200-NK	nd	0.038 (0.024-0.052)
Walker et al. (1992)	Sandy loam to loamy sand	C_2H_2 (10% v/v)	11-42 % WC v/v		5-13	0.026 (0.024-0.028)
Xu et al. (2008)	Sandy loam	C_2H_2 (10% v/v)	4-12% WC	Grazing intensity	5-25	0.0001
Zaman and Nguyen (2010)	Silt loam	C_2H_2 (5% v/v)	60-70% WFPS	400-U, KN	10-16	0.015 (0.0009-0.003)
Zaman et al. (2007)	Silt loam	C_2H_2 (10% v/v)	81% WFPS	200-KN	25	1.48 (0.80-2.23)

[a] no data available

Method used for quantifying N_2O: C_2H_2 refers to the use of acetylene to inhibit the reduction of N_2O to N_2; ^{15}N refers to the use of ^{15}N isotopic tracers.

N application: AN: ammonium nitrate; AS ammonium sulphate; PS: Pig slurry; KN: potassium nitrate; U urea.

Unit of N application was converted to kg N ha^{-1} if data are presented at mg N g^{-1} soil

2.4.3 N_2O from abiotic reactions

Some researchers have identified N_2O formation from abiotic pathways (Bremner, 1997; Stevens et al., 1998; Santoro et al., 2011). As chemical reactions normally occur at very high temperature (> 200 °C) (Tsang and Herron, 1991; Zhu et al., 2004), abiotic N_2O formation has often been neglected in studies measuring N_2O production from soil, while a reaction of hydroxylamine (NH_2OH) or nitrite (NO_2^-) can release N_2O at temperatures below 50 ºC. During nitrification, ammonium oxidising bacteria intermediately produce NH_2OH (Stüven et al., 1992; Schmidt et al., 2004), which can be rapidly oxidised by several soil constituents to form N_2O (Bremner, 1997). The production of this abiotic N_2O pathway was found to be positively correlated with the amounts of NH_2OH amendments and temperature in the range 10 °C to 50 °C (Liu et al., 2014; Heil et al., 2015). The oxidation of NH_2OH to form N_2O occurred faster at high temperatures than lower temperatures (Heil et al., 2015). Another chemical reaction producing N_2O production is chemo-denitrification, which is defined as a fast reaction of NO_2^- produced during denitrification in the presence of ferrous iron (Fe^{2+}) in soil, forming N_2O(Jones et al., 2013).

2.5 Temperature patterns in Australia

Australian agricultural soils experience particularly high diurnal and seasonal temperature fluctuations. In the northern to eastern regions, the highest mean monthly temperature (T_{max}) in 10 years (2004 – 2014) ranged from 30 °C to 35 °C in summer, particularly between November and February (Meteorology, 2015). The hottest day in these regions was recorded at 45 °C. Monthly mean temperature decreases from March to August with the lowest monthly temperature (T_{min}) at 11 – 24 °C (Meteorology, 2015). The lowest daily temperature was 5 °C in July or August. In southern Australia, T_{max}

ranged from 17 °C to 33 °C while T_{min} was around 5 – 18 °C. The highest daily temperature, observed in January or February, was 46.5 °C while the lowest temperature was 3 °C in August to September (Meteorology, 2015). In western regions, T_{max} experienced between December and February was 20 °C to 34 °C. The daily temperature could reach 45 °C in summer and down to 0.5 °C in winter (Meteorology, 2015). These data indicate that environments in Australia have large fluctuating temperatures among seasons and regions. Air temperature was predicted to increase of 1.0 – 2.0 °C by 2050 in almost regions of Australia (Hendon et al., 2007). T_{max} is expected to rise 1.0 – 1.5 °C in the southern half and 1.5 – 2.0 °C in the northern half Australia by 2050 (Hendon et al., 2007). Thus, the impact of high temperatures, particularly above 35 °C, on biological N_2O production requires further investigation.

Laboratory experiments have often used daily constant temperature (Table 2.1 & Table 2.2) to assess the thermal response of N_2O emissions although diurnal temperature actually occurs on field sites. Soil biological processes, particularly N mineralisation and nitrification, under these controlled conditions may differ from those under daily fluctuating temperature. For example, the rates of N mineralisation were significantly higher under the fluctuating temperatures than at a constant temperature having the same mean temperature in some experiments (Campbell and Biederbeck, 1972; Sierra, 2002). It was noted by Goodroad and Keeney (1984) that a fluctuating temperature may induce higher N_2O emissions than under a constant temperature. The result from that study suggests that the actual rates of N_2O production might be underestimated from the laboratory experiments through the use of the daily mean temperatures. The data, however, are limited to a narrow range of experimental conditions and temperature regimes, thus the results cannot be generalised. Therefore, the response of biological N_2O emissions to the fluctuating temperature needs to be better understood.

2.6 Summary and critical knowledge gaps

In summary, there are some uncertainties in understanding N_2O and N_2 production from soils at high temperature:

- The effect of fluctuating temperature and ammonium supply on nitrification and N_2O emissions in soil.
- The effect of fluctuating temperature on nitrification and N_2O emissions in soil
- The response of N_2O emission from nitrification to high temperatures.
- The production of N_2O in soils from different climatic regimes in response to temperature variations.
- The production of N_2O and N_2 from denitrification in soil as influenced by temperature and soil moisture.
- The production of N_2O and N_2 production from denitrification in soil as influenced by temperature and NO_3^- content.

Laboratory experiments were designed to address these issues, using the techniques outlined above.

2.7 Thesis objectives

The general aim of this project is to evaluate nitrification, denitrification and N_2O emissions from soils in response to temperature and to provide a detailed mechanistic understanding of the processes. The main objectives of the work presented in this thesis will:

- Evaluate if fluctuating temperature or ammonium concentration influences the nitrification process and the production of N_2O in a dairy soil (Chapter 3)

- Understand N_2O production from nitrification in response to a range of soil temperatures (**Chapter 4**)
- Determine the temperature response of N_2O emissions in soils from contrasting climatic regimes (**Chapter 5**)
- Evaluate the effect of temperature and soil moisture content on the production of N_2O and N_2 from denitrification (**Chapter 6**)
- Evaluate the effect of temperature and nitrate availability on the total denitrification and the N_2O/N_2 ratio from denitrification (**Chapter 7**)
- Summarise the effect of temperature on N_2O and N_2 production from biological processes through a conceptual model and highlight some questions for future research (**Chapter 8**).

Chapter 3 Are nitrification and N_2O emissions influenced by ammonium supply or fluctuating temperatures?

3.1 Introduction

Ammonium (NH_4^+) is a substrate for the initial step of nitrification, which produces nitrate (NO_3^-) and releases nitrous oxide (N_2O) as intermediate product. The influence of NH_4^+ concentration on the rate of nitrification has been investigated in some studies (Jones and Hedlin, 1970; Malhi and McGill, 1982; Avrahami et al., 2003) but the data are equivocal. A positive relationship between NH_4^+ availability and nitrification rate was reported by Jones and Hedlin (1970) but decreased NO_3^- formation by nitrification was found with increasing NH_4^+ supplied, above 200 µg NH_4–N g^{-1} soil in another study (Malhi and McGill, 1982). The production of N_2O from nitrification was reported to be limited by soil NH_4^+ availability (Avrahami et al., 2003; Blagodatskaya et al., 2014a) while N_2O production from nitrification was less affected by increased NH_4^+ amendments from 50 to150 µg N g^{-1} soil in another study (Vermoesen et al., 1996). Thus, it is important to investigate the effect of NH_4^+ concentrations on the rates of nitrification and associated N_2O production, particularly when investigating responses to high temperature.

Soil temperature can influence the growth of soil microorganisms and the enzymatic reactions they carry out, influencing N_2O production from soil. The rates of N transformations and N_2O production from soils have been described to exponentially increase with rising temperature between 5 °C and 25 °C (Focht and Verstraete, 1977; Goodroad and Keeney, 1984; Smith, 1997). Most laboratory studies have used constant temperatures to measure the thermal responses of nitrification, denitrification and N_2O emissions. The rates of these processes under constant temperatures (CT) may differ from those that occur under a fluctuating temperature pattern (FTP), typical of field

environments, which vary diurnally in temperature (Campbell and Biederbeck, 1972; Goodroad and Keeney, 1984; Sierra, 2002). For example, the rate of nitrification was significantly slower under a FTP that varied between 3 ºC and 14 °C compared with a CT of 10 ºC (Campbell and Biederbeck, 1972). Conversely, a FTP between 10 ºC to 30 ºC induced a higher rate of N_2O emissions than a CT of 20 ºC over 5 days of incubation in a study by Goodroad and Keeney (1984), suggesting that N_2O emissions may be underestimated when using a CT in the laboratory, rather than a more realistic FTP. However, the impact of FTP on N_2O production was only explored in a silt loam soil and a certain temperature regime (10 – 30 ºC), limiting the generalisations that can be made across regions due to variations in soils and climates. N_2O production in soil is thought to be influenced by the amplitude of temperature changes (Das et al., 1995); these fluctuations are typically experienced in soils from southern Australia, but the influence of FTP on N_2O production is poorly understood. Therefore, an investigation of the effects of FTP on N_2O emissions in soil will improve our knowledge of varying temperature in different environments.

The aims of this work were therefore to (1) determine the rates of nitrification and associated N_2O emission responses to different amounts of substrate (NH_4^+) under a constant temperature; and to (2) investigate if fluctuating temperature induces higher N_2O production compared with a constant temperature.

3.2 Materials and methods

3.2.1 Study site and sample collection

The soil used in this study was obtained at a non-irrigated commercial dairy farm (38° 14' S, 142°55'E) in south west Victoria, Australia, and was referred to as a 'chromosol' (Isbell, 1996). The mean annual rainfall of this site is about 745mm. Low soil temperatures (10 – 20 ºC) recorded at 10 cm topsoil were associated with high soil water contents (0.3 – 0.5 mm^3 mm^{-3}) between May and November. Higher soil temperatures (20 – 45 ºC) were associated with lower water contents (0.1 – 0.3 mm^3 mm^{-3}) between December and April as shown in Figure 3.1.

Experimental soil was collected from a plot of 100 m^2 that had been bare for 6 months prior to soil sampling. The soil is a brown chromosol [59.8% sand, 24.4% silt, 15.8% clay, pH-$CaCl_2$ 4.9, 4.06% organic carbon, 6.76 µg NH_4–N g^{-1} and 11.52 µg NO_3–N g^{-1} soil] according to the Australian soil classification (Isbell, 1996).

Experimental soil samples were collected from the top 10 cm layer of a 2 m^2 area in February 2013. Intact soil cores were used to measure bulk density (BD) and gravimetric water content at field capacity (FC) at –33 kPa. After sampling, the soil was sieved to remove the > 2 mm fraction, and thoroughly mixed to ensure uniformity. Sieved soil was stored in sealed containers at 4 ºC to retain the field moisture and minimise microbial activity until use in March 2013.

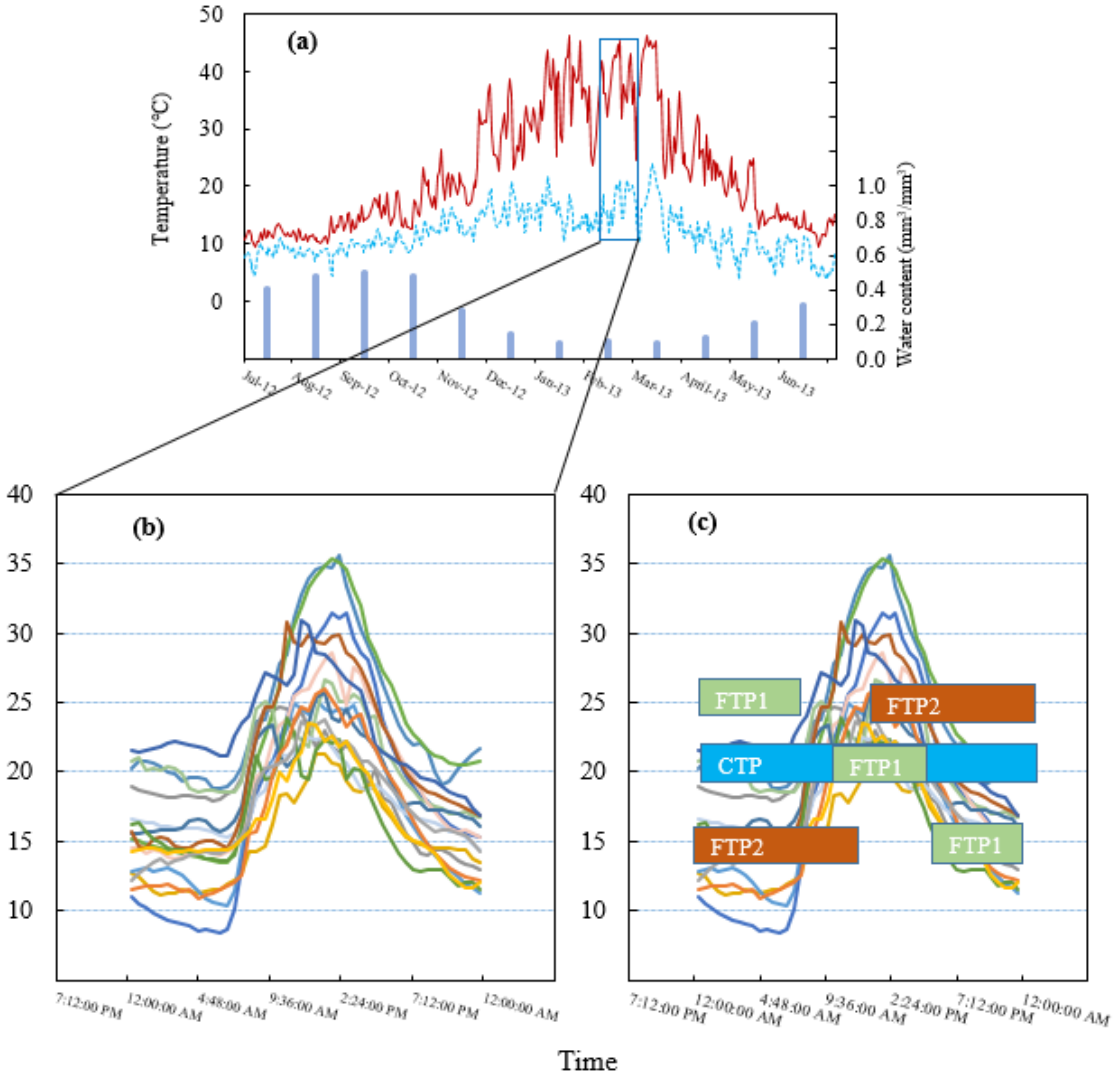

Figure 3.1 (a) The variation in soil temperature (50mm top layer) and water content in study site from June 2012 until July 2013 (unpublished data from Kevin Kelly, Department of Agriculture, VIC.): Lines present daily soil maximum (red line) and minimum temperature (blue line), bars present the monthly volumetric water content; (b) The variation in daily soil temperature captured automatically every 30 minutes in February 2013, (each line represents one day); and (c) three temperature patterns tested (FTP_1: 8h -15 °C, 8h- 20 °C, 8h - 25 °; FTP_2: 12h -15 °C and 12h - 25 °C; CT maintained continually at 20 °C).

3.2.2 Measurement of water field capacity and bulk density

"Field capacity" (FC) is a generic agronomic term used to describe the amount of water remaining in field soil after having been wetted to saturation and then being allowed to drain freely in the absence of evaporation for several days (GSST, 2008). In the laboratory, 'field capacity' was estimated by placing a soil sample onto a porous ceramic plate, saturating it and allowing it to come to hydraulic equilibrium at a static water potential of –33 kPa.

To determine the FC of our soil, four undisturbed soil cores were removed from the field in stainless steel rings (50 mm diameter × 50 mm high), trimmed and fitted with a nylon mesh (38 µm holes) on the base of the sample, and placed in a tray of distilled water filled to the top of the rings. After 24 hours, the samples were placed on ceramic pressure plates, which were then transferred into steel chambers and pressurized with N_2 gas to generate an equivalent soil water potential of –33 kPa. The weight of each sample was checked repeatedly every day until the weight stopped changing significantly (usually 4 days), at which point the samples were deemed to have come to equilibrium at FC. Samples were weighed then dried at 105 ºC for 24 hours to determine the gravimetric water content (g water per g dry soil). The bulk density (BD) of the soil samples was also calculated from the dry mass of the soil and the volume of the ring containing the soil, and this allowed the gravimetric water contents to be converted to volumetric water contents (cm^3 water per cm^3 bulk volume).

3.2.3 Laboratory incubations

3.2.3.1 Pre-incubation

Soil samples were wetted to 60% FC–33 kPa (0.23 g water g^{-1} soil) and placed into a plastic bag with holes to allow gas exchange with the atmosphere. All soil samples were pre-incubated for 7 days at 25 ºC to recover biological activity after sampling and processing (Davidson, 1991). Soil moisture was checked every 3 days and water added, if necessary, to maintain 60% FC. After this pre-incubation, soil moisture was maintained at 50% FC and soil subsamples (50 g dry weight equivalent) were repacked into cores (37 mm diameter × 42 mm height; 10.75 cm^2 surface area) to a bulk density of 1.02 g cm^{-3}, equivalent to that in the field.

The experimental set up is presented in Figure 3.2.

3.2.3.2 Incubation

- *Experiment 1: The effect of NH_4^+ supply*

The objective of this experiment was to investigate the effect of NH_4^+ on nitrification rate and associated N_2O production.

After seven days of pre-incubation, N solution, $(NH_4)_2SO_4$ was added to soil to provide final additions of NH_4^+ of 0, 50, 100 and 150 µg N g^{-1} soil. This N solution was added slowly onto the top of upright soil cores with duplicate times to promote uniformity of NH_4^+ through the soil and reach a final water content of 60% FC (0.22 g water g^{-1} soil). Each soil core was placed into a 250 ml jar with a gas-tight lid equipped with a rubber septum (Suber-seal#25, Sigma-Aldrich, St Louis, MO, USA). The jars were flushed with synthetic air for 2 minutes and acetylene (C_2H_2) was added though a 25 mL gas-tight syringe (Hamilton Company, Reno, NV, USA) to provide a final partial pressure of 10 Pa (0.01%v/v) in half of the jars, which is known to inhibit autotrophic nitrification

(Yoshinari et al., 1977; Klemedtsson et al., 1988). After injecting C_2H_2, the syringe was pushed forward and backward a few times to aid in mixing and promote diffusion of the C_2H_2 in the soil. Half of the jars were not amended with C_2H_2. All jars were incubated at 20 ºC for 11 days using four replicates (Figure 3.2).

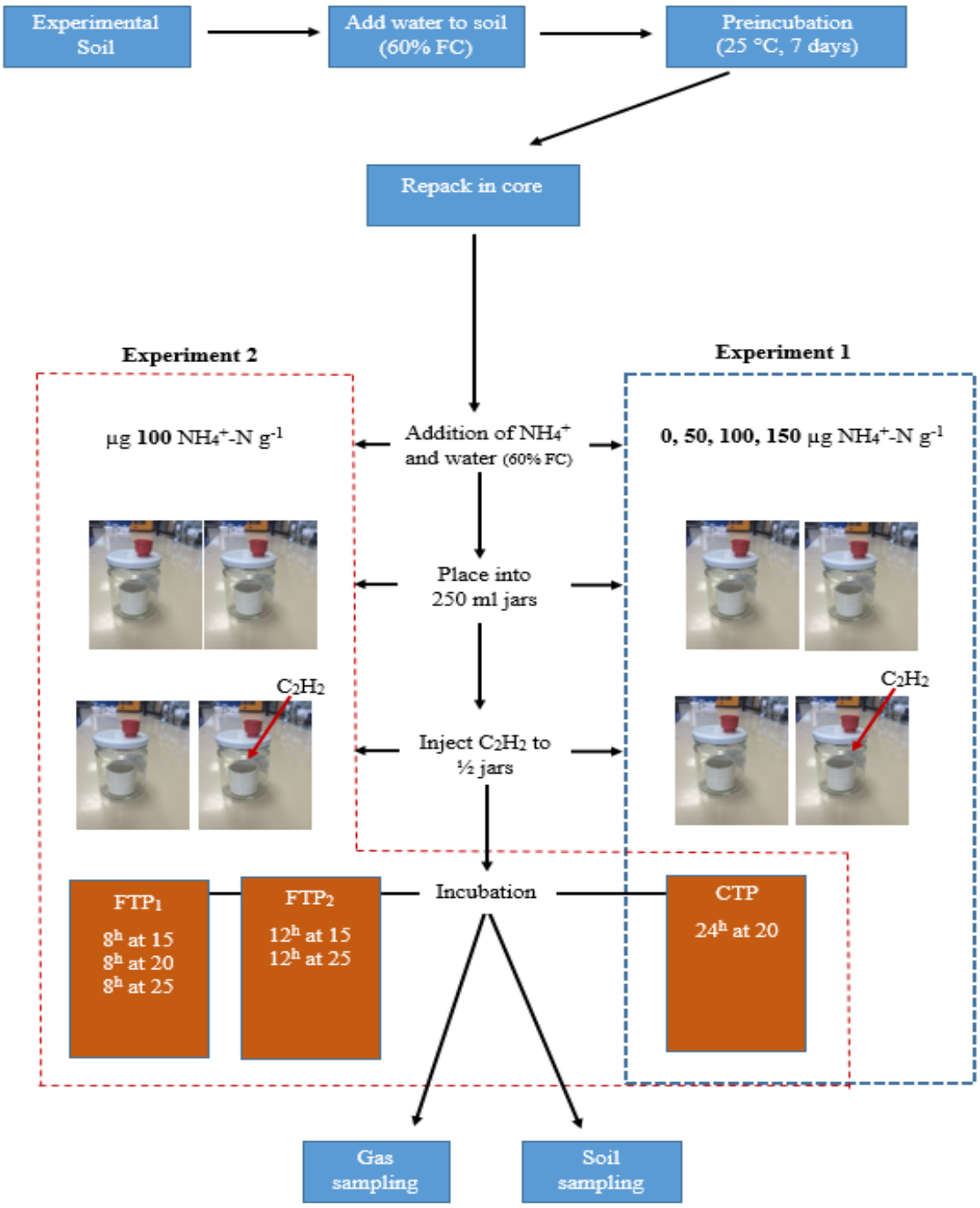

Figure 3.2. Experimental procedures outlined for experiment 1, testing different N supply and experiment 2, testing different temperature patterns during incubation.

- *Experiment 2: The effect of fluctuation temperature*

The objective of this experiment was to assess the influence of fluctuating temperature on total N_2O emissions and NO_3^- production from soil. Although a range of temperatures are experienced in field conditions of the site where soil was collected (Figure 3.1a & b), two FTPs, namely FTP_1 and FTP_2, and one CT at 20 °C were tested (Figure 3.1c). The characteristics of the temperature patterns were FTP_1: 8h at 15 °C, 8h at 20 °C, 8h at 25 °C, mean 20 °C; FTP_2: 12h at 15 °C and 12h at 25 °C, mean 20 °C. FTP_1 and FTP_2 were chosen in this study as they mimicked typical temperature amplitudes experienced at the site (Figure 3.1). The incubation temperature patterns were obtained using an incubator with a programmed temperature controller (UP150, Yokogawa, Lascar, UK). After addition of N as $(NH_4)_2SO_4$ solution to obtain 100 µg N g^{-1} soil at 60% FC, each temperature pattern was repeated daily for 30 days (Figure 3.2). The jars were opened after each gas sampling, aerated for 5 minutes, and the water content was checked by weighing and adjusting, as necessary.

Two sets of soil incubations were established to monitor gas production (the first set incubations) and mineral N transformations (the second set incubations) in both experiments.

3.2.4 Gas sampling and analysis

Gas samples were taken from the headspace of jars in the first set of incubations every three days after addition of the N solution. Briefly, a 20 ml sample of headspace gas was taken using a Hamilton 50 ml gas-tight syringe (Hamilton Company, Reno, NV, USA) and transferred into a pre-evacuated 12 ml glass exetainer (Labco, Ceredigion, UK) for measurement of N_2O and CO_2. After gas sampling, the headspace of all jars was refreshed

using a mini-fan for one minute. Acetylene was injected back into appropriate jars to maintain uniform pressure.

The CO_2 concentration of headspace gas samples was measured using a gas Chromatograph (Hewlett Packard 5890, Bremen, Germany) fitted with a thermal conductivity detector (TCD) at 60 °C. The separation was performed on a 1.5 m column (80 ºC) packed with Porapak Q, 50-80 mesh using grade A helium (BOC) as carrier gas (40 ml min^{-1}).

N_2O was analysed on a Varian 450 gas chromatograph (Agilent 7890, Bremen, Germany) equipped with a ^{63}Ni electron capture detector (ECD) at 360 ºC, using Pureshield Argon as the carrier gas (35 ml min^{-1}). Separation was carried out on a 1 m column (55 ºC) packed with HayeSep Q 60-80 mesh (Haye Seperations, Bandera, TX).

3.2.5 Soil mineral N and analysis

Soil samples were taken from the second set of incubations at 0, 7, 14 and 30 days in the first experiment and at 0, 3, 7 and 11 days in the second experiment, to measure mineral N contents (NH_4^+ and NO_3^-). NH_4^+ and NO_3^- were extracted from 10 g of moist soil by shaking for one hour with 50 ml of 2M KCl. The analyses of mineral N were performed colorimetrically with an AutoAnalyser (AA3 HR, SEAL, West Midlands, UK) using the hydrazine reduction method for NO_3^- (Kamphake et al., 1967) and the nitroprusside method for NH_4^+ (Kaplan, 1965).

3.2.6 Most probable number count

The numbers of ammonium oxidising bacteria (AOB) in the three temperature patterns were estimated from soils without C_2H_2 on day 0, 7, 14 and 30 days after incubation. The most probable number count (MPN) described by Schmidt and Belser (1994) was applied to estimate AOB population. Briefly, 2.0 g of dry soil was mixed with 18 ml of 1mM

sterile phosphate buffer to make the initial solution (10^{-1}). Serial dilutions of soil (from 10^{-2} to 10^{-6}) were made with 1mM sterile phosphate buffer. One ml of each dilution was inoculated into 4 ml of MPN media tubes containing the 1 mM ammonium solution and each inoculation dilution (from 10^{-1} to 10^{-6}) was replicated five times. Inoculated MPN tubes were incubated at 28 ºC in the dark. At intervals of one week for a maximum of 12 weeks, each tube was tested for NO_3 production by observing colour change from blue green to yellow due to pH drop, which indicated acid production during the nitrification process. A Griess–Ilosvay colorimetric spot test and a Merckoquant strip test were also carried out to confirm the nitrification process in the positive MPN tubes (Schmidt and Belser, 1994). The AOB population (cells g^{-1} dry soil) was estimated using population estimates derived by Woomer (1994).

3.2.7 Calculation of nitrogen transformation rates

Autotrophic nitrification was inhibited in the samples with C_2H_2 addition (0.01%v/v) (Yoshinari et al., 1977; Klemedtsson et al., 1988), so the production of N_2O (µg N_2O-N g^{-1} d^{-1}) in these samples was attributed to denitrification and heterotrophic nitrification (N_2O-$_{DH}$). The production of N_2O in samples without C_2H_2 addition was emitted from denitrification and from autotrophic and heterotrophic nitrification (N_2O_{DN}). Therefore, the difference in N_2O production between samples with and without C_2H_2 addition was attributed to autotrophic nitrification (N_2O_A) (Davidson et al., 1986; Klemedtsson et al., 1988).

$$N_2O_A = N_2O_{DN} - N_2O_{DH} \quad \text{(Equation 3.1)}$$

The autotrophic nitrification rate (N_{nit}, µg NO_3-N g^{-1} d^{-1}) was estimated by the difference in NO_3^- concentration in the samples with (+ace) and without C_2H_2 addition (-ace) divided by the time of incubation (t, days).

$$N_{nit} = (NO_{3(-ace)} - NO_{3(+ace)})/t \quad \text{(Equation 3.2)}$$

3.2.8 Statistical analysis

The pooled ANOVA for measurement over time (Gomez and Gomez, 1984) was applied to determine the rates of change over time (N_2O and mineral N contents). One-way ANOVA was used to determine the difference between treatments at the end of the experiments. The Tukey ($HSD_{0.05}$) test was applied *post hoc* to identify any significant differences among treatments. All statistical analyses were performed using Statistix 10 (Tallahassee, USA).

3.3 Results

3.3.1 Experiment 1: The effect of NH_4^+ supply

3.3.1.1 Soil mineral N

In the absence of C_2H_2, NH_4^+ concentrations decreased at all treatments throughout the experiment, except in the control (A_0), where changes were negligible (Table 3.1). Following amendment with of C_2H_2, NH_4^+ contents in the first 3 days of incubation remained close to stable as soil was either added with 100 (A_{100}) or 150 (A_{150}) µg NH_4–N g^{-1} soil but increased at A_0 and A_{50}. In the subsequent measurements, cumulative NH_4^+ in presence of C_2H_2 occurred in all NH_4^+ treatments (A_0 – A_{150}) (Table 3.1).

In the absence of C_2H_2, NO_3^- in the control was accumulated in the first 3 days of incubation (Table 3.1). NO_3^- contents in the subsequent measurements did not differ to

that measured on day 3 in this treatment. Following NH_4^+ addition (A_{50}, A_{100}, A_{150}), the concentration of NO_3^- increased with incubation time in the samples without C_2H_2, indicating the production of NO_3^- from nitrification. In the presence of C_2H_2, the concentration of NO_3^- remained stable in the first 3 days but decreased in all treatments over the period 3 – 11 days (Table 3.1).

Table 3.1. The concentrations of NH_4^+ and NO_3^- over time following amendment with different amounts of NH_4^+ at time 0 (A_0: 0, A_{50}: 50, A_{100}: 100 & A_{150}: 150 µg N g^{-1} soil) with (0.01% v/v) and without C_2H_2 addition. Soil moisture was maintained at 60% of the gravimetric water content at field capacity. Each value is followed by the standard error of the mean (n=4).

Treatments	N form		Without C_2H_2				With C_2H_2 (0.01%v/v)			
		Day 0	Day 3	Day 7	Day 11	ANOVA	Day 3	Day 7	Day 11	ANOVA
A_0	**Ammonium** (µg N g^{-1} dry soil)	**2.92** ± *1.2*	**2.88** ± *0.5*	**3.59** ± *0.6*	**2.02** ± *0.1*	*ns*	**9.55** ± *0.4*	**14.05** ± *0.5*	**17.29** ± *2.3*	*
A_{50}		**53.42** ± *1.9*	**49.68** ± *1.8*	**44.71** ± *2.8*	**35.98** ± *1.5*	*	**64.26** ± *0.9*	**69.45** ± *0.5*	**69.57** ± *11.7*	*
A_{100}		**102.45** ± *5.9*	**92.54** ± *4.5*	**85.73** ± *4.8*	**81.01** ± *7.9*	*	**102.78** ± *3.8*	**115.31** ± *2.5*	**125.54** ± *2.1*	*
A_{150}		**153.93** ± *1.0*	**151.11** ± *1.9*	**147.05** ± *9.7*	**136.37** ± *10.0*	*	**156.76** ± *2.4*	**164.67** ± *2.3*	**169.92** ± *6.0*	*
ANOVA		*	*	*	*		*	*	*	
A_0	**Nitrate** (µg N g^{-1} dry soil)	**11.50** ± *0.4*	**13.47** ± *0.5*	**12.22** ± *1.1*	**12.10** ± *0.6*	*ns*	**11.40** ± *0.4*	**8.98** ± *0.8*	**7.90** ± *0.6*	*
A_{50}		**11.32** ± *0.6*	**13.67** ± *0.9*	**15.66** ± *0.6*	**16.87** ± *1.2*	*	**10.90** ± *0.7*	**10.53** ± *0.5*	**8.98** ± *0.7*	*
A_{100}		**11.56** ± *0.5*	**13.70** ± *1.3*	**15.58** ± *0.3*	**16.60** ± *0.3*	*	**11.28** ± *1.2*	**10.03** ± *0.4*	**8.59** ± *0.4*	*
A_{150}		**11.62** ± *0.4*	**12.96** ± *0.2*	**15.60** ± *0.5*	**16.42** ± *0.2*	*	**11.01** ± *0.4*	**10.08** ± *0.2*	**8.68** ± *0.3*	*
ANOVA		*ns*	*ns*	*	*		*ns*	*ns*	*ns*	

^{ns}: non-significant difference ; * significant difference among NH_4^+ treatments (column) and sampling time (row) at α =0.05

3.3.1.2 Production of N_2O

During the first three days of incubation, 2.2 to 3.3 ng N_2O–N g^{-1} d^{-1} was emitted from nitrification and denitrification (Table 3.2). In the control, A_0, the daily rate of N_2O emissions decreased over the period 3 to 11 days. The total N_2O varied from 3.3 to 3.8 ng N_2O–N g^{-1} d^{-1} in the soil in treatments A_{50} – A_{150} over this period and these rates of total N_2O production were significantly higher than in A_0 (Table 3.2).

The rate of N_2O from denitrification and/or heterotrophic nitrification (N_2O_{DH}) was unaffected by NH_4^+ supply during the experiment ($p > 0.05$,Table 3.2). The average rate of N_2O_{DH} was 1.3 to 2.2 ng N_2O–N g^{-1} d^{-1}.

Autotrophic nitrification released from 0.6 to 1.1 ng N_2O–N g^{-1} d^{-1} in the first 3 days and there was no significant difference among treatments ($p > 0.05$,Table 3.2). The daily rate of N_2O from autotrophic nitrification (N_2O_A) reduced after 3 days in the A_0 while N_2O_A produced 1.6 – 2.3 ng N_2O–N g^{-1} d^{-1} in soils with A_{50} – A_{150}, although the amounts of N_2O_A did not differ among NH_4^+ treatments (Table 3.2).

3.3.1.3 Autotrophic nitrification rate

Net rates of autotrophic nitrification (N_{nit}) ranged from 0.65 to 0.92 µg NO_3–N g^{-1} d^{-1} soil in the first 3 days of incubation but there was no significant difference among NH_4^+ treatments ($p > 0.05$,Table 3.2). After 3 days, N_{nit} were significantly higher in the treatments with A_{50} – A_{150} than in A_0. From days 7 –11, N_{nit} was limited by low NH_4^+ content in the A_0 but contributed 0.58 – 0.69 µg NO_3–N g^{-1} d^{-1} in the soils with N additions, although there was no difference between among these NH_4^+ additions (Table 3.2).

Table 3.2. The rate of total N_2O, N_2O from denitrification and/or heterotrophic nitrification (N_2O_{DH}), N_2O from autotrophic nitrification (N_2O_A), and autotrophic nitrification (N_{nit}) in the chromosol soil after additions at time 0 of 0 (A_0), 50 (A_{50}), 100 (A_{100}), & 150 µg N g^{-1} soil (A_{150}) as $(NH_4)_2SO_4$. Soil moisture was maintained at 60% of the gravimetric water content at field capacity. Each value is followed by the standard error of the mean (n=4).

	Day 0-3				**Day 3-7**				**Day 7-11**			
	Total N_2O	N_2O_{DH}	N_2O_A	N_{nit}	Total N_2O	N_2O_{DH}	N_2O_A	N_{nit}	Total N_2O	N_2O_{DH}	N_2O_A	N_{nit}
	ng N $g^{-1}d^{-1}$	*ng N $g^{-1}d^{-1}$*	*ng N $g^{-1}d^{-1}$*	*µg N $g^{-1}d^{-1}$*	*ng N $g^{-1}d^{-1}$*	*ng N $g^{-1}d^{-1}$*	*ng N $g^{-1}d^{-1}$*	*µg N $g^{-1}d^{-1}$*	*ng N $g^{-1}d^{-1}$*	*ng N $g^{-1}d^{-1}$*	*ng N $g^{-1}d^{-1}$*	*µg N $g^{-1}d^{-1}$*
A_0	**2.9** ± *0.1*	**1.9** ± *0.1*	**0.9** ± *0.2*	**0.69** ± *0.13*	**1.9** ± *0.1*^{b}	**1.6** ± *0.1*	**0.3** ± *0.1*^{c}	**0.29** ± *0.02*^{b}	**1.5** ± *0.1*^{b}	**1.3** ± *0.2*	**0.3** ± *0.0*^{b}	**0.24** ± *0.14*^{b}
A_{50}	**2.2** ± *0.3*	**1.7** ± *0.2*	**0.6** ± *0.2*	**0.92** ± *0.09*	**3.3** ± *0.2*^{a}	**1.5** ± *0.2*	**2.0** ± *0.1*^{a}	**0.66** ± *0.08*^{a}	**3.5** ± *0.4*^{a}	**1.4** ± *0.1*	**1.9** ± *0.2*^{a}	**0.69** ± *0.01*^{a}
A_{100}	**2.6** ± *0.2*	**1.7** ± *0.6*	**1.1** ± *0.3*	**0.81** ± *0.12*	**3.8** ± *0.1*^{a}	**1.5** ± *0.1*	**2.3** ± *0.3*^{a}	**0.78** ± *0.07*^{a}	**3.3** ± *0.4*^{a}	**1.4** ± *0.1*	**1.8** ± *0.3*^{a}	**0.61** ± *0.06*^{a}
A_{150}	**3.3** ± *0.2*	**2.2** ± *0.1*	**1.1** ± *0.2*	**0.65** ± *0.08*	**3.4**± *0.1*^{a}	**1.8** ± *0.2*	**1.6** ± *0.1*^{a}	**0.89** ± *0.15*^{a}	**3.3** ± *0.8*^{a}	**1.7**± *0.1*	**1.7** ± *0.1*^{a}	**0.58** ± *0.10*^{a}
Sig.	*ns*	*ns*	*ns*	*ns*	*	*ns*	*	*	*	*ns*	*	*
$HSD_{0.05}$	**-**	**-**	**-**	**-**	**0.8**	**-**	**0.7**	**0.35**	**0.9**	**-**	**0.9**	**0.32**

*ns: non-significant difference (p>0.05); *: significant difference (p<0.05). Different letters in each column indicate significant difference among NH_4 treatments.*

3.3.2 Experiment 2: The effect of fluctuating temperatures

3.3.2.1 Soil mineral N

The changes in mineral N under different incubation temperature patterns are presented in Figure 3.3. In samples without C_2H_2 addition, the concentration of NH_4^+ declined with incubation time for all three temperature patterns (Figure 3.3a). In contrast, the contents of NH_4^+ increased slightly in the samples with C_2H_2 addition.

In the samples without C_2H_2, NO_3^- contents increased throughout the experiment, particularly from 7 – 14 days ($p < 0.05$, Figure 3.3b). The temperature patterns affected cumulative NO_3^- during the experiment. Cumulative NO_3^- contents were significantly greater under FTP_2 than those under FTP_1 and CT at all sampling time (Figure 3.3b). In the presence of C_2H_2, NO_3^- decreased in the first 7 days but generally remained stable after days 7 under CT and FTP_1. Under FTP_2, the amount of NO_3^- did not change in first 14 days but slightly reduced in the second 14 days ($p < 0.05$, Figure 3.3).

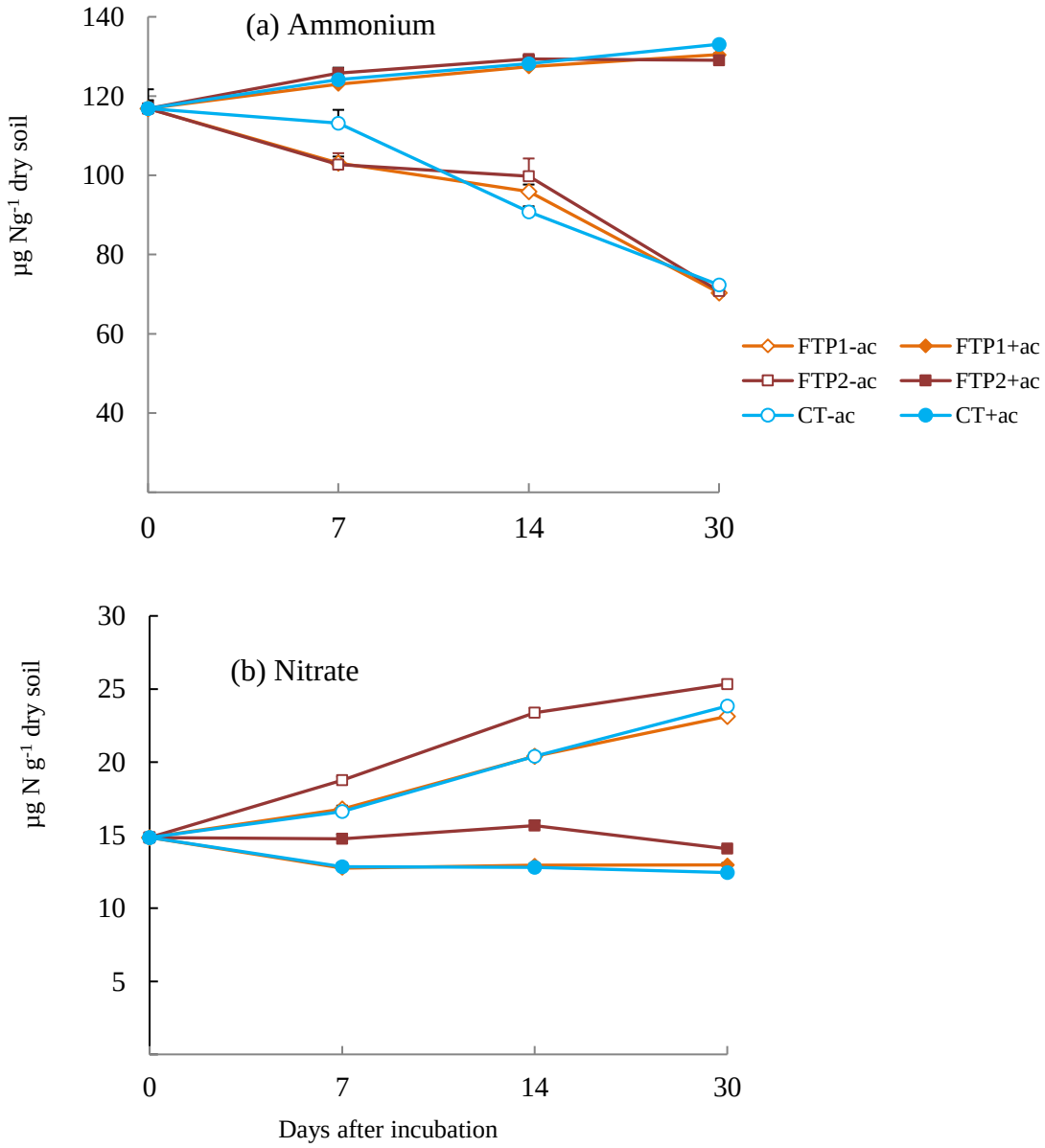

Figure 3.3 Variation in concentrations of soil ammonium and nitrate concentrations over time when subjected to different temperature patterns (FTP_1: 8h -15 °C, 8h- 20 °C, 8h - 25 °; FTP_2: 12h -15 °C and 12h - 25 °C; CT maintained continually at 20 °C) and C_2H_2 pressure (without C_2H_2 -open symbols and with C_2H_2 -filled symbols). Soil moisture was maintained at 60% field capacity. Vertical bars are +1SE.

3.3.2.2 Production of N_2O

In the samples without C_2H_2 amendment, N_2O production was emitted from both denitrification and nitrification, and is referred to here as total N_2O emissions. Total N_2O production occurred slowly over the period 0 – 3 days (Figure 3.4). Cumulative N_2O was 0.35 µg N_2O–N g^{-1} d^{-1} after 3 days of incubation and did not differ among temperature patterns. N_2O was produced faster after 7 days at all temperature patterns. There was no interaction between temperature patterns and sampling time ($p > 0.05$, Figure 3.4). Cumulative N_2O production in the last 14 days was significantly higher under FTP_2 than FTP_1 and CT ($p < 0.05$). The patterns of N_2O in samples with C_2H_2 addition were similar to those without C_2H_2 (Figure 3.4). The production of N_2O in the presence of C_2H_2, which was attributed to denitrification and/or heterotrophic nitrification, were lower than those without C_2H_2 over 30 days at all the temperature patterns.

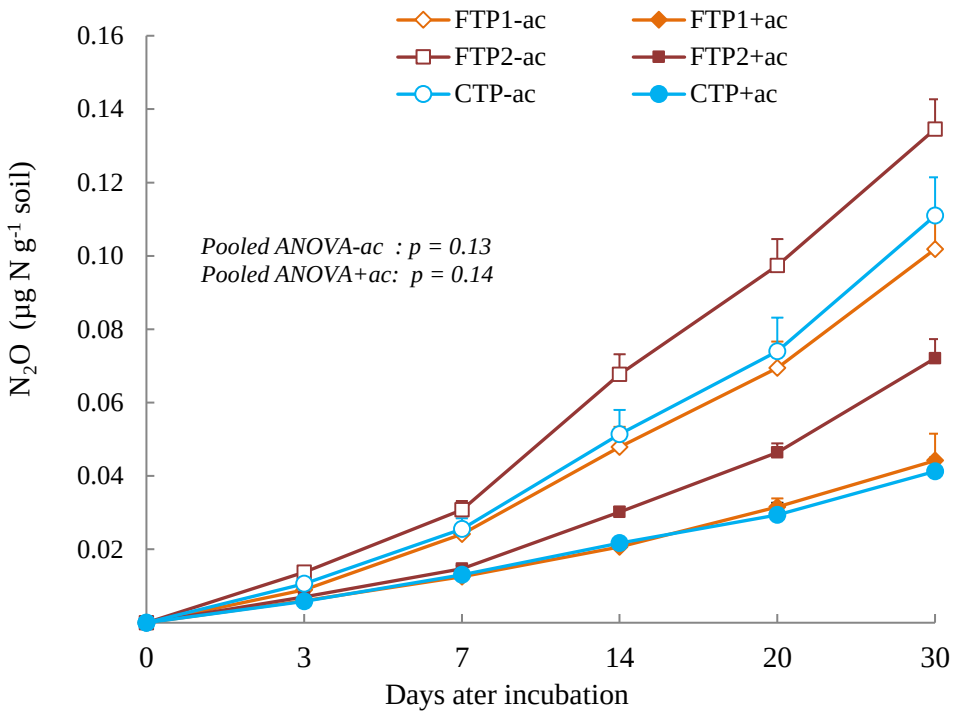

Figure 3.4. Cumulative N_2O production in the chromosol soil under different temperature patterns (FTP_1: 8h -15 °C, 8h- 20 °C, 8h - 25 °; FTP_2: 12h -15 °C and 12h - 25 °C; CT maintained continually at 20 °C) and C_2H_2 treatments (without C_2H_2 - open plots and with C_2H_2 - filled plots). Soil moisture was maintained at 60% field capacity. Vertical bars are +1SE. Pooled ANOVA indicated no interaction between temperature patterns and sapling time ($p > 0.05$).

3.3.2.3 N_2O from autotrophic, heterotrophic nitrification and denitrification

Autotrophic nitrification produced 1.9 to 2.5 ng N_2O–N g^{-1} d^{-1} over 30 days of incubation and there was no significant difference among temperature patterns (Table 3.3). N_2O from denitrification and/or heterotrophic nitrification (N_2O_{DH}) was unaffected by the temperature patterns in the first 14 days of incubation. In the second 14 days, N_2O_{DH} was significantly higher under FTP_2 than CT ($p < 0.05$,Table 3.3) but there were no significant difference between FTP_2 and FTP1. A high variability (se) associated with the average rate of N_2O_{DH} was estimated under FTP_2 in the last two weeks of incubation (Table 3.3).

3.3.2.4 Autotrophic nitrification rate

In first two weeks of incubation, net autotrophic nitrification rate (N_{nit}) varied from 0.5 to 0.6 µg NO_3–N g^{-1} d^{-1} with the temperature patterns but there was no significant difference among these patterns ($p > 0.05$,Table 3.3). N_{nit} slowed over the period 14 – 30 days of incubation, at 0.2 – 0.3 µg NO_3–N g^{-1} d^{-1}, under all the temperature patterns. The FTP did not trigger a higher N_{nit} compared to a CT in the last 14 days of incubation ($p > 0.05$,Table 3.3).

3.3.2.5 Ammonium oxidising bacteria (AOB) population

The AOB population was estimated at 1632 to 1985 cells g^{-1} in the first two weeks of incubation and increased to 1961 to 2335 cells g^{-1} in the last two weeks of incubation (Table 3.3). The numbers of AOB were not affected by the temperature patterns used during the experiment ($p > 0.05$,).

Table 3.3. Total N_2O emissions, N_2O production from different sources (autotrophic and heterotrophic nitrification, denitrification), autotrophic nitrification rate (N_{nit}), ammonium oxidising bacteria (AOB) population and CO_2 production in the chromosol soil after 4 weeks of incubation under different temperature patterns (FTP_1: 8h -15 °C, 8h- 20 °C, 8h - 25 °C; FTP_2: 12h -15 °C and 12h - 25 °C; CT at 20 °C). Soil sample was maintained at 60% of the gravimetric water content at field capacity (- 33 kPa). Each value is followed by the standard error of the mean (n=4).

	From 0-14 days						From 14-30 days					
	Total N_2O	N_2O_{DH}[1]	N_2O_A[2]	N_{nit}	AOB	CO_2	Total N_2O	N_2O_{DH}	N_2O_A	N_{nit}	AOB	CO_2
	ng N g^{-1} d^{-1}	*ng N g^{-1} d^{-1}*	*ng N g^{-1} d^{-1}*	*µg N g^{-1} d^{-1}*	*cells g^{-1}*	*µg C g^{-1} d^{-1}*	*ng N g^{-1} d^{-1}*	*ng N g^{-1} d^{-1}*	*ng N g^{-1} d^{-1}*	*µg N g^{-1} d^{-1}*	*cells g^{-1}*	*µg C g^{-1} d^{-1}*
FTP_1	**3.4** ± *0.2*	**1.5** ± *0.6*	**1.9** ± *0.2*	**0.5** ± *0.02*	**1985** ± *282*	**4.9** ± *0.1*^{b}	**3.4** ± *0.1*^{b}	**1.5** ± *0.1*^{ab}	**1.9** ± *0.1*	**0.2** ± *0.01*	**2335** ± *159*	**7.1** ± *0.1*^{a}
FTP_2	**4.5** ± *0.7*	**2.2** ± *0.2*	**2.3** ± *0.3*	**0.6** ± *0.03*	**1772** ± *461*	**8.5** ± *0.2*^{a}	**4.2** ± *0.6*^{a}	**2.6** ± *0.5*^{a}	**1.6** ± *0.5*	**0.2** ± *0.01*	**2198** ± *203*	**4.9** ± *0.3*^{b}
CT	**3.7** ± *0.2*	**1.6** ± *0.6*	**2.1** ± *0.2*	**0.5** ± *0.02*	**1632** ± *412*	**5.8** ± *0.6*^{b}	**3.7** ± *0.1*^{b}	**1.2** ± *0.4*^{b}	**2.5** ± *0.1*	**0.3** ± *0.01*	**1961** ± *134*	**8.0** ± *0.2*^{a}
ANOVA	*ns*	*ns*	*ns*	*ns*	*ns*	*	*	*	*ns*	*ns*	*ns*	**
$HSD_{0.05}$	**-**	**-**	**-**	**-**	**-**	**1.9**	**0.4**	**1.3**	**-**	**-**	**-**	**0.9**

*ns: non-significant difference (p>0.05); *: significant difference (p<0.05). Different letters in each column indicate significant difference among temperature patterns*

[1] Denitrification and/or heterotrophic nitrification

[2] Autotrotphic nitrification

3.3.2.6 Soil respiration rate

The production of CO_2, a measure of the soil respiration rate, was influenced by the temperature pattern in the soil tested (Table 3.3). The rate of CO_2 was significantly greater under FTP_2 than under FTP_1 and CT in the first two weeks of incubation. In contrast, soil respiration rates under FTP_1 and CT exceeded that under FTP_2 in last two weeks of incubation (Table 3.3).

3.4 Discussion

Our results demonstrate that soil NH_4^+ concentration is vital for the activity of autotrophic nitrification but it does not appear to determine the magnitudes of NO_3^- and N_2O production from autotrophic nitrification. The production of NO_3^- and N_2O from autotrophic nitrification were relatively low in the soil without N addition. This finding supports the previous results by Gödde and Conrad (1999) and Avrahami et al. (2003) that low NH_4^+ (< 5 μg N g^{-1} soil) limits the thermal response of nitrification rate and associated N_2O production. In agreement with Vermoesen et al. (1996), our data show that autotrophic nitrification was unaffected by increased NH_4^+ addition above 50 μg N g^{-1} soil. These results suggest that the activity of autotrophic nitrification is likely to be regulated by other factors such as oxygen availability rather than simply NH_4^+ content. Oxygen availability in the soil microsite is known to be influenced by soil moisture (Smith et al., 2003) and temperature via soil microbial respiration (de Klein and Van Logtestijn, 1996). Once the threshold of NH_4^+ was met for autotrophic nitrification, the production of NO_3^- and N_2O from this biological process were constant with additional soil NH_4^+.

Autotrophic nitrification rate and associated N_2O production were unaffected by the temperature pattern tested throughout the experimental period. This result supports the

finding by Sierra (2002) that NO_3^- production from nitrification occurred consistently under FTPs and a CT of the same mean temperature. The growth of ammonium oxidising bacteria (AOB) was unaffected by FTPs, potentially explaining the consistent rate of autotrophic nitrification and associated N_2O achieved under different temperature patterns. Despite that, ammonium oxidising archaea (AOA) might have been involved in nitrification in agricultural soils (Di et al., 2010). However, the growth and activity of AOA are often limited under high soil mineral N contents (Di et al., 2009; Schauss et al., 2009; Di et al., 2010), thus cumulative NO_3^- in the chromosol with addition of 100 µg N g^{-1} soil was likely attributed to AOB. The same rate of autotrophic nitrification under different temperature patterns indicates that a relatively constant NH_4^+ was biologically nitrified to produce NO_3^-, according to the mean of daily temperature rather than diurnal temperature between 15 to 25 ºC. An FTP with a wider diurnal fluctuation in temperature may potentially influence nitrification rate. Campbell and Biederbeck (1972) showed that shifting temperature between 3 ºC and 14 ºC (12h -12h) had a detrimental effect on the growth of nitrifiers, which resulted in a lower NO_3^- production under FTP than under a CT of 10 ºC. However, air temperatures below 5 ºC were not experienced in the field site where the experimental soil was collected for the current work, so temperature regimes below 5 ºC were not relevant for the present study. With daily temperature variation from 15 ºC to 25 ºC, the production of NO_3^- and N_2O from autotrophic nitrification were primarily determined by the mean of daily temperature. The results suggest that daily constant temperature can be used to assess the thermal response of nitrification.

Our result identifies that the rate of total N_2O emission was significantly higher under FTP_2 than under CT after two weeks of incubation. This finding does not support the result by Goodroad and Keeney (1984), who reported a significant difference in total N_2O production between FTP ranging from 10 ºC to 30 ºC and a CT of 20 ºC over 5 days of

incubation in a silt loam soil. That result, also at a 10 °C temperature differential as tested using FTP_2, suggests that N_2O production may be underestimated by using the CT in the laboratory study rather than a more realistic FTP. Our data indicate that total N_2O production from soil is unlikely to be affected by diurnal temperature variation of up to 10 °C in first 14 days of incubation. It has been explained that the thermal acclimation and/or adaptation of soil microbial organisms only occur after 2 weeks (Campbell and Biederbeck, 1972) or 6 weeks (Avrahami et al., 2003). FTP ranging from 15 ºC to 25 ºC (12h – 12h) impacted on the activity of denitrification and/or heterotrophic nitrification, resulting in higher total N_2O production in last two weeks of incubation. However, this should be treated with caution due to the high variability of emissions observed in FTP_2. Isotope techniques (^{15}N) should be applied to further distinguish N_2O production from heterotrophic nitrification and denitrification, which was not achieved with the use of C_2H_2 (0.01% v/v) in our experiments. Despite this, the magnitude of N_2O and NO_3^- production was lower in the soils with C_2H_2 than those without C_2H_2 addition, reflecting the efficiency of C_2H_2 to inhibit autotrophic nitrification. We conclude that total N_2O emissions from soil are likely to be insensitive to fluctuating temperature in short term experiments using the types of soil examined in these experiments. Daily mean constant temperature, therefore, can be used to assess the thermal response of N_2O production from these soils.

3.5 Conclusions

The findings in the present study demonstrate that autotrophic nitrification was limited in the soil without addition of NH_4^+, resulting in lower NO_3^- and N_2O production than those from the soils amended with NH_4^+. The activity of autotrophic nitrification was not influenced by NH_4^+ amendments once the threshold had met (≥ 50 μg NH_4-N g^{-1} soil).

Fluctuating temperature patterns did not trigger higher rates of autotrophic nitrification, associated N_2O production and AOB population over the 30 days of incubation. Cumulative N_2O production was significantly greater under FTP_2 than under CT in the second 14 days of incubation, due to denitrification and/or heterotrophic nitrification. However, this should be treated with caution due to the high variability of N_2O production associated with FTP_2. The results suggests that there is a requirement to supply ≥ 50 µg NH_4-N g^{-1} soil to avoid the limitation of NH_4^+ when testing nitrification responses to environmental factors such as temperature. The use of daily constant temperature should be suitable for short-term experimentation to assess the thermal responses of N_2O production.

Statement of Authorship

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Publication Details	

Principal Author

Name of Principal Author (Candidate)	Thang Lai
Contribution to the Paper	Conducted experiments, performed analysis on samples interpreted data, wrote manuscript and acted as corresponding author
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date: 10/9/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Matthew Denton
Contribution to the Paper	Supervised development of work; helped in data interpretation and manuscript evaluation.
Signature	Date: 14/9/16

Name of Co-Author	
Contribution to the Paper	
Signature	Date:

Please cut and paste additional co-author panels here as required.

Chapter 4 **Nitrous oxide production from nitrification in soils and its dependence on temperature**[*]

4.1 Introduction

Nitrous oxide (N_2O) is a potent greenhouse gas that contributes to global warming and the depletion of ozone in the stratosphere (IPPC, 2006). The primary N_2O emissions in the atmosphere come from soil, resulting largely from the conversion of ammonium to nitrate during nitrification and the reduction of NO_3^- to N_2 during denitrification (Conrad, 1996). The production of N_2O in soils is influenced by environmental conditions such as soil moisture, organic carbon, N concentration, pH and temperature. Increasing temperature affects soil moisture content, microbial activity, N pools, OC and pH, thus it has been regarded as a key factor regulating N_2O fluxes between soil and the atmosphere. Global warming was estimated at 0.8 °C over the past century and is predicted to increase at an average rate of +0.2 °C per decade in the 21th century (IPCC, 2001; Hansen et al., 2006). In a recent report (Holthaus, 2016), average temperature across the northern hemisphere increased +2 °C in comparison with pre-industrial levels. This could have a large effect on plant and soil systems by enhancing the rates of biological processes. Nitrification and denitrification under global warming scenarios are expected to increase (Davidson and Janssens, 2006), and could impact N_2O production (Smith, 1997; Castaldi, 2000). Therefore, it is important to understand the impact that increased soil temperature will have on the loss of N as N_2O emissions.

The effect of temperature on N_2O production from nitrification in soil has been investigated in many studies, which were typically conducted at temperatures below 30

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°C. The decrease in N_2O production from nitrification was observed with rising incubation temperature between 5 °C to 25 °C (Maag and Vinther, 1996; Avrahami et al., 2003), while a positive correlation between N_2O production and temperature from 15 °C to 30 °C was demonstrated in other studies (Goodroad and Keeney, 1984; Smith, 1997). The impact of high temperatures (above 35°C) on N_2O production is poorly understood. As the temperature increases above 35 °C, it may influence the soil bacterial / archaeal nitrifying communities, and /or change soil microsite conditions such as oxygen and OC availability, which may alter N_2O emissions and the relative contributions that autotrophic and heterotrophic nitrification make to the production of N_2O from nitrification.

Autotrophic nitrification has often been considered to be the predominant source of N_2O production from nitrification but recent studies have reported a significant contribution from heterotrophic micro-organisms to nitrification. Depending on soil properties, autotrophic nitrification accounted for 60 – 90% of N_2O produced from nitrification during the oxidation of NH_4^+ or NO_2^- to NO_3^- (Goreau et al., 1980; Abbasi and Adams, 2000; Wrage et al., 2001). The ability of heterotrophic micro-organisms to nitrify both NH_4^+ and organic-N compounds has been previously observed (Papen et al., 1989; Anderson et al., 1993; De Boer and Kowalchuk, 2001), indicating potential N_2O production from heterotrophic nitrification. For example, in soils incubated at 20 – 22 °C, heterotrophic nitrification accounted for 19% of N_2O from nitrification (Islam et al., 2007) and 20% of overall N_2O production (Bateman and Baggs, 2005). In a recent study, heterotrophic nitrification contributed up to 69% of NO_3^- production at 15 °C, but was reduced to less than 10% as temperature increased to either 25 °C or 35 °C in an acidic soil with 50 – 75% water filled pore space (WFPS) (Liu et al., 2015). However, the thermal response of N_2O production from heterotrophic nitrification has not been

documented. Thus, the relative contributions of autotrophic and heterotrophic nitrification to total N_2O production at high temperatures is the focus of this study.

The aim of this work was therefore to investigate N_2O emissions from nitrification as influenced by increasing temperature from 10 – 45 °C. A ^{15}N labelling technique was applied with acetylene (C_2H_2, 0.01% v/v) inhibition to determine the respective contributions of autotrophic and heterotrophic nitrification processes.

4.2 Materials and methods

4.2.1 Study site and sample collection

The soils used in this study were obtained from south west Victoria, Australia, at the TAFE Glenormiston College Campus, referred to as the 'dermosol', and a commercial dairy farm, referred to as the 'chromosol' (Isbell, 1996). These sites are not irrigated and have an average annual rainfall of 745 mm (chromosol) and 522 mm (dermosol). Low soil temperatures recorded in the top 10 cm of soil (8 – 20 °C) were associated with high soil moisture contents (0.3 – 0.5 mm^3/mm3) between May and November (Figure 4.1). High soil temperatures (20 – 45 °C) were associated with low moisture contents between December and April (0.1 – 0.3 mm^3/mm^3). Some characteristics of the top 10 cm of these soils are provided in Table 4.1. Experimental soil samples were collected from the upper 10 cm layer of a 2 m^2 area within each site, and soil cores were used to measure bulk density (BD) and gravimetric water content at field capacity (FC) at – 33 kPa (**Chapter 3**). The soils then were sieved to remove the fraction > 2mm, and thoroughly mixed to increase uniformity. Sieved soil was stored at field moisture in sealed containers at 4 °C to minimise microbial activity until use.

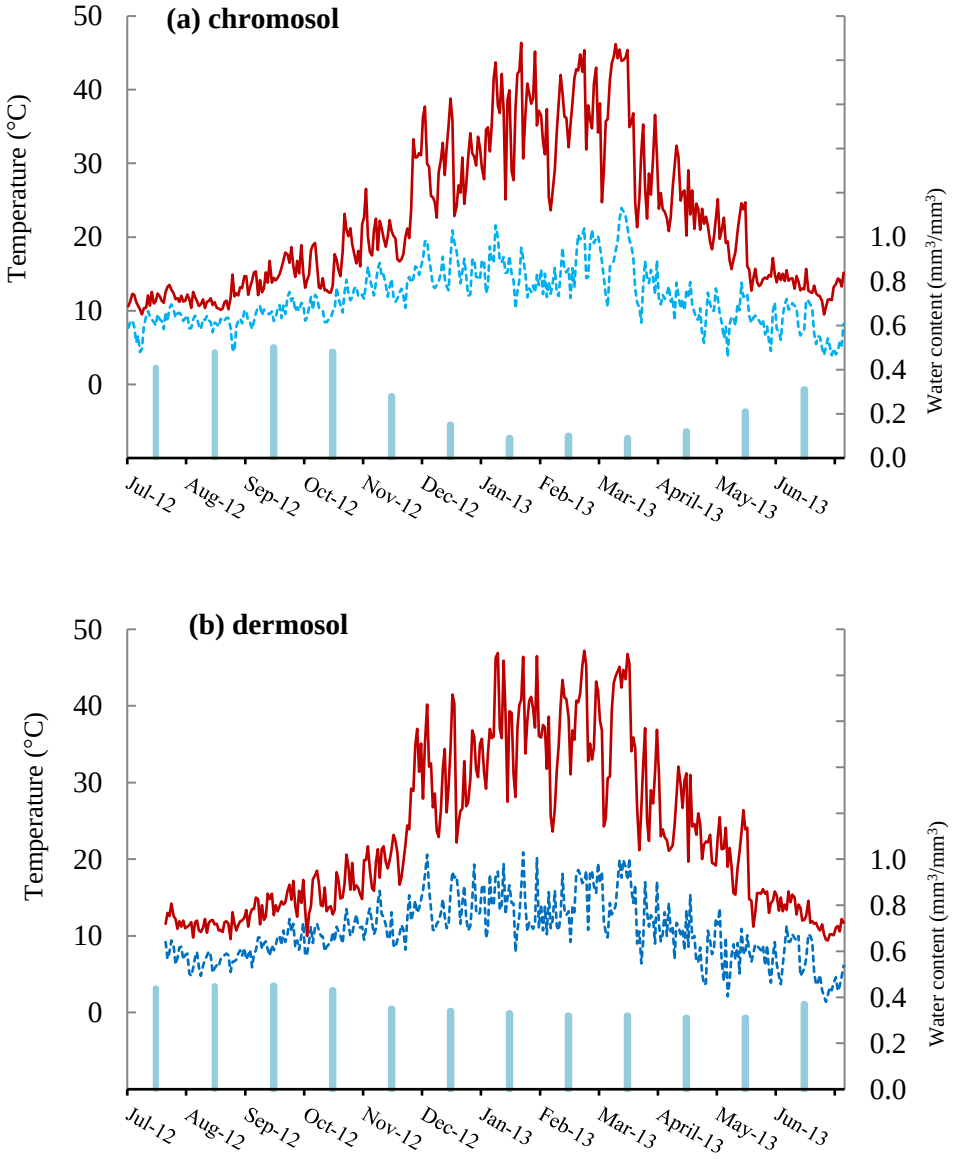

Figure 4.1. The variation in temperature and water content in study sites from June 2012 until July 2013. Lines indicate daily soil maximum (red line) and minimum temperature (blue line); bars present the monthly volumetric water content.

Table 4.1. Some properties of unamended experimental soils used in this study. Soils were sampled from 0-10 cm depth.

	chromosol	**dermosol**
General		
Location	38° 14’ S, 142°55’E	38°10’S, 142°58’E
Land use	Rainfed Dairy pasture	Rainfed Dairy pasture
Soil		
Soil type*	Brown chromosol	Black dermosol
Texture	Light fine sandy-clay loam	Clay loam
Clay (%)	15.7	37.0
Silt (%)	24.4	21.0
Coarse sand (%)	5.9	9.0
Fine sand (%)	52.8	16.0
Gravel (%)	1.2	17
Bulk Density (g/cm^3)	1.02	1.12
Moisture content at $FC_{33\ kPa}$ (g water/g soil)	0.38	0.37
Moisture content at 60%FC (g water/g soil)	0.23	0.22
pH- $CaCl_2$	4.9	5.5
NH_4^+- N (mg N kg^{-1} d.w)	16.5	12.8
NO_3^-- N (mg N kg^{-1} d.w)	6.2	8.5
Organic carbon (%)	4.06	5.24

*Australian Soil Classification (Isbell, 1996)

4.2.2 Laboratory incubations

4.2.2.1 Experiment 1

The objective was to assess N_2O emissions in two soils at temperatures ranging from 10 °C to 45 °C over 5 days. Soil was placed in plastic bags and wetted to achieve 60% FC (at 0.23 g water g^{-1} soil for the chromosol and 0.22 g water g^{-1} soil for the dermosol). All soil samples were pre-incubated at 25 °C for 7 days to recover biological activity after sampling and processing (Davidson, 1991). Soil samples (50 g dry weight) were repacked into cores (42 × 36 mm) to achieve the original bulk density measured at field sites (1.02 and 1.12 g cm^{-3} for the chromosol and dermosol respectively). After the pre-incubation, soil water was maintained at 50% of FC. To avoid the limitation of substrate (NH_4^+) on nitrification (**Chapter 3**), 2 ml of the nitrogen solution [0.09 mM $(NH_4)_2SO_4$] was added to each soil core to provide the final concentration of NH_4^+ at 100 µg N g^{-1} soil. This solution was added slowly onto the top of upright soil cores with duplicate times to promote uniformity of NH_4^+ through the soil and reach a final water content of 60% FC. Each soil core was placed into a 250 ml jar with a gas-tight lid equipped with a rubber septum (Subaseals#25, Sigma-Aldrich, St Louis, MO, USA). The jars were flushed with atmospheric air for 2 minutes and, in half set of the jars, acetylene (C_2H_2) was then added though a 25ml gas-tight syringe (Hamilton Company, Reno, NV, USA) to provide a final partial pressure of 10 Pa (0.01%v/v), to inhibit autotrophic nitrification (Yoshinari et al., 1977; Klemedtsson et al., 1988). After injecting C_2H_2, the syringe was pushed forward and backward a few times to aide mixing and increase diffusion of the C_2H_2 into the soil. Jars were incubated at constant temperatures of 10, 20, 25, 30, 35, 40 or 45 °C for 5 days using four replicates.

4.2.2.2 Experiment 2

The objective was to assess the production of N_2O from autotrophic and heterotrophic nitrification as influenced by temperatures between 35 °C and 45 °C, where an asymptotic maximum of N_2O production from nitrification likely occurs (based on data of the experiment 1). A ^{15}N labelled technique was coupled with C_2H_2 to determine the pathways of N_2O from denitrification, autotrophic nitrification and heterotrophic nitrification, as described by Bateman and Baggs (2005). Briefly, $^{15}N\text{-}N_2O$ derived from the $^{15}NH_4{}^{15}NO_3$ amendment is attributed to denitrification and nitrification (N_2O_{DN}). The production of $^{15}N\text{-}N_2O$ emitted from $^{15}NH_4{}^{15}NO_3$ with C_2H_2 addition (0.01% v/v) is attributed to denitrification and heterotrophic nitrification (N_2O_{DH}). $^{15}N\text{-}N_2O$ production from the $^{14}NH_4{}^{15}NO_3$ amendment is attributed to denitrification (N_2O_D). Therefore, the difference in $^{15}N\text{-}N_2O$ between $^{15}NH_4{}^{15}NO_3$ and $^{15}NH_4{}^{15}NO_3$ plus C_2H_2 (0.01% v/v) amendments is attributed to autotrophic nitrification (N_2O_A). Likewise, the difference in $^{15}N\text{-}N_2O$ between $^{14}NH_4{}^{15}NO_3$ and $^{15}NH_4{}^{15}NO_3$ + C_2H_2 (0.01% v/v) amendments is attributed to heterotrophic nitrification (N_2O_H) (Table 4.2).

Table 4.2. Outline of the ^{15}N fertiliser and C_2H_2 inhibition treatments used to estimate the contribution of denitrification, autotrophic nitrification and heterotrophic nitrification to $^{15}N\text{-}N_2O$ production (Baggs et al., 2003; Bateman and Baggs, 2005).

Treatment	**Source of $^{15}N\text{-}NO_2$**
(a) $^{14}NH_4{}^{15}NO_3$	Denitrification (N_2O_D)
(b) $^{15}NH_4{}^{15}NO_3$	Denitrification and nitrification (N_2O_{DN})
(c) $^{15}NH_4{}^{15}NO_3$ + C_2H_2 (0.01% v/v)	Denitrification and heterotrophic nitrification (N_2O_{DH})
(c) minus (a)	Heterotrophic nitrification ($N_2O_{H)}$
(b) minus (c)	Autotrophic nitrification (N_2O_A)

Two sets of incubations were used in this experiment, one to monitor gas sampling and the other to monitor soil mineral nitrogen concentrations. The first set of incubations included 36 jars (three temperatures × three labelled ^{15}N + C_2H_2 amendments × four replicates) which were used for gas sampling during the experiment. In this set, three different amendments with ^{15}N labelled NH_4NO_3 (100 µg N g^{-1} soil, 10 atom% excess ^{15}N) and C_2H_2 were applied: $^{14}NH_4^{15}NO_3$, $^{15}NH_4^{15}NO_3$ and $^{15}NH_4^{15}NO_3$ + C_2H_2 (0.01 % v/v) (Table 4.2). For the samples with C_2H_2, the jars were flushed with air for two minutes and C_2H_2 was then injected though a syringe (50 mL, PTFE, SGE, Australia) to provide a final partial C_2H_2 pressure of 10 Pa. All jars were incubated at 35, 40 or 45 °C in the dark for 10 days. Soil moisture was maintained at 60% FC on a weight for weight basis after each sampling time. The soil used in this experiment was the dermosol (pH 5.4) to minimise the production of N_2O from nitrifier denitrification (where the oxidation of NH_4^+ to NO_2^- is followed by the denitrification of NO_2^- to N_2O), as was previously observed in our early study (**Chapter 2**), and is often active under acidic soils (Wrage et al., 2001). The ^{15}N labelled technique applied in the present study was not able to distinguish N_2O production from nitrifer-denitrification (Wrage et al., 2005).

The second set of incubations were included for mineral N measurement in soil treated with the above ^{15}N labelled NH_4NO_3 source ($^{14}NH_4^{15}NO_3$). This set included 48 jars (three temperatures × four replicates × four sampling times) for subsoil sampling on days 0, 3, 7 and 10 after N addition for measurement of NH_4^+ and NO_3^- contents.

4.2.3 Gas sampling and analysis

A 20 ml gas sample was taken from the headspace of each jar using a Hamilton 50 ml gas-tight syringe (Hamilton Company, Reno, NV, USA) on days 0, 3, 5 and stored in a pre-evacuated 12 ml glass exetainer (Labco, Ceredigion, UK) for analysis of N_2O and CO_2 production in the first experiment. In the second experiment, the headspace of each

jar was sampled twice on days 0, 3, 7, 10 after incubation: one for analysis of total N_2O, $^{15}N\text{–}N_2O$ and $^{15}N\text{–}N_2$, and the other for measurement of CO_2 production. After collecting gas samples, all jars were aerated with the atmosphere by a mini fan for two minutes and C_2H_2 was injected back into appropriate jars to maintain C_2H_2 pressure. Gas samples were sent to The Stable Isotope Facility (UCDAVIS, California, USA) for gas analyses. The methods for measurement of N_2O and CO_2 production were presented at section **3.2** in **Chapter 3**.

Trace gas isotope ratios ($\delta^{15}/\delta^{14}N$) were determined using a ThermoFinnigan GasBench + PreCon trace gas concentration system interfaced to a Delta V^{TM} Isotope-ratio Mass Spectrometer (Thermo-Scientific, Bremen, Germany).

4.2.4 Soil mineral N and pH measurement

A 10 g sample of moist soil was taken from the second incubation set on days 0, 4, 7 and 10 after incubation. Soil NH_4^+ and NO_3^- were extracted from 10 g of moist soil by shaking for one hour in 50 ml of 2M KCl. The analyses of mineral N were performed colorimetrically with an AutoAnalyser (AA3 HR, SEAL, West Midlands, UK) using the hydrazine reduction method for NO_3^- (Kamphake et al., 1967) and the nitroprusside method for NH_4^+ (Kaplan, 1965).

For pH measurement, 5.0 g of dry soil sample was mixed with 25 ml of calcium chloride ($CaCl_2$) (0.01M), shaken for one hour. After allowing sediment to settle for 30 minutes, pH in the supernatant was measured using an electrode. The measurements of pH were done on 4 replicates at the beginning and the end of incubation in each treatment to evaluate if incubation temperature impacted on soil pH.

4.2.5 Most probable number count

The numbers of ammonium oxidising bacteria (AOB) in the different temperature treatments were estimated from soil samples without the addition of C_2H_2, at the beginning and end of the experiment. The most probable number (MPN) count described by Schmidt and Belser (1994) was applied to estimate AOB populations. Briefly, 2.0 g of dry soil was mixed with 18 ml of 1 mM sterile phosphate buffer to make the initial solution (10^{-1}). Serial dilutions of soil (from 10^{-2} to 10^{-6}) were made with 1mM sterile phosphate buffer. One ml of each dilution was inoculated into 4 ml of MPN media tubes containing the 1 mM ammonium solution and each inoculation (10^{-1} to 10^{-6}) was replicated five times. Inoculated MPN tubes were incubated at 28 °C in the dark. At intervals of one week for a maximum of 12 weeks, each tube was tested for NO_3 production by observing colour change from blue green to yellow due to pH drop, which indicated acid production during the nitrification process. A Griess–Ilosvay colorimetric spot test and a Merckoquant strip test were also carried out to confirm the nitrification process in the positive MPN tubes (Schmidt and Belser, 1994). The AOB population was estimated using population estimates following Woomer (1994).

4.2.6 Statistical analysis

The pooled ANOVA for measurement over time (Gomez and Gomez, 1984) was applied to determine the rate of change over time of N_2O production and mineral N concentrations. One-way ANOVA was used to determine the significant difference between temperature treatments at the end of experiment. Tukey's Honestly Significant Difference ($HSD_{0.05}$) test was applied *post hoc* to identify a specific difference among treatments. Statistical analysis of percentage values such as $\%N_2O$ from different sources was based on Arcsine transformed data, as described by Ireland (2010). All statistical analyses were performed on Statistix 10.0 (Tallahassee, US).

4.3 Results

4.3.1 Experiment 1: N_2O emissions in response to temperature

Total N_2O production in the samples without C_2H_2 addition occurred slowly at 10 – 20 °C but increased at temperatures above 25 °C in the two tested soils (Figure 4.2). The total production of N_2O was greatest at 40 °C in the chromosol and between 30 °C to 40 °C in the dermosol, and N_2O production declined at 45 °C in the two soils (Figure 4.2).

The production of N_2O in the samples with C_2H_2 addition, which was attributed to denitrification and/or heterotrophic nitrification, was generally consistent across temperatures in the chromosol soil. N_2O production from denitrification and/or heterotrophic nitrification increased within the temperature range 10 – 30 °C and decreased above 35 °C in the dermosol (Figure 4.2). The rates of N_2O production from autotrophic nitrification (indicated by the difference between the treatments with and without C_2H_2) increased with rising temperature and peaked at 40 °C then decreased with further increases in temperature in both soils tested (Figure 4.2).

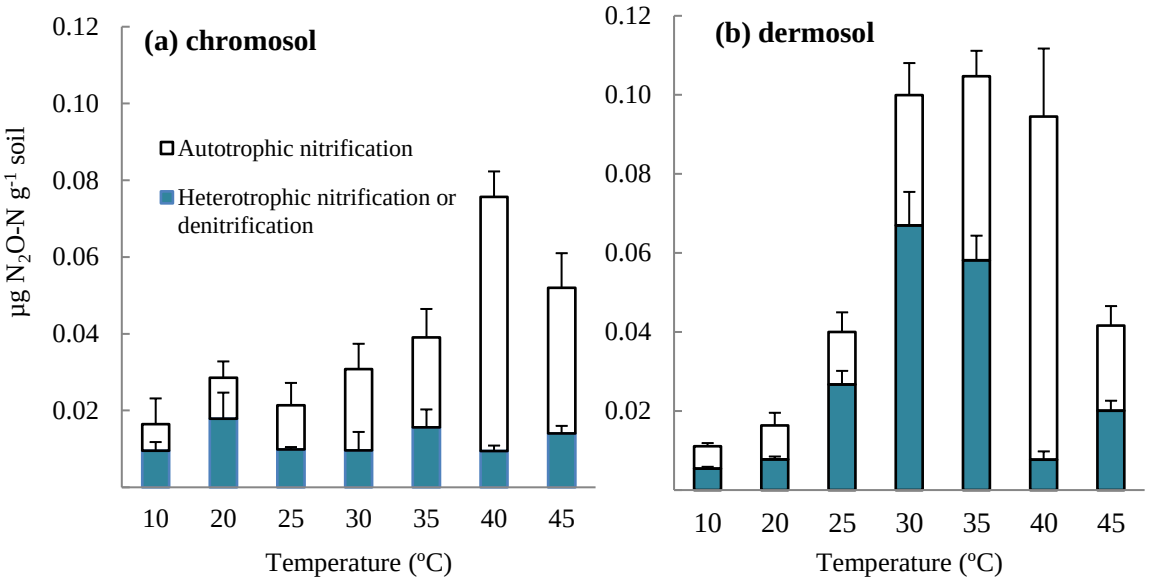

Figure 4.2. The production of N_2O from autotrophic nitrification and heterotrophic nitrification or denitrification in soils incubated at different temperatures for 5 days after application of 100 µg NH_4-N g^{-1} soil. Total bar height represents total N_2O production (all processes). Grey bar represents N_2O arising from heterotrophic nitrification and/or denitrification in C_2H_2-amended treatments. The size of the open bar represents ^{15}N-N_2O from autotrophic nitrification determined from the difference in N_2O from C_2H_2 and non-C_2H_2 treatments. *Vertical bars* are +1 SE of four replicates.

4.3.2 Experiment 2: Effect of high temperature

4.3.2.1 Production of N_2O

The production of N_2O in the absence of C_2H_2 ranged from 0.06 to 0.18 μg N_2O–N g^{-1} with incubation temperatures over the period 0 – 3 days (Figure 4.3a). The average of N_2O production was significantly greater at 40 °C than at 35 °C and 45 °C over this period ($p < 0.05$). N_2O production increased rapidly at 35 °C and 40 °C from days 3 to 10, while N_2O production was emitted at a lower rate with increasing temperature to 45 °C. A similar pattern of temperature response of N_2O production was found in the presence of C_2H_2 (Figure 4.3b) and the magnitudes of N_2O production were lower than those without C_2H_2 addition. Cumulative N_2O production at 35 °C and 40 °C was much greater than at 45 °C over the 10 days, regardless of C_2H_2 treatment (Figure 4.3).

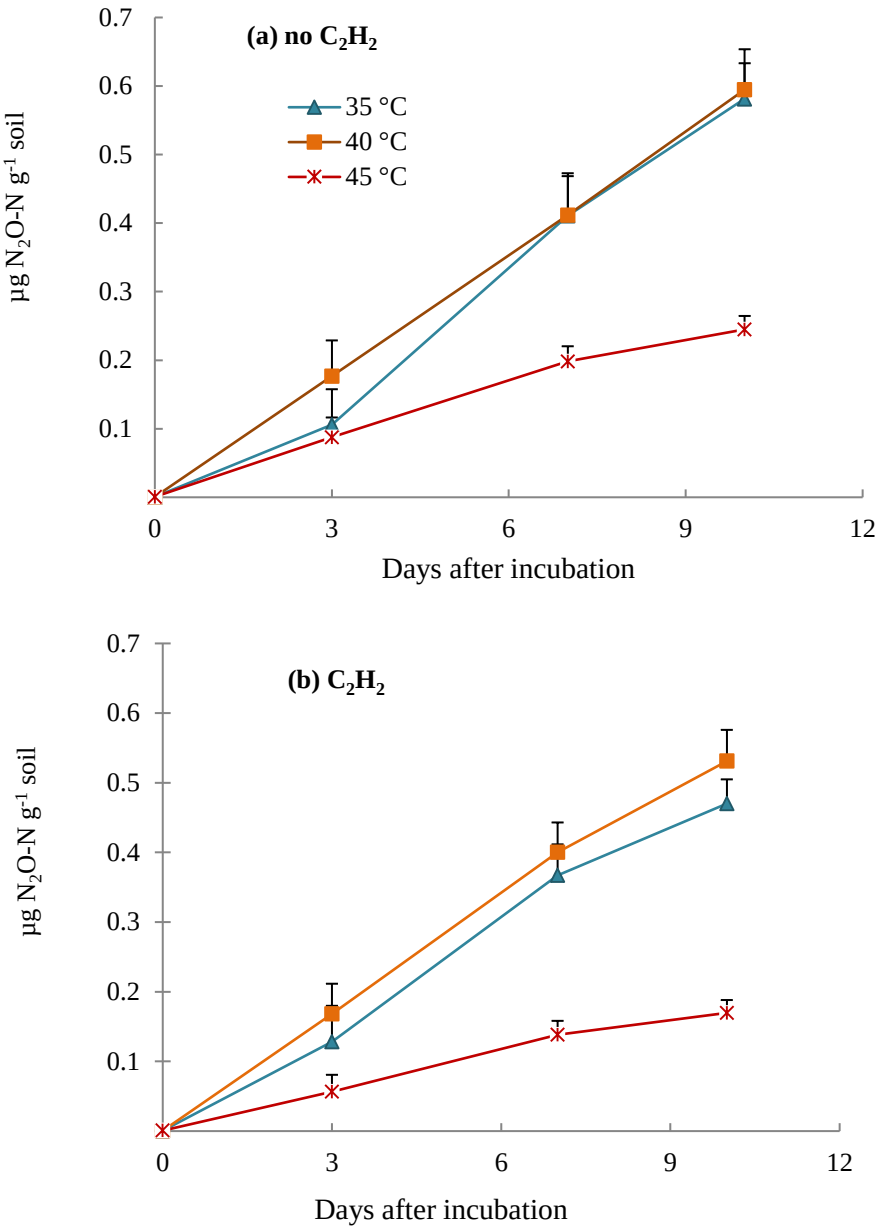

Figure 4.3. The response of N_2O emissions to different temperatures and C_2H_2 pressures over the 10 days of incubation. Soil samples were amended with NH_4NO_3 and maintained at 60% FC. *Vertical bars* are +1 SE of four replicates.

4.3.2.2 Production of $^{15}N_2O$

The amounts of $^{15}N\text{-}N_2O$ production from samples amended with $^{15}NH_4{}^{15}NO_3$ were attributed to nitrification and denitrification processes. The production of $^{15}N\text{-}N_2O$ from the soil over the 10 days of incubation differed between 35 °C to 45 °C (Table 4.2). The daily rates of $^{15}N\text{-}N_2O$ production for the first three days of incubation varied from 0.9 – 2.2 ng $^{15}N\text{-}N_2O\text{-}N$ g^{-1} d^{-1} but there was no difference among incubation temperatures (Table 4.3). From days 3 – 7, the highest rate of $^{15}N\text{-}N_2O$ production was measured at 35 °C, followed by 40 °C and the lowest rate of was emitted at 45 °C ($p < 0.001$, Table 4.3). After day 7, the daily rates of $^{15}N\text{-}N_2O$ production were 2.2 – 2.4 ng $^{15}N\text{-}N_2O\text{-}N$ g^{-1} d^{-1} between 35 °C to 40 °C and 0.3 ng $^{15}N\text{-}N_2O\text{-}N$ at 45 °C (Table 4.3).

4.3.2.3 The production of $^{15}N_2O$ from autotrophic and heterotrophic nitrification

The differences observed in $^{15}N\text{-}N_2O$ between $^{14}NH_4{}^{15}NO_3$ and $^{15}NH_4{}^{15}NO_3$ + C_2H_2 (0.01% v/v) (Figure 4.4) indicate that heterotrophic microorganisms nitrified $^{15}NH_4$ and released $^{15}N_2O$, since autotrophic nitrifiers were inhibited by C_2H_2.

The production of N_2O from nitrification at 35 – 40 °C was 2 – 3 fold that at 45 °C (Table 4.3). N_2O production from autotrophic and heterotrophic nitrification responded dissimilarly to temperatures between 35 °C to 40 °C. Increased temperature from 35 to 40 °C reduced the production of N_2O from autotrophic nitrification but did not affect N_2O release from heterotrophic nitrification. Cumulative N_2O from heterotrophic nitrification at 40 °C was similarly high as at 35 °C. At 45 °C, there was a rapid decrease in N_2O from heterotrophic nitrification (Table 4.3).

Nitrification accounted for 54 – 68% of total N_2O production at 35 – 45 °C over the 10 days (Table 4.3). The percentage of N_2O production from heterotrophic nitrification increased from 31% to 46% as temperature increased from 35 °C to 40 °C, while

autotrophic nitrification declined from 25% to 8% of total N_2O production. The contribution of autotrophic and heterotrophic nitrification to N_2O emissions were 33 – 35% at 45 °C (Table 4.3).

4.3.2.4 AOB population

The size of the population of ammonium oxidising bacteria (AOB) was affected by incubation temperature. Although AOB population in tested soil at 45 °C did not change after 10 days of incubation in comparison with the initial AOB abundance, the populations of AOB increased at 35 °C and 40 °C ($p < 0.05$, Table 4.3).

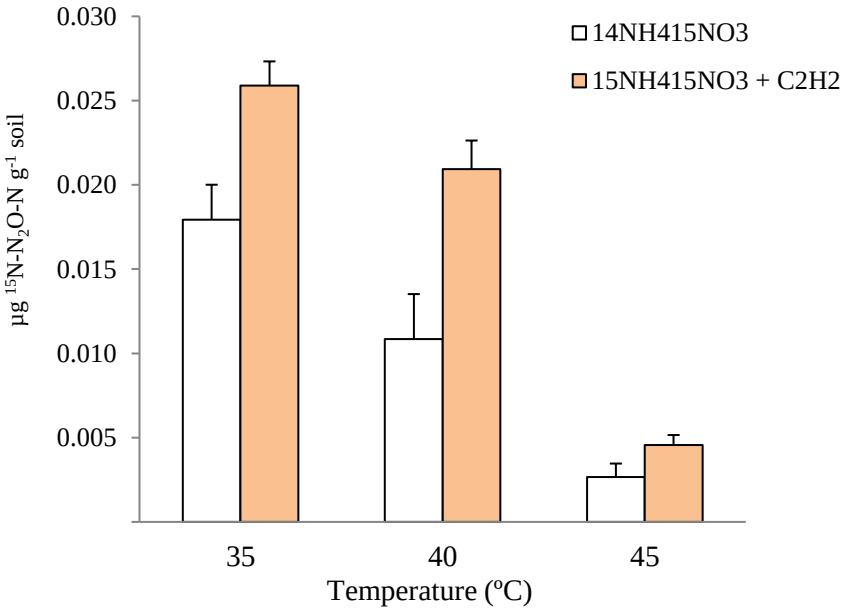

Figure 4.4. Production of ^{15}N-N_2O emissions in soil incubated at different temperatures for 10 days after application of NH_4NO_3. Open bars represent ^{15}N-N_2O from denitrification, grey bars represent ^{15}N-N_2O from denitrification and heterotrophic nitrification (since autotrophic nitrification was blocked with C_2H_2). *Vertical bars* are +1 SE of four replicates.

Table 4.3. Daily rate of ^{15}N-N_2O emissions, cumulative ^{15}N-N_2O from different sources and ammonium oxidising bacteria (AOB) population after 10 days of the incubation in the dermosol. Soil was applied 100µg N g^{-1} as $^{14}NH_4{}^{15}NO_3$ or $^{15}NH_4{}^{15}NO_3$ and maintained at 60% FC. Each value has a standard error of the mean indicated under it (n=4) .

	Temperature (ºC)			$HSD_{0.05}$
	35	**40**	**45**	
Daily rate of N_2O emission *(ng $^{15}N_2O$-N g^{-1} d^{-1})*				
0-3	**1.99**	**2.17**	**0.90**	*ns*
	± 0.9	*± 0.7*	*± 0.3*	
3-7	**3.82** *a*	**2.29** *b*	**0.77** *c*	**0.76**
	± 0.3	*± 0.02*	*± 0.09*	
7-10	**2.46** *a*	**2.16** *a*	**0.31** *b*	**0.43**
	± 0.20	*± 0.05*	*± 0.03*	
Cumulative N_2O emissions over the 10 days *(ng $^{15}N_2O$-N g^{-1})*				
Overall	**28.62** *a*	**22.14** *b*	**6.68** *c*	**2.1**
	± 1.8	*± 2.2*	*± 0.6*	
Denitrification (N_2O_D)	**12.73** *a*	**10.35** *a*	**2.16** *b*	**8.0**
	*± 2.1(44)**	*± 2.7(47)*	*± 0.8 (32)*	
Nitrification (N_2O_N)	**15.90** *a*	**11.80** *a*	**4.58** *b*	**6.22**
	± 3.5(56)	*± 1.1(53)*	*± 0.5 (68)*	
Autotrophic nitrification (N_2O_A)	**7.05** *a*	**1.68** ***b***	**2.35** *b*	**4.05**
	± 2.5 (25)	*± 0.8 (8)*	*± 0.7 (35)*	
Heterotrophic nitrification (N_2O_H)	**8.89** *a*	**10.09** *a*	**2.23** *b*	**5.13**
	± 1.6 (31)	*± 1.0 (45)*	*± 0.8 (33)*	
AOB** *(cell g^{-1}dry soil)*	**220545** *a*	**7998** *b*	**718** *c*	**3617**
	± 0	*± 1264*	*± 104*	

*Contributions of autotrophic and heterotrophic nitrification to total $^{15}N_2O$ are given in parentheses (%). Different letters indicate significant differences in total ^{15}N-N_2O-N among temperatures.

**: The initial AOB size was 688 ± 138 cells g^{-1} dry soil. The counts of AOB population at different temperatures were estimated from samples without C_2H_2 amendment after 10 days of incubation.

4.3.2.5 Soil mineral nitrogen and pH

Changes in soil mineral N during the incubations indicated that little change in NH_4^+ at 35 °C and at 40 °C, and an increase in NH_4^+ at 45 °C during the experiment (Figure 4.5). From days 0 – 7, soil NO_3^- concentration increased from 21 to 28 and 32 µg NO_3–N g^{-1} dry soil at 35 °C and 40 °C, respectively (Figure 4.5). The concentrations of NO_3^- at these temperatures continuously increased over the period 7 – 10 days and reached 33 – 35 µg NO_3 –N g^{-1} soil at the end of experiment (Figure 4.5). The soil NO_3^- concentration at 45 °C did not change in the first 7 days of incubation but decreased in the last 3 days of the experiment (Figure 4.5).

At the beginning of the experiment, soil pH_{CaCl_2} was measured at 5.45 (± 0.005). At day 10, the pH was 5.50 (± 0.009) in the treatment at 45 ºC. The pH decreased in soil samples incubated at 35 or 40 ºC. The largest decrease in the pH was observed in the treatments at 35 ºC, which had a mean value of 5.29 (± 0.003) at the end of the experiment. The differences in pH change could be attributed to the difference in the rate of nitrification process ((Bolan and Hedley, 2003).

4.3.2.6 Soil respiration rate

The production of CO_2, a measure of the soil respiration, was affected by incubation temperatures in both soils in the present study (Figure 4.6). Soil respiration rates were significantly lower at 10 – 25 °C than at the range of 30 – 45 °C over the 5 days of incubation in both tested soils ($p < 0.05$, Figure 4.6). Cumulative CO_2 production in the dermosol over the 10 days in the second experiment was significantly less at 35 °C than at 40 °C and 45 °C ($p < 0.05$, Figure 4.6).

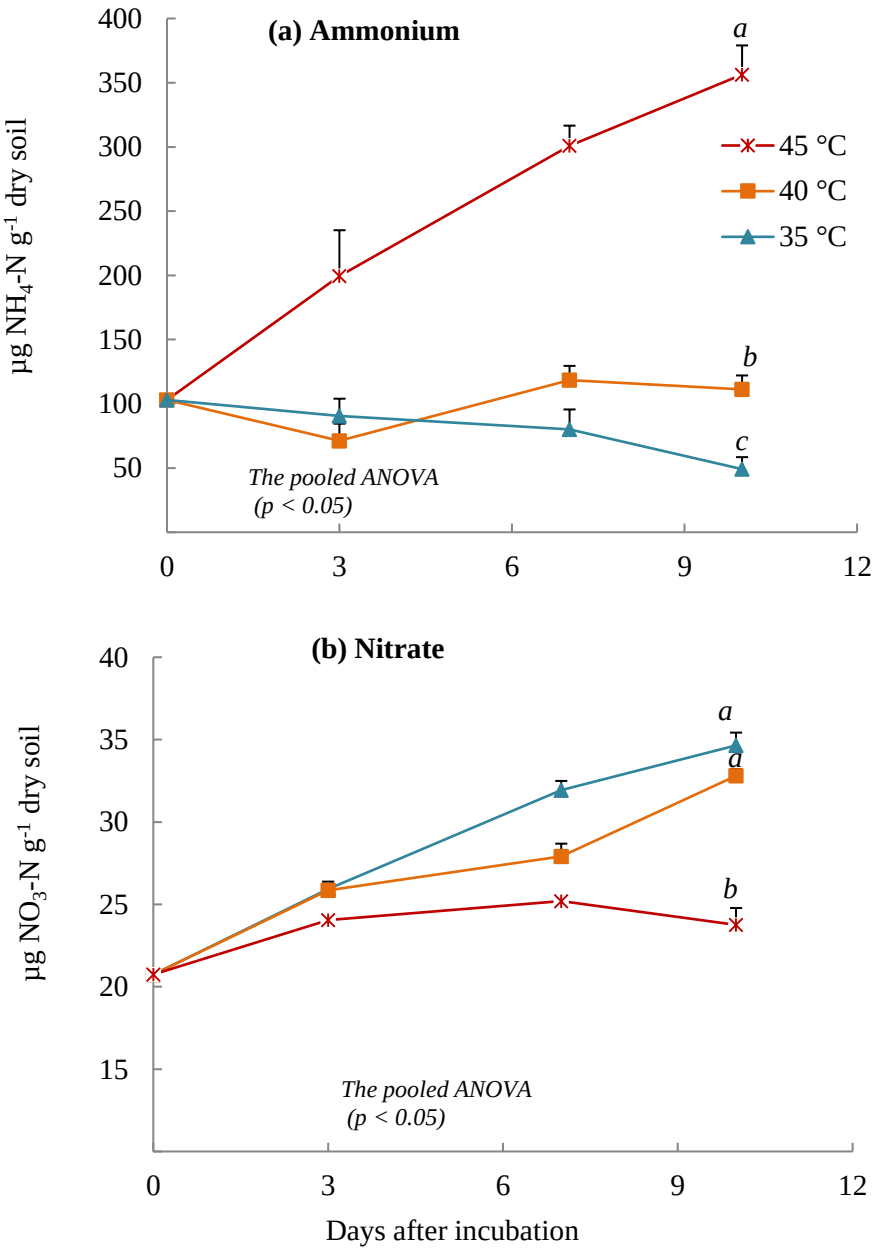

Figure 4.5. The concentrations of ammonium and nitrate in the dermosol soil exposed at different temperatures after application of 100 µg N g^{-1} as NH_4NO_3 to soil on day 0. Soil samples were maintained at 60% FC during the incubation time. The pooled ANOVA was applied to determine the different rates of mineral N change over incubation time. Different letters indicate significant difference among temperatures. *Vertical bars* are +1 SE of four replicates.

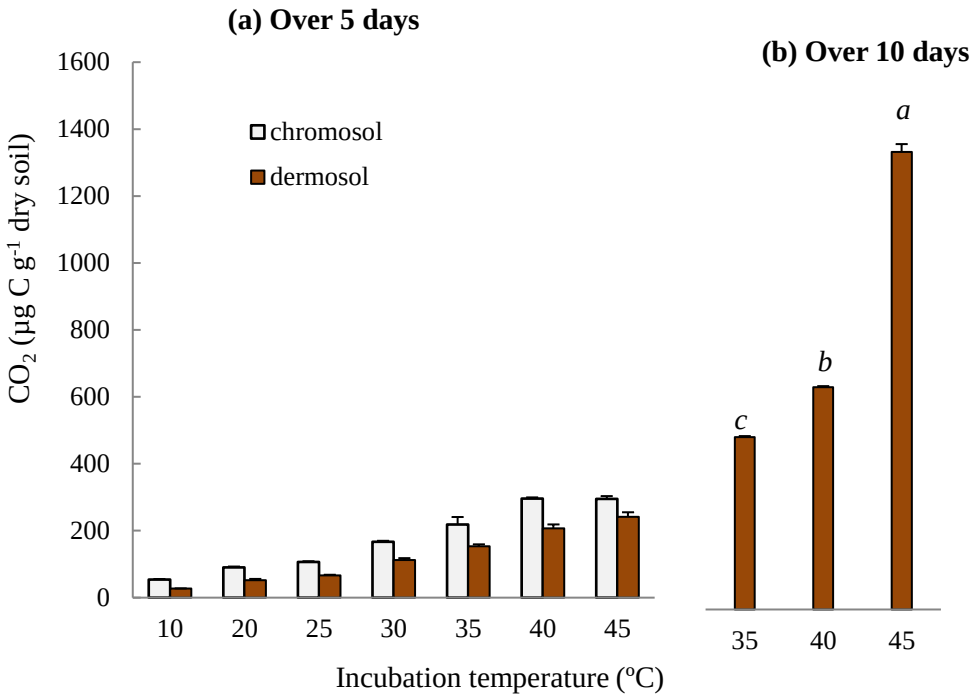

Figure 4.6. The production of CO_2 in response to different incubation temperatures. Soil moisture was maintained at 60% field capacity (33kPa). Different letters indicate significant differences in CO_2 concentration among temperature treatments for the dermosol ($p < 0.05$). *Vertical bars* are +1 SE of four replicates.

4.4 Discussion

Our results demonstrated that N_2O emission from nitrification increased between 10 °C and 40 °C in two soils tested and accounted for more than 50% of total N_2O emissions in the dermosol. This suggests an important role of nitrification as the pathway of N_2O in dry conditions (~ 0.25 g water cm^{-3}) and supports the assumption that high nitrification activity likely occurs at temperatures between 35 °C and 40 °C in soils during summer time. In previous studies, the highest nitrification rate (NO_3^- production) was observed at 35 °C in tropical soils (Myers, 1975; Liu et al., 2015) while in another study, which did not specifically measure heterotrophic nitrification, N_2O production from nitrification increased with rising temperature from 13 °C to 35 °C (Gödde and Conrad, 1999). In our study, N_2O production from nitrification (both autotrophic and heterotrophic nitrification) peaked between 35 °C and 40 °C, then declined at 45 °C. This suggests that an optimum function may describe the relationship between temperature and N_2O production from nitrification, such as is used in the DAYCENT model (Parton et al., 2001). The optimal temperature for N_2O production appeared to be similar in the two tested soils, reflecting that the activity of nitrification relative to N_2O production may be largely affected by climate rather than soil type for samples arising from similar climatic environments. In such a case, the pattern of N loss as N_2O via nitrification may be principally controlled by temperature although soil moisture and oxygen concentration may affect the magnitude of N_2O production from nitrification (Khalil et al., 2004; Bateman and Baggs, 2005).

Heterotrophic nitrification produced more N_2O production than autotrophic nitrification at temperatures between 35 °C to 40 °C. Autotrophic nitrification has been considered to be more important than heterotrophic nitrification, as the pathways of N_2O production in soil (Goreau et al., 1980; Inubushi et al., 1996; Abbasi and Adams, 2000; Wrage et al.,

2001). However, we found that autotrophic nitrification only contributed 25% of N_2O at 35 °C and reduced to 8% of total N_2O emissions at 40 °C. This reduction was potentially linked to the size of AOB population, which was lower at temperatures above 35 °C. Interestingly, heterotrophic nitrification produced the predominant N_2O emission at 40 °C and therefore supports the recent findings (Bateman and Baggs, 2005; Islam et al., 2007) that heterotrophic nitrification contributes a significant source of N_2O production. The relatively high contribution of heterotrophic nitrification to total N_2O production at 35 – 40 °C, however, contrasts with the finding by Liu et al. (2015) that heterotrophic nitrifiers decreased their activity, according to NO_3^- production, with increased temperature from 15 °C to 35 °C. Higher N_2O production from heterotrophic nitrification with increasing temperature from 30 °C to 40 °C suggests an increasing role of heterotrophic nitrification in total N_2O emissions from agricultural soils. Studies that applied C_2H_2 to all treatments would underestimate N_2O from nitrification, by neglecting N_2O from heterotrophic nitrification (Knowles, 1990; Bateman and Baggs, 2005). This N_2O produced would be mistakenly attributed to denitrification even though our study showed that heterotrophic nitrification was one of the dominant processes of N_2O emissions at the temperature range of 35 to 40 °C.

There has been some controversy in the past about the substrates for heterotrophic nitrification. A general concept is that NH_4^+ is not considered as important as organic-N compounds for the activity of heterotrophic nitrifiers (Schimel et al., 1984; Islam et al., 2007). Our result indicated that heterotrophic nitrification used NH_4^+ as a prime substrate for nitrification, particularly at 35 °C to 40 °C, as evidenced by high $^{15}N_2O$ production in the presence of C_2H_2. It was suggested by Bateman and Baggs (2005) that N_2O emitted by heterotrophic nitrification from organic N is negligible if the soil has sufficient inorganic N. Thus, the addition of 100 μg N g^{-1} soil in the present study may influence

the substrate selection of heterotrophic nitrifiers by oxidising the NH_4^+ supply, in preference to organic N. This is similar to the results of Liu et al. (2015), who demonstrated that NH_4^+ was predominantly converted to NO_3^- by heterotrophic nitrification in soil with 100 µg NH_4^+ added. Another possible effect on the oxidation of NH_4^+ by heterotrophic nitrifiers may relate to oxygen (O_2) concentration in the soil microsites as an indirect impact of temperature. High respiration rates at temperatures above 30 °C resulted in low O_2 concentrations due to the consumption of O_2 by soil microbial respiration (de Klein and Van Logtestijn, 1996). As O_2 concentration declines in soil microsites, N_2O production from heterotrophic nitrification tends to increase (Anderson et al., 1993; Zhu et al., 2013). High rates of N_2O from heterotrophic nitrification at 35 °C to 40 °C in the present study suggested the large proportion of NH_4^+ subject to heterotrophic nitrification at these temperatures. Increasing the temperature to 45 °C reduced N_2O production both from autotrophic and heterotrophic nitrification, contributing to maintain a higher NH_4^+ availability than at lower temperatures. Soil NH_4^+ availability is primarily determined by other microbial processes such as N mineralisation and immobilisation. Net N mineralisation rate, a difference between gross N mineralisation and immobilisation (Schmidt et al., 1999), has been reported to increase rapidly with rising temperature from 25 °C to 40 °C (Zaman and Chang, 2004) and to 50 °C (Sierra and Marban, 2000) due to increased substrate decomposition. Thus, high NH_4^+ availability measured at 45 °C in the present experiment suggested an increasing role of high temperatures in regulating net N mineralisation as the present study provided an evidence of low autotrophic nitrification at 45 °C. More research will be needed to thoroughly understand the rates of gross N mineralisation, which provides the information of microbial activities (Hart et al., 1994), and nitrification at temperatures between 40 °C to 50 °C.

The other pathways of N_2O production in soil, namely denitrification, nitrifier-denitrification or an oxidation of hydroxylamine (NH_2OH), could be important subjects for future work. Denitrification accounted for 44% to 47 % total N_2O production at temperature range of 35 to 40 °C in the dermosol and it is expected to be higher if the soil moisture content is greater than 60% FC (Sexstone et al., 1988; Renault and Stengel, 1994). Nitrifier-denitrification, a reduction of NO_2^- following the oxidation of NH_4^+ in nitrification, could produce 30% of total N_2O production in acidic soils associated with low OC and O_2 concentration (Wrage et al., 2001; Zhu et al., 2013). This process was presumably limited by pH 5.3 to 5.5 and high OC, as indicated by soil respiration rates in the present study. However, it is imperative to quantify N_2O production from nitrifier-denitrification in many acidic soils in southern Australia by applying the isotope techniques (^{15}N and ^{18}O) described by Wrage et al. (2005). In addition, chemical oxidation of NH_2OH could result in N_2O formation (Bremner, 1997; Heil et al., 2015). The release of NH_2OH from AOB has been reported by previous studies (Stüven et al., 1992; Schmidt et al., 2004), thus potentially enhancing this abiotic N_2O production, particularly at the temperature range 30 – 50 °C (Heil et al., 2015). Therefore, further research will be needed to measure the proportion of N_2O from the oxidation of NH_2OH during nitrification, to have a better understanding of the mechanisms of N_2O production in soil.

Although the growth and activity of AOA were presumably negligible in the soils with the additions above 50 µg NH_4-N g^{-1} in the experiments presented in the present studies, the thermal response of AOA needs to be understood, particularly in low fertilised or un-fertilised soils. The abundance of Archaeal *amo*A genes can be quantified using primers crenamoA23F and crenamoA616R (Tourna et al., 2008) for amplification in QuantiFastTM qPCR master mix (Qiagen). Amplification efficiency for archaeal *amo*A

qPCR helps improve the knowledge of the role of AOA in N cycle and the thermal response of AOA relative to N_2O production. Ammonium oxidising bacteria populations estimated by MPN are often variable in response, and time-consuming. Amplification of bacterial *amo*A genes can be performed similarly to archaeal *amo*A qPCR, except for the primers. The primers for bacterial *amo*A can be A-1R and amoA-2R (Rotthauwe et al., 1997). Future research should apply these qPCR techniques to precisely evaluate the growth and activity of bacterial *amo*A genes and archaeal *amo*A genes in response to high temperatures.

4.5 Conclusions

Our results indicated that the rate of N_2O production from nitrification increased with rising temperature and peaked between 35 °C to 40 °C, where nitrification accounted for 55 % of total N_2O emissions. Heterotrophic nitrifiers used NH_4^+ as prime substrate during nitrification and produced the dominant N_2O production at 35 °C to 40 °C, while autotrophic nitrification declined above 35 °C. The activity of autotrophic nitrification appeared to relate to the growth of ammonium oxidising bacteria, as influenced by incubation temperature. Temperature might have indirect effects on N_2O production from nitrification, by reducing O_2 concentration in the soil microsite as indicated by high soil respiration rates above 30 °C. Denitrification was the dominant pathway of N_2O production between 35 °C and 40 °C, suggesting high potential N losses as N_2O emissions at these temperatures. At 45 °C, high soil NH_4^+ concentration could have resulted from low nitrification rate and/or high gross N mineralisation rate, indicating a great impact of high temperatures on N cycle.

Chapter 5 **Soils from two contrasting climates differ in their thermal patterns of N_2O and nitrification**

5.1 Introduction

The magnitude of N_2O production from soils across climatic regions has been related to soil properties, climate and land management, since these factors influence the abundance and activity of soil microorganisms responsible for the production of N_2O. Microorganisms that mediate N have been observed to adapt to local temperature regimes in some studies (Powlson et al., 1988; Malhi et al., 1990; Stark and Firestone, 1996; Schimel and Gulledge, 1998). The thermal adaptation of these microorganisms may influence their capacities to produce N_2O with seasonal change and climatic zones. However, the available data of N_2O production are based on studies conducted under different experimental conditions (Goodroad and Keeney, 1984; Maag and Vinther, 1996; Gödde and Conrad, 1999; Holtan-Hartwig et al., 2002). Therefore, it is difficult to make comparisons of the thermal responses of N_2O production among different climatic regions because the effect of temperature may be confounded with other factors such as soil moisture or substrate (NH_4^+ and NO_3^-) availability. Thus direct investigation of the thermal response of N_2O production in soils from different climates is, therefore, a novel approach.

Nitrification is one of the major biological processes of N_2O emissions from soils (Bremner, 1997; Groffman et al., 2006), particularly in dry conditions such as southern Australia. It was reported that bacterial growth is very responsive above 30 °C (Bárcenas-Moreno et al., 2009) but the growth rates of nitrifiers, i.e., ammonium oxidising bacteria (AOB) (Schmidt, 1982), have not been previously examined at high temperatures. The evidence that bacteria meditating N mineralisation in soils originating from different

climates respond dissimilarly to temperature (Dessureault-Rompré et al., 2010) suggests that this may be similar for AOB. Thus, it is important to evaluate the thermal responses of AOB, nitrification rate and associated N_2O production in soils sourced from contrasting climates.

In this study, we evaluated the magnitudes of N_2O emissions in soils from two contrasting climatic zones following incubation between 20 °C to 40 °C over two weeks. In addition, the thermal responses of AOB to temperature and NO_3^- and N_2O production from autotrophic nitrification were investigated in the soils.

5.2 Materials & methods

5.2.1 Study sites and sample collection

The soils used in this study were collected from two contrasting environments in July 2014. A loam soil collected from a sugar cane system in Queensland (18°35' S, 146°08' E) represented the tropical climate while a sandy loam collected from an apple orchard system in Tasmania (42° 57' S, 147°05' E) represented a temperate climate (Figure 5.1). The variations in daily air temperature and soil moisture (0-10 cm) from these sites are shown in Figure 5.2.

After sampling, the soil samples were kept cool and immediately transported to the laboratory. Soils were sieved to remove the > 2 mm fraction to remove any stone and plant's roots then thoroughly mixed to ensure uniformity.

Temperate soil		Tropical soil
42° 57' S, 147°05'E	Location	18°35' S, 146°08'E
Apple orchard	Land use	Sugar Cane
Sandy loam	Texture	Silt loamy
10.4	Clay (%)	22.0
16.8	Silt (%)	16.0
72.8	Sand (%)	62.0
1.04	Bulk Density (g/cm^3)	1.10
0.35	$FC_{33\ kPa}$ (g water/g soil)	0.40
0.21	FC60% (g water/g soil)	0.24
6.0	pH-H_2O	4.82
11.7	NH_4 - N (mg N kg^{-1} d.w)	14.5
8.05	NO_3 - N (mg N kg^{-1} d.w)	4.25
2.42	Organic carbon (%)	1.76

Figure 5.1. Study sites and major characteristics (0-10 cm depth) of unamended experimental soils used in this study.

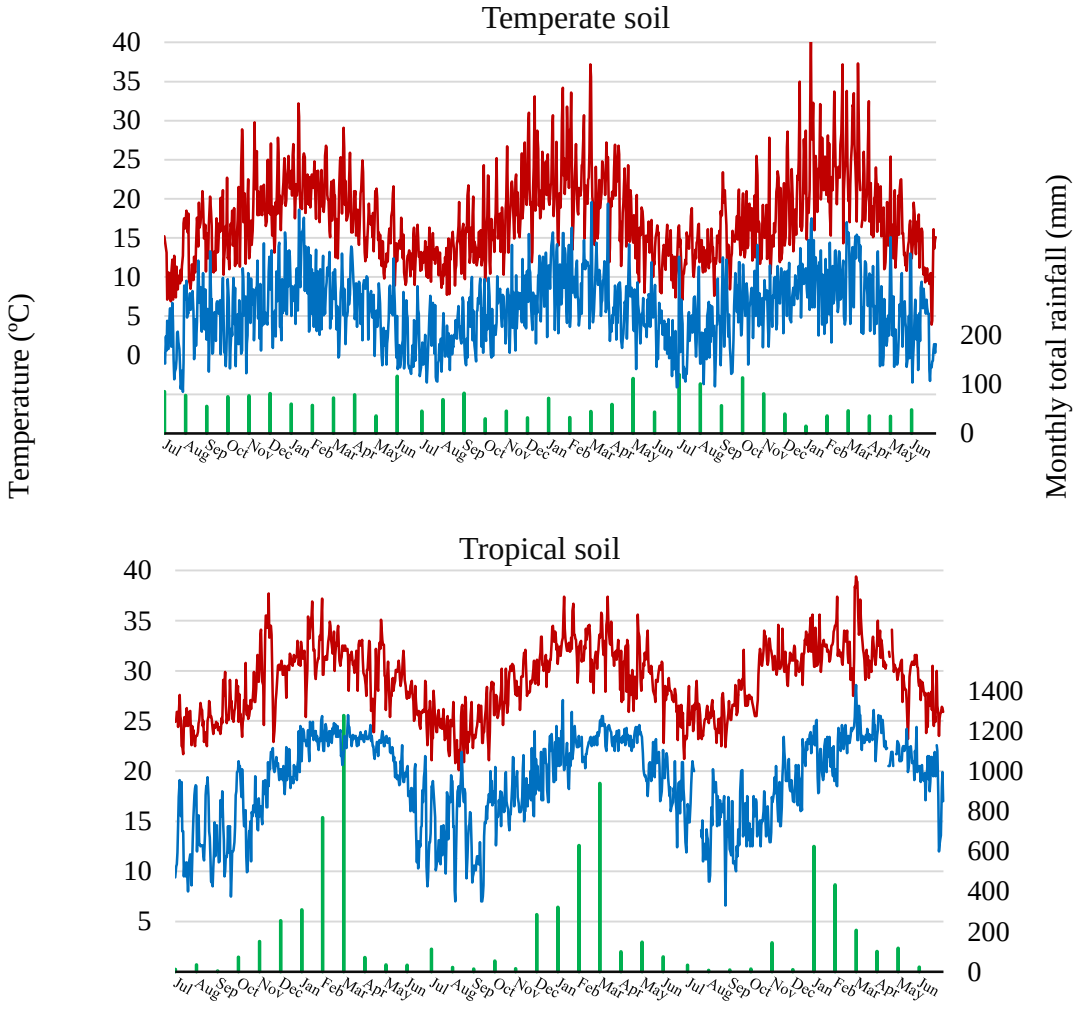

Figure 5.2. The variation in daily air temperature with maximum temperature (red line), minimum temperature (blue line) and monthly total rainfall in two study sites (green vertical bars) from 2012-2014 (unpublished data from Nigel Swarts, The University of Tasmania for temperate soil and Melissa Royle, HCPSL for tropical soil).

5.2.2 Laboratory incubation

The procedures of laboratory incubation are shown in Figure 5.3. Briefly, soil samples were wetted up to 60% FC then placed into a plastic bag with holes to allow gas exchange with the atmosphere. All soil samples were pre-incubated 7 days at average monthly temperatures of sampling time, 8 °C for the temperate and 22 °C for the tropical soil to re-establish soil microbial activity. Soil moisture was checked every 3 days and water added, if necessary, to maintain at 60% FC. After this pre-incubation, soil samples (50 g dry weight equivalent) were repacked into cores (37 mm diameter × 42 mm height; 10.75 cm^2 surface area) to achieve the original bulk density from field sites at 1.04 and 1.10 g soil cm^{-3} for the temperate and tropical soil, respectively. To avoid the limitation of NH_4^+ on nitrification (**Chapter 3**), 2ml of 0.09 mM of $(NH_4)_2SO_4$ was added to each soil core to provide final addition of NH_4^+ of 100 µg N g^{-1} soil. This solution was added slowly onto the top of upright soil cores to reach a final water content of 60% FC, which reflects the soil moisture in the field at certain times during summer, between December to March (Figure 5.2). Each soil core was placed into a 250ml jars with a tight seal and a lid equipped with a rubber septum (Subaseals#25, Sigma-Aldrich, St Louis, MO, USA). The air in the jars was refreshed for two minutes using a mini fan before being sealed.

Acetylene (C_2H_2) was applied to distinguish N_2O production from autotrophic nitrification and N_2O from other sources (heterotrophic nitrification and denitrification), as described in section **3.2 (Chapter 3)**. All jars were incubated at constant temperatures of 20, 25, 30, 35 and 40 ºC for 14 days using four replicates (Figure 5.3).

5.2.3 Sampling and analysis

Two sets of incubations were used in this experiment, one to monitor gas sampling and the other to monitor soil mineral nitrogen concentrations. In the first set of incubations,

the headspace of 40 jars (two soils × five temperatures × four replicates) was sampled on days 0, 3, 7 and 10 after incubation. A 20 ml sample of headspace gas was taken using a Hamilton 50 ml gas-tight syringe (Hamilton Company, Reno, NV, USA) and transferred into a pre-evacuated 12 ml glass Exetainer (Labco, Ceredigion, UK) for measurement of N_2O and CO_2 production, as presented in section **3.2** in **Chapter 3**. After gas sampling, the headspace of all jars were refreshed using a mini-fan for two minutes and re-injected C_2H_2 to the relevant jars for re-establishing the inhibition of autotrophic nitrification.

In the second set of incubations, soil samples were taken on 0, 7 and 14 days for measurement of NH_4^+ and NO_3^- concentrations, as described in section **3.2** (**Chapter 3**). Subsoil samples (10 g of dry soil) were taken on day 0 and 14 for estimation of the populations of ammonium oxidising bacteria (AOB) using the Most Probable Number (MPN) method as described in **Chapter 4.**

5.2.4 Calculation of nitrogen transformation rates

The estimation of autotrophic nitrification (N_{nit}, µg NO_3-N g^{-1} d^{-1}) and associated N_2O (N_2O_A, µg N_2O-N g^{-1} d^{-1}) was described in section 3.2 (**Chapter 3**).

The proportion (P_n) of N_2O to NO_3^- by autotrophic nitrification was calculated by dividing the amount of N_2O and NO_3^- production.

$$P_n = \frac{N_2O}{NO_3} \times 100 \qquad \text{(Figure 5.1)}$$

The change in the rate of a biological process with increasing the temperature can be assessed by using the function of Q_{10} (Van't Hoff, 1884)

The influence of temperature on the growth of AOB population could be expressed as:

$$\boldsymbol{P_t} = P_0 \times e^{\mu t} \qquad \text{(Figure 5.2)}$$

where p_t (cells g^{-1}) is the counts of AOB at time t (day), p_0 is the initial AOB at t = 0 and μ (d^{-1}) is the growth rate.

5.2.5 Statistical analysis

The pooled ANOVA for measurement over time (Gomez and Gomez, 1984) was applied to determine the rates of change over time of N_2O and mineral N contents in each soil. Two-way ANOVA was used to determine the significant differences in N_2O and CO_2 production or mineral N between soils and temperatures over the 14 days of incubation. The Tukey ($HSD_{0.05}$) test was applied *post hoc* to determine any significant differences between treatments. Statistical tests on ratios like N_2O/NO_3^- was based on Arcsine transformed data, as described by Ireland (2010). Correlations (Pearson) were applied to determine the relationships among temperature and nitrification products. All statistical analysis was performed using Statistix 10 (Tallahassee, USA).

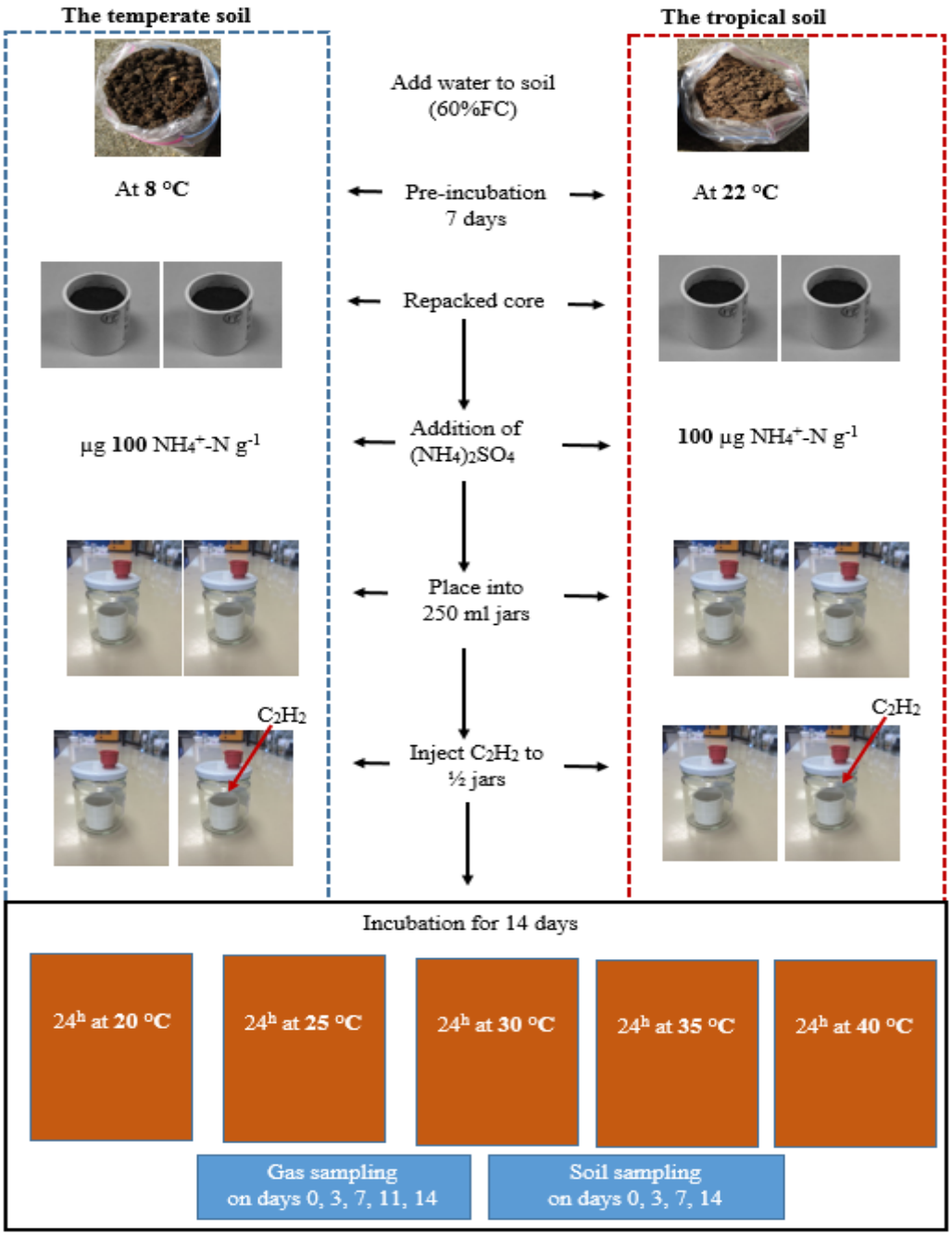

Figure 5.3. Experimenal procedures, including: Pre-incubation, repacking soil core, addition of NH_4^+ solution and C_2H_2 (0.01% v/v) then incuabtion at different temperatures.

5.3 Results

5.3.1 Soil mineral N

5.3.1.1 NH_4^+ content

In the temperate soil, the concentrations of NH_4^+ decreased over the period 0 – 7 days in the absence of C_2H_2, then remained relatively stable after day 7, except for 40 °C where NH_4^+ increased in the last 3 days of incubation (Figure 5.4). Following amendment with C_2H_2, NH_4^+ contents remained stable during the first 7 days then increased after that at all temperatures, except for 30 °C.

In the tropical soil, the amount of NH_4^+ did not change at 20 °C and 40 °C throughout the experimental period in the absence of C_2H_2 (Figure 5.4). The reduction of NH_4^+ was observed after 3 days at 25 °C and 35 °C and after 7 days at 30 °C. Ammonium increased at 35 °C to 40 °C in last three days of incubation while NH_4^+ remained consistent between 20 °C and 30 °C in the sample without C_2H_2. In the presence of C_2H_2, the concentration of NH_4^+ remained stable during the first 7 days at all temperatures (Figure 5.4).

5.3.1.2 NO_3^- content

The concentration of soil NO_3^- in the temperate soil increased rapidly in the first 7 days of incubation at 25 – 30 °C without C_2H_2 addition (Figure 5.5). Nitrate increased slowly at 20 °C and 35 °C over the period 0 – 7 days while NO_3^- did not change over this period in the 40 °C treatment. After day 7, the concentrations of NO_3^- were remained similar at 25 – 30 °C but increased at other temperatures (Figure 5.5). In the samples with amendment of C_2H_2, the concentrations of NO_3^- declined at all temperatures through the experiment to a similar extent at different temperatures (Figure 5.5).

In the tropical soil, NO_3^- concentrations increased rapidly at 30 – 35 °C in soil without C_2H_2 addition over the period 0 – 7 days and steadily at other temperatures during the experiment (Figure 5.5). The highest NO_3^- concentration after 14 days of incubation was found at 30 – 35 °C and followed by 25 °C and 40 °C while the lowest NO_3^- concentration was measured at 20 °C (Figure 5.5). In samples with C_2H_2 addition, NO_3^- declined from days 0 – 3 at all temperatures then remained at 0.4 µg N g^{-1} soil over the period 3 – 14 days (Figure 5.5).

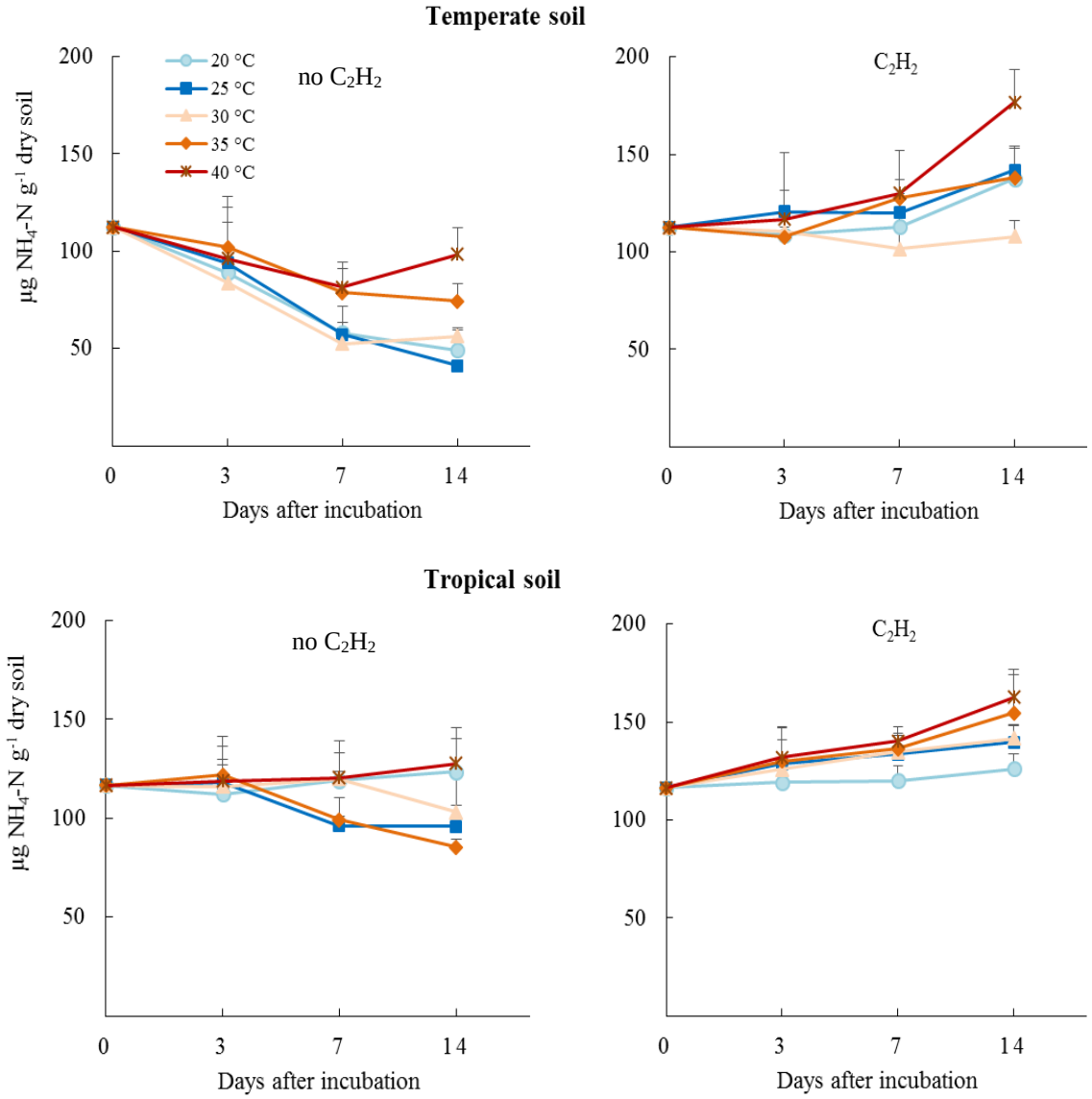

Figure 5.4. Soil ammonium concentrations at different temperature and with and without acetylene addition in two tested soils after N application of 100µg N g^{-1} soil, as $(NH_4)_2SO_4$. Soil moisture was maintained at 60% FC during incubation. Error bars represent +1 *SE* (n = 4).

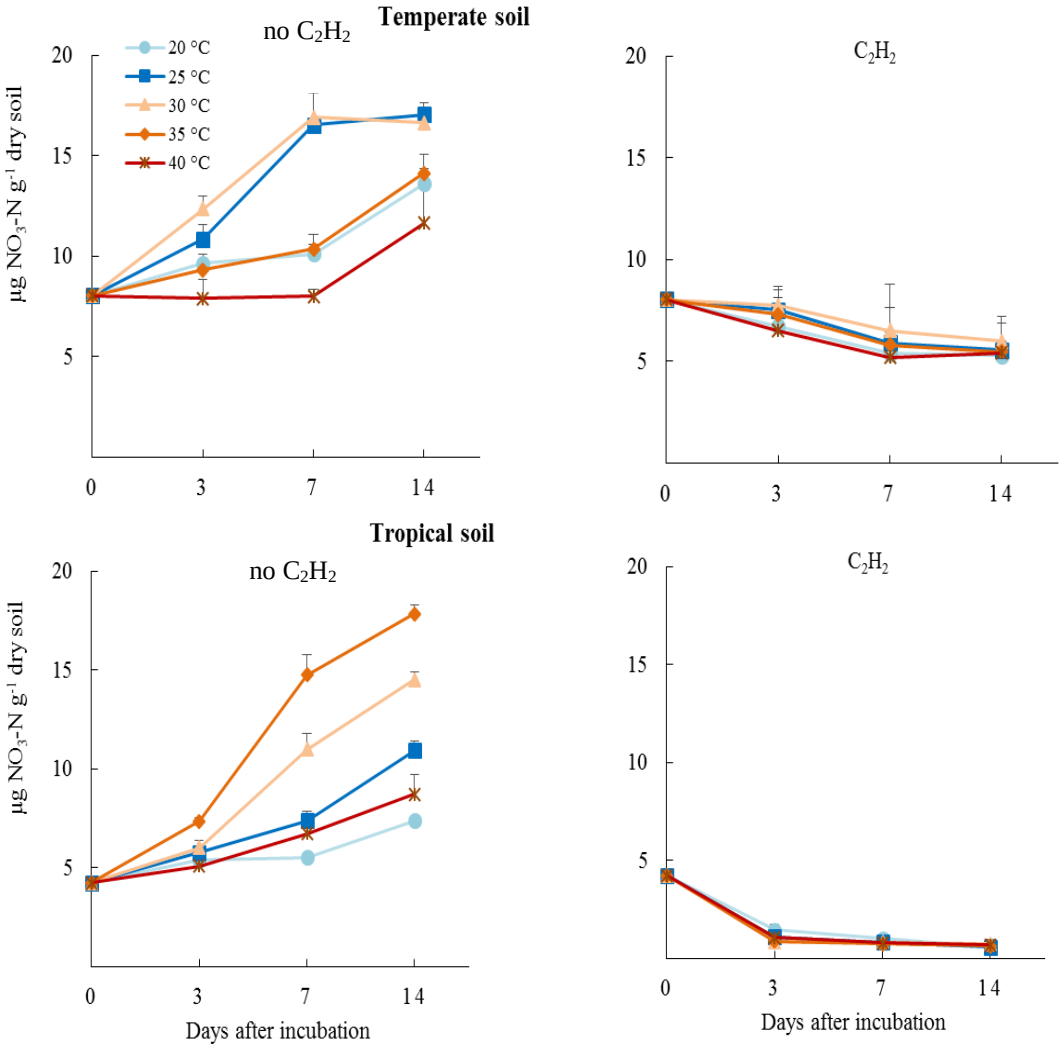

Figure 5.5. Soil nitrate concentrations at different temperature and with and without acetylene amendment in two tested soils after N application at 100 µg N g^{-1} soil, as $(NH_4)_2SO_4$. Soil moisture was maintained at 60% FC during incubation. Error bars represent +1 *SE* (n = 4).

5.3.2 Total N_2O emissions

The concentration of N_2O in the absence of C_2H_2 was attributed to both nitrification and denitrification, and is referred to here as total N_2O production (Figure 5.6). Total N_2O production differed with incubation time among temperatures (Pooled ANOVA, $p < 0.05$). In the temperate soil, total N_2O emissions were produced slowly over the period 0 to 3 days but increased rapidly after 3 days of incubation at all temperatures, except for 20 °C, where total N_2O increased steadily (Figure 5.6). The highest cumulative N_2O production after 14 days was measured at 30 °C and 35 °C, followed by 40 °C and 25 °C; the lowest N_2O production was at 20 °C. In the tropical soil, total N_2O production occurred fastest at 35 °C and 40 °C than at 20 °C to 30 °C (Figure 5.6). The production of N_2O at 35 – 40 °C accumulated rapidly in the first 7 days then slowed from 7 to 14 days. Cumulative N_2O production was threefold less between 20 °C and 30 °C than at 35 °C to 40 °C (Figure 5.6).

Over the 14 days of incubation, the rates of total N_2O production in the tropical soil were significantly higher than in the temperate soil at all sampling times ($p < 0.05$). Cumulative N_2O production over the 14 days ranged from 0.06 – 0.20 µg N_2O-N g^{-1} soil in the temperate soil and from 0.12 – 0.79 µg N_2O - N g^{-1} soil in the tropical soil (Figure 5.6).

5.3.3 N_2O from heterotrophic nitrification and/or denitrification

The amounts of N_2O produced in the soils with addition of C_2H_2 (0.01% v/v) were attributed to denitrification and/or heterotrophic nitrification (Figure 5.6). In the temperate soil, the rates of N_2O from these processes were low in the first 3 days of incubation and there was no significant difference among temperatures. Cumulative N_2O emissions were higher at 30 °C to 40 °C than at lower temperatures in subsequent measurements.

In the tropical soil, the thermal response of N_2O production from heterotrophic nitrification and/or denitrification followed a similar pattern to total N_2O production (Figure 5.6). The low rates of N_2O production from heterotrophic nitrification and/or denitrification in this soil over the period 3 –14 days were presumably due to low soil NO_3^- concentration, which inhibited the production of N_2O from denitrification in the presence of C_2H_2 (Figure 5.6).

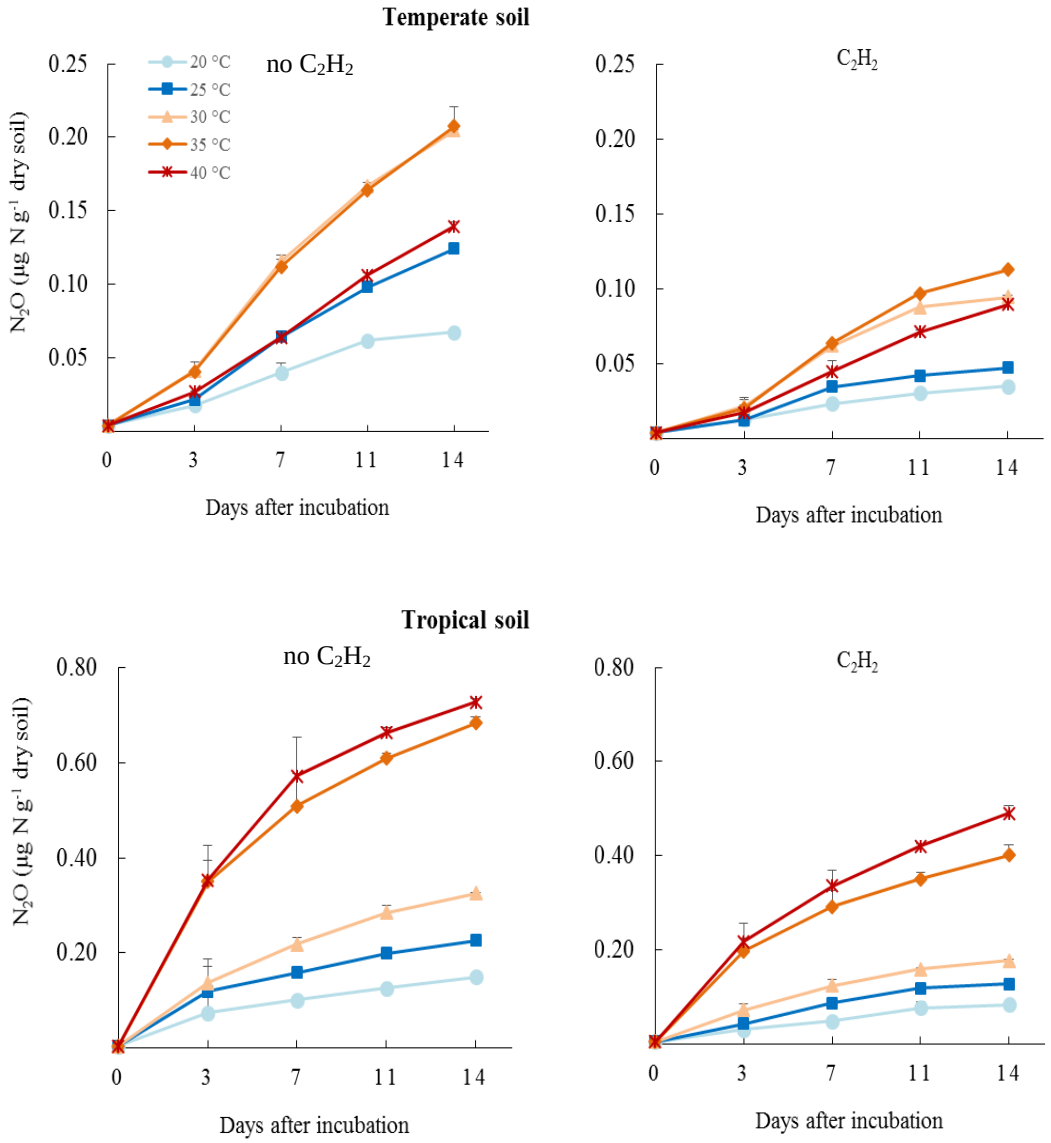

Figure 5.6. Cumulative N_2O production at different temperatures and with and without acetylene amendment in two soils after N application at 100µg N g^{-1} soil as $(NH_4)_2SO_4$. Soil moisture was maintained at 60% FC. Bars represent +1 *SE* (n = 4).

5.3.4 Net rate of autotrophic nitrification

The daily net rate of nitrification (N_{nit}) in first 7 days of incubation varied from 0.47 to 1.68 µg NO_3^--N g^{-1} d^{-1} in the temperate soil (Table 5.1). N_{nit} increased with rising temperature from 20 °C to 30 °C, then declined above 30 °C, indicating the T_{opt} at 25 – 30 °C in this soil from 3 to 7 days (Table 5.1).

In the tropical soil, N_{nit} occurred slowly at 20 °C, at 1.31 µg NO_3^--N g^{-1} d^{-1}, but increased to 2.15 µg NO_3^--N g^{-1} d^{-1} at 35 °C. N_{nit} at 40 °C was as low as 20 °C, suggesting that T_{opt} for autotrophic nitrification rate was between 30 °C and 35 °C (Table 5.1).

5.3.5 N_2O from autotrophic nitrification

The differences in N_2O production between samples with and without C_2H_2 indicated N_2O from autotrophic nitrification (Figure 5.6). Differential responses of N_2O from autotrophic nitrification to temperatures were primarily observed after 7 days in the temperate soil but 3 days in the tropical soil (Figure 5.6). Estimation of N_2O from autotrophic nitrification was likely to be overestimated from 3 to 14 days in the tropical soil because N_2O arising from denitrification (Figure 5.6) was potentially limited by low NO_3^- contents in the presence of C_2H_2 (Figure 5.5). The thermal response of N_2O from autotrophic nitrification were clearly determined in the first 3 days in the tropical soil. Thus, the data reported in Table 5.1 focus on the thermal response of N_2O from autotrophic nitrification in the first 7 days in the temperate soil and the first 3 days in the tropical soil.

In the temperate soil, daily rate of N_2O from autotrophic nitrification was 0.002 – 0.008 µg N_2O-N g^{-1} d^{-1} in the first 7 days of incubation (Table 5.1). Autotrophic nitrification produced 0.004 – 0.051 µg N_2O-N g^{-1} d^{-1} in the tropical soil in the first 3 days of incubation (Table 5.1). The highest daily rate of N_2O from autotrophic nitrification,

referred to as the optimal value (T_{opt}), was found between 30 °C and 35 °C in the temperate soil but between 35 °C and 40 °C in the tropical soil (Table 5.1).

5.3.6 The proportion of N_2O to NO_3^- from autotrophic nitrification

In the temperate soil, the proportion of N_2O to NO_3^- (P_n) production from autotrophic nitrification varied from 0.1% to 0.4% between 20 °C to 30 °C but there was no significant difference among temperatures (Table 5.1). Above 30 °C, P_n were above 1.0% the first 7 days of incubation due to a faster decline of NO_3^- than N_2O production from autotrophic nitrification.

In the tropical soil, P_n did not differ between 20 °C and 30 °C although P_n ranged from 0.3% to 1.3% in the first 3 days of incubation (Table 5.1). P_n was above 2.0% in the treatments at 35 °C and 40 °C (Table 5.1) in this period.

5.3.7 Ammonium oxidising bacteria (AOB) population

Incubation temperature had a great influence on the growth of AOB in two soils after 14 days of incubation (Table 5.1). The growth rate of AOB was greater at 20 –30 °C than at higher temperatures in the temperate soil while the growth rate of AOB in the tropical soil was greatest at temperatures of 25 – 30 °C. Higher temperatures slowed the abundance of AOB in the tropical soil and had a detrimental effect on the growth of AOB in the temperate soil.

Table 5.1. Daily rate of NO_3^- production (N_{nit}), nitrous oxide (N_2O_A) from autotrophic nitrification, the proportion of N_2O to NO_3^-(P_n) in the first 7 days of incubation and the growth rate of AOB population (d^{-1}) after 14 days in soils added wtith 100 µg NH_4-N g^{-1} soil. Soil was maintained at 60% of field capacity (−33 kPa).

	From 0 – 3 days			From 3 – 7 days			From 0 – 14 days
	N_2O_A	**N_{nit}**	**P_n**	**N_2O**	**N_{nit}**	**P_n**	**µ-AOB**
	µg N g^{-1} d^{-1}	*µg N g^{-1} d^{-1}*	*%*	*µg N g^{-1} d^{-1}*	*µg N g^{-1} d^{-1}*	*%*	*(d^{-1})*
Temperate soil							
20	**0.002** ± 0.001 *c*	**0.97** ± 0.31 *ab*	**0.18** *b*	**0.003** ± 0.001 *b*	**0.68** ± 0.05 *b*	**0.43** *b*	**0.42** ± 0.03 *a*
25	**0.003** ± 0.001 *c*	**1.12** ± 0.27 *ab*	**0.27** *b*	**0.005** ± 0.001 *b*	**1.52** ± 0.15 *a*	**0.34** *b*	**0.35** ± 0.01 *a*
30	**0.007** ± 0.002 *a*	**1.55** ± 0.15 *a*	**0.42** *b*	**0.008** ± 0.001 *a*	**1.49** ± 0.19 *a*	**0.57** *b*	**0.25** ± 0.10 *a*
35	**0.007** ± 0.002 *a*	**0.68** ± 0.32 *ab*	**1.01** *a*	**0.007** ± 0.002 *ab*	**0.65** ± 0.07 *b*	**1.04** *ab*	**-0.10** ± 0.04 *b*
40	**0.005** ± 0.001 *b*	**0.47** ± 0.17 *b*	**1.05** *a*	**0.005** ± 0.00. *b*	**0.40** ± 0.09 *b*	**1.19** *a*	**-0.20** ± 0.07 *b*
$HSD_{0.05}$	**0.001**	**1.03**	**(5.24)***	**0.002**	**0.53**	**(3.26)**	**0.28**
Tropical soil							
20	**0.004** ± 0.001 *c*	**1.31** ± 0.16 *b*	**0.28** *b*	**0.005** ± 0.001 *c*	**0.65** ± 0.04 *c*	**0.82** *b*	**0.24** ± 0.09 *b*
25	**0.011** ± 0.004 *c*	**1.55** ± 0.11 *b*	**0.70** *b*	**0.009** ± 0.001 *bc*	**0.94** ± 0.07 *c*	**0.94** *b*	**1.19** ± 0.03 *a*
30	**0.022** ± 0.009 *c*	**1.72** ± 0.13 *ab*	**1.27** *ab*	**0.021** ± 0.006 *b*	**1.45** ± 0.09 *b*	**1.42** *ab*	**1.22** ± 0.03 *a*
35	**0.051** ± 0.006 *a*	**2.15** ± 0.07 *a*	**2.39** *a*	**0.039** ± 0.005 *a*	**2.00** ± 0.14 *a*	**1.94** *a*	**0.13** ± 0.04 *b*
40	**0.045** ± 0.008 *ab*	**1.33** ± 0.08 *b*	**3.40** *a*	**0.041** ± 0.005 *a*	**0.85** ± 0.12 *c*	**4.85** *a*	**0.19** ± 0.13 *b*
$HSD_{0.05}$	**0.017**	**0.51**	**(6.80)**	**0.015**	**0.51**	**(6.91)**	**0.34**
Two-way ANOVA	*	*	*ns*	*	*ns*	*ns*	*

* *significant difference between two soils at $p < 0.05$;* ^{ns} *non-significant difference.*

The same letters within one column and soil indicate no significant difference at $\alpha = 0.05$; Values in parentheses were based on Arcsine transformed data

5.3.8 Production of CO_2

The production of CO_2, indicated soil microbial respiration, increased with rising temperatures in both soils (Figure 5.7). The daily rates of soil respiration were significantly lower at 20 – 25 °C than at higher temperatures over the first 7 days of incubation in both tested soils ($p < 0.05$, Figure 5.7).

5.3.9 Inter-relationships

The relationships among temperature, total N_2O production, autotrophic nitrification products and CO_2 production from two soils are shown at Table 5.2. Total N_2O production was positively correlated with temperature ($r = 0.54$, $p < 0.01$). N_{nit} was closely related to the growth rate of AOB ($r = 0.42$, $p < 0.05$). P_n was strongly influenced by the rate of N_2O from autotrophic nitrification ($r = 0.90$), indicating the impact of temperature on P_n by regulating N_2O production (Table 7.4). The rate of N_2O_A was negatively correlated with the rate of CO_2 ($r = – 0.43$, $p < 0.05$).

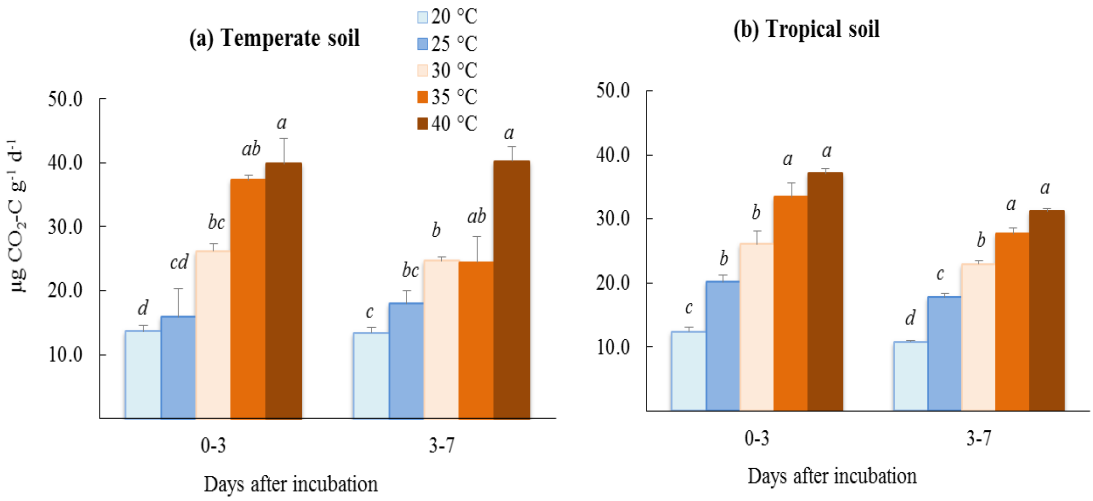

Figure 5.7. The rate of CO_2 production at different temperature in two tested soils after N application at 100 μg N g^{-1} soil as $(NH_4)_2SO_4$. Soil moisture was maintained at 60% FC during incubation. Bars represent +1 *SE* (n = 4).

Table 5.2. Correlation coefficients (r values) among temperature, total N_2O, CO_2 production, autotrophic nitrification (N_{nit}), N_2O from autotrophic nitrification (N_2O_A), ammonium oxidising bacteria (AOB) and the proportion of NO_3^- to N_2O (P_n) (n = 30).

	Temperature	Total N_2O	N_{nit}	AOB	N_2O_A	P_n
Total N_2O	0.54**					
N_{nit}	-0.10	0.49**				
AOB	-0.43*	-0.07	0.42*			
N_2O_A	0.43*	0.94**	0.48**	-0.02		
P_n	0.57**	0.84**	0.17	-0.13	0.90**	
CO_2	0.62**	-0.18	-0.53**	-0.60**	-0.43*	-0.05

* significant at $P < 0.05$

** significant at $P < 0.01$

5.4 Discussion

Our study revealed that the tropical soil was more responsive to temperature in total N_2O production than the temperate soil. This finding supports Bouwman's scaling model (Bouwman et al., 2002) that N_2O emissions in tropical climates exceed those in temperate climates. The results reflect that relative N_2O production from soil microorganisms in contrasting climates respond dissimilarly to temperature. Above 30 °C, the AOB population increased in the tropical soil but declined in the temperate soil. The distinct growth rate of AOB in each climate could be a result of the physiological adaptation of microorganisms to local temperature regimes, which has been identified by previous studies (Powlson et al., 1988; Malhi et al., 1990; Stark and Firestone, 1996; Schimel and Gulledge, 1998). The different capacities of soil microorganisms in responding to high temperatures influenced the magnitudes of N_2O production in two tested soils. We postulate that the higher N losses as N_2O emissions in response to high temperatures are more likely to occur in tropical climate soils rather than in temperate climate soils.

Our results confirm that an optimum function (T_{opt}) can be used to describe the response of autotrophic nitrification to temperature, although it may vary between regions. T_{opt} for NO_3^- production from autotrophic nitrification was 20 – 30 °C in the temperate soil and was at 30 – 35 °C in the tropical soil. This finding supports the suggestion that different T_{opt} for nitrification are observed for different climatic regions, as described by Stark (1996) and employed in the DayCent model (Parton et al., 2001). A comparison of soils from different regions indicates that nitrifiers in warm regions have higher T_{opt} than those from cooler areas. For example, nitrifiers from temperate soils in Denmark (Maag and Vinther, 1996) and Canada (Malhi and McGill, 1982) had T_{opt} of 20 °C while those from tropical Australian soils (Myers, 1975) had T_{opt} at 35 °C (Figure 5.8). The differences in T_{opt} for nitrification rate may suggest that ecosystem models predicting N_2O should

consider this characteristic for a better understanding of N_2O production across climatic regions.

The proportion of N_2O to NO_3^- production from autotrophic nitrification (P_n) increased with rising temperature ($r = 0.57$, $p < 0.01$) and was above 1.0% at 35 ºC in both tested soils. P_n was often reported to be less than 1.0% in previous studies at the temperature range 5 ºC to 30 ºC (Table 2.3, **Chapter 2**). There was a significant effect of temperature on P_n, with a greater P_n at high temperatures, due to a faster decline of NO_3^- than N_2O production from nitrification. Decreased NO_3^- and N_2O production above 35 ºC was not only influenced by the growth of AOB ($r = 0.42$, $p < 0.05$) but also affected by the presence of high anaerobic zones caused by high microbial respiration rates ($r = -0.53$, $p < 0.01$). Increased soil respiration rate results in reduced O_2 availability in soil microsites (Smith, 1997; Öquist et al., 2004; Butterbach-Bahl and Dannenmann, 2011), which increases P_n. As the partial pressure of O_2 dropped from 20 kPa to 0.7 kPa and 0.5 kPa, P_n increased from 0.1% to 1.4% and up to 6.9 %, respectively (Khalil et al., 2004; Zhu et al., 2013). The mechanism of the effect of O_2 on P_n was hypothesised to relate to the use of nitrite (NO_2^-) as the terminal electron acceptor, since O_2 becomes limiting in the soil microsites (Poth and Focht, 1985; Remde and Conrad, 1990). Wrage et al. (2001) confirmed this hypothesis by observing the production of N_2O from NO_2^- following nitrifier-denitrification. Since P_n increased above 35 °C, the loss of N as N_2O during nitrification should be observed even under low nitrification rate (low NO_3^- production). An effective function describing the dependence of P_n on temperature may allow a quick estimate of N_2O through measurement of NO_3^- produced during nitrification across regions. However, greater replicates will be needed to validate the relationship between P_n and temperature, identified in the present study.

The use of C_2H_2 to distinguish N_2O released by autotrophic nitrification from denitrification has been used in many previous studies (Maag and Vinther, 1996; Gödde and Conrad, 1998; Garrido et al., 2002; Avrahami et al., 2003). In the soil with a low NO_3^-content, N_2O from denitrification is likely to have been underestimated due to lack of NO_3^- supply from autotrophic nitrification in the presence of C_2H_2 (0.01 % v/v) (Mosier, 1980; Knowles, 1990; Malone et al., 1998; Groffman et al., 2006). In other words, the amount of N_2O from autotrophic nitrification may be overestimated. In the tropical soil, low NO_3^- contents after 3 days in the presence of C_2H_2 appeared to limit the production of N_2O from denitrification, and may have led to an overestimation of N_2O from autotrophic nitrification from days 3 to14. However, this did not have a big impact on the thermal response of autotrophic nitrification since N_2O from autotrophic nitrification among temperatures diverged primarily within the first 3 days in the tropical soil. Generally, the magnitudes of N_2O and NO_3^- were lower in the soils treated with C_2H_2 (0.01% v/v) than those without C_2H_2 treatments (Figure 5.4), indicating the efficient inhibition of autotrophic nitrification by the C_2H_2 method in the present study. Total N_2O production was estimated from the soils without C_2H_2 addition, so the thermal response of total N_2O production was unaffected by the C_2H_2 method.

It is acknowledged that this study was based on just two soils from contrasting climates and did not take into account variations in soil properties, which may affect total N_2O production and thermal responses of N_2O emissions (Davidson, 1991; Aulakh et al., 1992). Thus, future research should be conducted with greater samples from each zone to confirm the important findings on the influence of climate on total N_2O production and the optimal temperatures for nitrification.

Heterotrophic nitrification and/or denitrification were very responsive above 35 °C, evidenced from the amounts of N_2O from soils with the presence of C_2H_2. Stable isotope

techniques (^{15}N) should be employed to measure N_2O production from heterotrophic nitrification and denitrification, which cannot be distinguished using amendments of C_2H_2 (Hynes and Knowles, 1982; Bateman and Baggs, 2005), to better understand thermal responses of biological processes of N_2O at a range of climatic regimes.

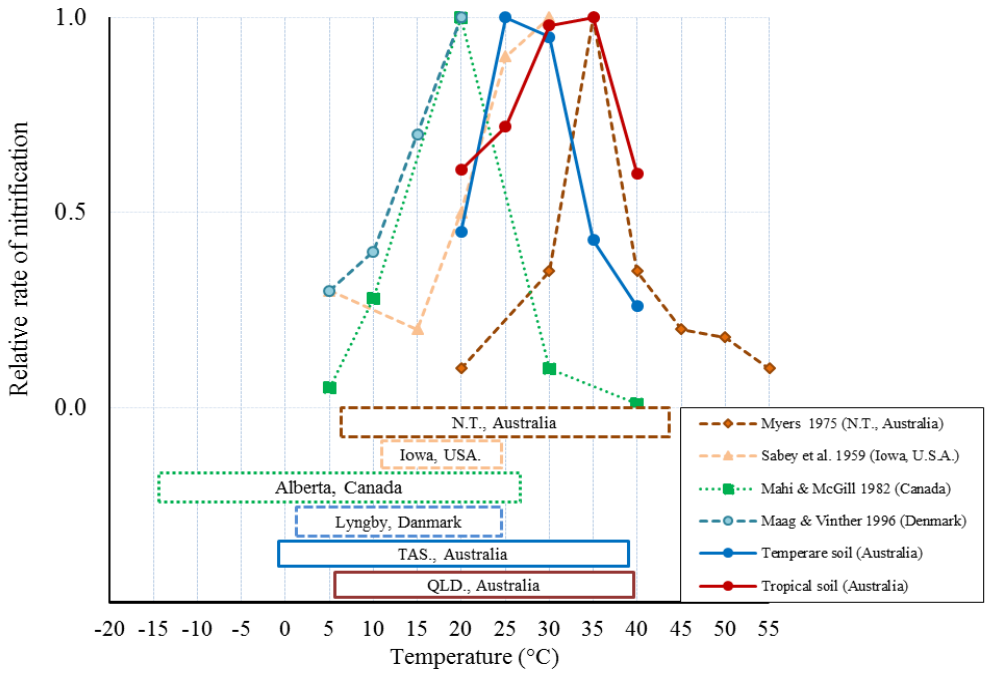

Figure 5.8. The effect of soil temperature on relative nitrification rate in this study compared with results from published results: relative nitrification rates (lines) and variation in mean of monthly air temperatures (bars).

5.5 Conclusions

Our results indicate that total N_2O production in soils from two contrasting climates responded dissimilarly to rising temperature. The production of total N_2O was higher in the tropical than in the temperate soil. An optimum function can be used to describe the response of autotrophic nitrification to temperature, although it may vary with climates. The temperature optima for autotrophic nitrification (NO_3^-) were higher in the tropical (30 – 35 °C) than the temperate climate (25 – 30 °C). N_2O from autotrophic nitrification peaked at 30 – 35 °C in the temperate soil and was (35 – 40 °C) at in the tropical soil. The proportion of N_2O released to NO_3^- by nitrification increased with rising temperature and was over 1.0% at 35 °C, regardless of climatic zone. The results suggest that nitrogen losses as N_2O emissions are likely to be higher in the tropical than temperate climate in response to rising temperature. Heterotrophic nitrification and/or denitrification appear to be important sources of N_2O production from agricultural soils exposed to high temperatures.

Chapter 6 **Soil moisture affects the thermal response of denitrification**[*]

6.1 Introduction

The rates of denitrification in soil are controlled by multiple factors including climate, soil properties (pH, percentage of clay) and agricultural practices (tillage, fertiliser supply, stubble retention, irrigation, etc). Many studies have attempted to understand the influence of these factors on the activity of denitrification from agriculture soil. It has been shown that soil moisture plays an important role in regulating denitrification products (NO, N_2O and N_2). An increase in soil moisture tends to increase the rate of denitrification and change the proportions of denitrification products, particularly above 60% water-filler pore space (WFPS). At high soil water contents, denitrification appears to be the key process related to N_2O and N_2 production (de Klein and Van Logtestijn, 1996; Hefting et al., 2003; Bateman and Baggs, 2005), while NO production is very small as a result of its high reactivity and low diffusion from soil to the atmosphere (Firestone and Davidson, 1989).

Soil moisture content controls oxygen (O_2) availability, gas and nitrate (NO_3^-) diffusion into soil microsites (Smith et al., 2003), which regulates the total N denitrified and denitrification product ratios, i.e., the N_2O/N_2. Increasing soil water content above 60% WFPS results in an increasing proportion of the soil volume that is anaerobic (Sexstone et al., 1988; Renault and Stengel, 1994), promoting the activity of heterotrophic denitrification (Dobbie and Smith, 2001) and increasing the N_2O/N_2 ratio. As O_2 content drops below 0.02 atm (~ 2 kPa), the reduction of N_2O to N_2 is likely to be inhibited

[*] To be submitted to *Science of the Total Environment*

(Morley et al., 2008; Zhu et al., 2013), thus increasing the N_2O/N_2 ratio. Under anaerobic conditions, NO_3^- can act as a terminal electron acceptor (Strong and Fillery, 2002), potentially increasing the N_2O/N_2 ratio from denitrification.

Several studies measured denitrification at different temperatures (e.g., (Keeney et al., 1979; Maag and Vinther, 1996; Holtan-Hartwig et al., 2002; Schaufler et al., 2010) and observed that N_2O production exponentially increased with rising temperature, up to 25 °C. These studies, however, were conducted under constant moisture contents, while others (Dobbie and Smith, 2001; Bateman and Baggs, 2005; Senbayram et al., 2012) used the constant temperatures with varying moisture or O_2 contents to evaluate the change in N_2O/N_2 ratio. The interactive effect between soil moisture and temperature in regulating the total N denitrified and N_2O/N_2 ratio is not documented and therefore is the focus of this study. Futhermore, the measurements of N_2 production in previous studies (Keeney et al., 1979; Avalakki et al., 1995; Maag and Vinther, 1999; Rudaz et al., 1999; Dobbie and Smith, 2001)) were indirectly estimated by the inhibition of N_2O reductase using the C_2H_2 technique. Importantly, uneven distribution of C_2H_2 in soil can prevent the inhibition of N_2O reduction, causing an error in the calculation of N_2O/N_2 ratio. The use of an isotopic tracer (^{15}N) enables a more precise evaluation of the reduction of N_2O to N_2 and N_2O/N_2 responses to temperature and soil moisture.

The aim of this study was, therefore, to assess the total denitrification rate and the responses of N_2O and N_2 at different temperatures and soil moisture contents. The use of an isotopic tracer (^{15}N) was employed to measure gas transformations during denitrification at temperatures in the range 25 °C to 45 °C and at moisture contents of 60% and 75% of field capacity.

6.2 Material and methods

6.2.1 Study sites and sample collection

The soil used in this study was a black dermosol, according to the Australian soil classification (Isbell, 1996) with 45% clay, 25% silt, 30% sand, total carbon 5.2%, total N 0.2%, pH ($CaCl_2$) 6.9, bulk density 1.12 g cm^{-3}, field capacity at – 33 kPa ($FC_{33\ kPa}$) 0.37 g water g^{-1}. The annual variations in daily soil temperature and monthly water content from the field site are shown in Figure 4.1 (Chapter 4).

6.2.2 Laboratory incubation

6.2.2.1 Pre-incubation

Soil samples were wetted up to 60% $FC_{33\ kPa}$ (0.21 cm^3 cm^{-3} soil) then placed into a plastic bag with holes to allow gas exchange with the atmosphere. All soil samples were pre-incubated for 7 days at 25 °C to re-establish soil microbial activity.

6.2.2.2 Incubation

Soil moisture after the pre-incubation was maintained at 50% of FC. Soil subsamples (50 g dry weight equivalent) were repacked in a core (ø37 mm × 42 mm; 10.75 cm^2 surface area) to a bulk density of 1.12 g cm^{-3}, equivalent to that measured from the field. To avoid substrate limitation affecting denitrification (**Chapter 5**), isotopically labelled nitrate solutions as $^{14}NH_4{}^{15}NO_3$ were added slowly onto the top of upright cores. To reach final water contents of 60% FC (0.25 cm^3 cm^{-3}) and 75% FC (0.31 cm^3 cm^{-3}), 2 ml of $^{14}NH_4{}^{15}NO_3$ concentration at 0.09 mM and 5ml of $^{14}NH_4{}^{15}NO_3$ at 0.036 mM were used to achieve the final concentration of 100 µg N g^{-1}. These water contents reflect the range of soil moisture at the field site during summer. Soil cores were then placed into 250ml jars

equipped with a rubber septum (Subaseals#25, Sigma-Aldrich, St Louis, MO, USA) in the cap. All jars were then incubated at 25, 35, 40 or 45 ºC for 10 days using 4 replicates.

Two sets of soil incubations were established to monitor gas production and mineral nitrogen transformations during the experiment

6.2.3 Sampling and analysis

In the first set of incubations, the headspace of 32 jars (two levels of soil moistures × four temperatures × four replicates) was sampled on days 0, 3, 7 and 10 after addition of the N solution. A 20 ml sample of headspace gas was taken using a Hamilton 50 ml gas-tight syringe (Hamilton Company, Reno, NV, USA); and transferred into a pre-evacuated 12 ml Exetainer (Labco, Ceredigion, UK) for measurement of $^{14+15}N\text{–}N_2O$, $^{15}N\text{–}N_2O$, $^{15}N\text{–}N_2$ and CO_2. After gas sampling, the headspace of all jars was refreshed using a mini-fan for one minute.

In the second set of incubations, soil samples were taken on day 0 and 10 to measure NO_3^- concentrations as a substrate for denitrification. Briefly, NO_3^- was extracted from 10 g of moist soil by shaking for one hour in 50 ml of 2 M KCl. The analysis of NO_3^- was performed colorimetrically with an AutoAnalyser (AA3 HR, SEAL, West Midlands, UK) using the hydrazine reduction method (Kamphake et al., 1967).

6.2.4 Gas measurement

The measurement of CO_2 and N_2O production was described in **Chapter 3**.

Trace gas isotope ratios ($\delta^{15}/\delta^{14}N$) were determined a Delta V^{TM} Isotope-ratio Mass Spectrometer (Thermo-Scientific, Bremen, Germany) as described in section 4.2 (**Chapter 4)**.

6.2.5 Calculation of nitrogen transformation rates

Nitrogenous gas ($^{15}N_2O$ or $^{15}N_2$) was calculated using ^{15}N atom % excess determined by mass spectrometry and the amount of total N (N_2O or N_2), taking into account the natural abundance of ^{15}N in replicate control (blank) samples.

$$^{15}N_2O = {}^{(14+15)}N_2O \times \%\ \text{atom}\ ^{15}N\ \text{excess} \qquad \text{(Equation 6.1)}$$

The production of $^{15}N_2O$ and the reduction of $^{15}N_2O$ to $^{15}N_2$ (corresponding to $^{15}N_2$ production) were estimated from the accumulation of each nitrogenous gas during the experiment. The reduction of NO_3^- to N_2O and reduction of N_2O to N_2 assessed were;

$$^{15}NO_3^- \longrightarrow {}^{15}N_2O \longrightarrow {}^{15}N_2 \qquad \text{(Equation 6.2)}$$

As N_2 production was exclusively derived from N_2O, it was assumed that both gases have the same isotopic composition, i.e., that the ratio of N_2O/N_2 was equal to the measured ratio of $^{15}N_2O/^{15}N_2$.

6.2.6 Determining the activation energy (E_a) of a reaction

The rate of a reaction, i.e., N_2O reduction, depends on the temperature. As temperature increases, the molecules move faster and collide more frequently. The molecules, which carry kinetic energy, requires energy to start a reaction, defining as the 'activation energy (E_a). The relationship between temperature (T) and the rate of a reaction (k) was modelled by Arrhenius (1889), as:

$$k = A\, e^{\frac{-E_a}{RT}} \qquad \text{(Equation 6.3)}$$

where A is a constant, E_a (kJ mol $^{-1}$) the apparent activation energy, R is the universal gas constant (0.082 J mol^{-1} K^{-1}), and T is the absolute temperature (K).

The activation energies for a reaction (i.e., for N_2O reduction) were determined from Arrnenius equation by taking the natural logarithm of both side of equation.

$$\ln(k) = -\frac{E_a}{R}\left(\frac{1}{T}\right) + lnA \qquad \text{(Equation 6.4)}$$

According the *equation 6.4*, a plot of ln(k) versus 1/T should give a straight line with a slope of –Ea/R, as:

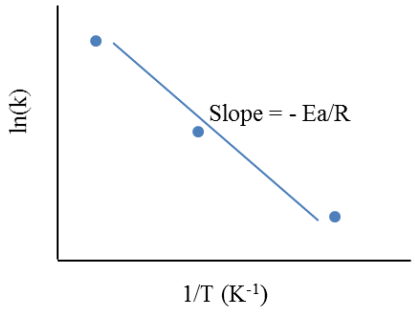

Figure 6.1. The relationship between the rate of a reaction (k) and temperature.

6.2.7 Calculation of oxygen (O_2) availability

The partial pressure of O_2 availability was calculated from the Universal Gas Law, as:

$$P = \frac{nRT}{V} \qquad \text{(Equation 6.5)}$$

Where P is the pressure at certain the volume (atm) , n is number of mole, R is the Universal Gas Constant (0.082 J mol^{-1} K^{-1}), T is the temperature (K) and V is the volume (L).

During soil respiration, microorganisms consume O_2 and release CO_2, as;

$$C_6H_{12}O_6 + 6O_2 = 6CO_2 + 6H_2O + \text{energy} \quad \text{(Equation 6.6)}$$

The partial pressure of O_2 is expected to reduce with rising CO_2 production; and O_2 consumption (mole) is equal to CO_2 production (mole) at each sampling time (t). Change in the partial pressure of O_2 (P_t) and its volume (V_t) can be expressed as;

$$\frac{P_t}{P_0} = \frac{V_t}{V_0} \quad \text{(Equation 6.7)}$$

Where P_0 and V_0 are, respectively, the pressure of O_2 (20.3 kPa) and volume of O_2 (0.21 L) at t = 0; P_t and V_t are the pressure (kPa) and volume of O_2 (L) at measurement time (t), respectively.

6.2.8 Statistical analysis

Two-way ANOVA was used to determine the interaction between water contents and temperature treatments. The Tukey's test was applied *post hoc* to identify significant differences among treatments and to determine whether N_2O production, reduction and total denitrification rates varied significantly with soil water content and temperature. Statistical tests on ratios such as N_2O/N_2 were based on log-transformed data. All statistical analysis was performed using Statistix 10 (Tallahassee, USA).

6.3 Results

The use of isotopically labelled nitrate in the incubation allowed measurement of three key gaseous products of denitrification: (i) total N_2O ($^{15}N_2O$ + $^{14}N_2O$), (ii) isotopically labelled N_2O ($^{15}N_2O$) and (iii) isotopically labelled N_2 ($^{15}N_2$) under different temperature and soil moisture content conditions.

6.3.1 The production of total N_2O

Total N_2O production was relatively low for the first 3 days of the incubation at all temperatures in the soil kept at 60% FC (Figure 6.2a). After 3 days, N_2O production increased at all temperatures, particularly at 35 °C and 40 °C. The average rate of N_2O production over the period 3 – 7 days was three times that over the period 0-3 days in the treatments at 35 °C and 40 °C. From day 7 – 10, N_2O production increased at these temperatures while rates of N_2O production did not change at 25 °C and 45 °C over this period. Cumulative N_2O production after10 days of incubation was greatest at 35 °C (0.75 µg N_2O-N g^{-1} soil), followed by 40 °C (0.55 µg N_2O-N g^{-1} soil), and around 2.0 µg N_2O-N g^{-1} soil at 25 °C and 45 °C (Figure 6.2a).

There was a significantly higher rate of N_2O production at 75% FC than at 60% FC all temperatures (Figure 6.2b). Divergence in N_2O production was observed at different temperatures after 3 days of incubation. In contrast to the slower rates of N_2O production at 60% FC, N_2O was produced at a constant rate at 25 °C over the course of the experiment at 75% FC. Rates of N_2O production at this FC increased at the higher temperatures after day 3. The average of N_2O production after10 days was 1.0 – 1.2 µg N_2O-N g^{-1} soil between 35 °C and 40 °C, 0.9 µg N_2O-N g^{-1} soil at 45 °C and 0.55 µg N_2O-N g^{-1} soil at 25 °C (Figure 6.2b).

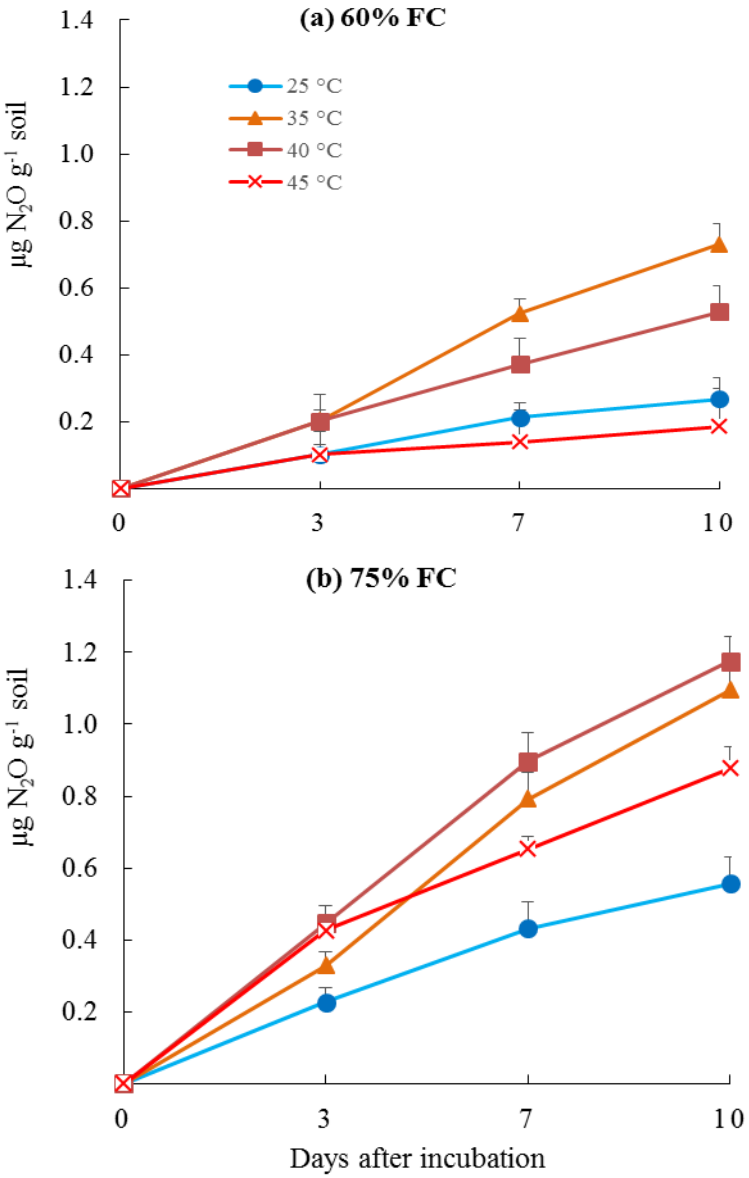

Figure 6.2. Cumulative N_2O production over time in response to temperature and soil moisture following addtion of N fertiliser to soil at100 µg N g^{-1} soil as $^{14}NH_4{}^{15}NO_3$. Soil moisture was maintained at 60 % FC (a) and 75% FC (b).

6.3.2 $^{15}N_2O$ production during denitrification

The rate of $^{15}N_2O$ during denitrification was influenced by both soil moisture and temperature over 10 days of incubation (Table 6.1).$^{15}N_2O$ was produced slowly at 25 °C and 45 °C over the first 7 days while the daily rate of $^{15}N_2O$ between 35 °C and 40 °C was 1.0 – 1.6 ng N. The greatest $^{15}N_2O$ production was at 35 °C and between 35 °C and 40 °C in soils treated at 60% and 75% FC, respectively. From days 7 – 10, $^{15}N_2O$ production slowed at all temperatures.

6.3.3 $^{15}N_2O$ reduction during denitrification

The rate of $^{15}N_2O$ reduction from denitrification (corresponding to $^{15}N_2$ production) was less than $^{15}N_2O$ production at all temperatures, except at 45 °C, where $^{15}N_2$ production was equal or higher than $^{15}N_2O$ production (Table 6.1). The magnitudes of N_2 production did not differ between 60% and 75% FC treatments at all temperatures (Table 6.1& Figure 6.3). Cumulative N_2 production after 10 days of incubation was positively correlated with temperature ($r = 0.68$, $p < 0.01$, Figure 6.3).

The activition energy (E_a) for N_2 production varied from 47 to 72 kJ mol^{-1} from 25 °C to 45 °C (Table 6.2). Activition energy for N_2O reduction estimated between 25 °C and 35 °C did not differ that at 35-45 °C ($p > 0.05$, Table 6.2). E_a was similar at the soil moisture contents (Table 6.2).

Table 6.1. Average of rates of $^{15}N_2O$ and ^{15}N production from denitrification in soil at different temperatures and water contents after addition 100 as $NH_4{}^{15}NO_3$.

Days	Temperature (°C)	60% FC		75% FC	
		$^{15}N_2O$ *(ng N g^{-1} d^{-1})*	**$^{15}N_2$** *(ng N g^{-1} d^{-1})*	**$^{15}N_2O$** *(ng N g^{-1} d^{-1})*	**$^{15}N_2$** *(ng N g^{-1} d^{-1})*
0 – 3	**25**	**0.52** ± 0.26 *bc*	**0.15** ± 0.05 *a*	**0.54** ± 0.03 ***b***	**0.14** ± 0.01 *c*
	35	**1.45** ± 0.22 *a*	**0.26** ± 0.05 *a*	**1.22** ± 0.28 ***a***	**0.28** ± 0.03 *c*
	40	**1.05** ± 0.25 *ab*	**0.26** ± 0.04 *a*	**1.27** ± 0.26 ***a***	**0.32** ± 0.04 *b*
	45	**0.20** ± 0.11 *c*	**0.25** ± 0.14 *a*	**0.58** ± 0.05 *b*	**0.52** ± 0.02 *a*
3 – 7	**25**	**0.44** ± 0.21 *c*	**0.27** ± 0.05 *b*	**0.48** ± 0.13 *b*	**0.22** ± 0.02 *c*
	35	**1.55** ± 0.26 *a*	**0.30** ± 0.11 *b*	**1.64** ± 0.24 ***a***	**0.27** ± 0.04 *bc*
	40	**1.02** ± 0.12 *b*	**0.34** ± 0.15 *b*	**1.56** ± 0.23 ***a***	**0.39** ± 0.03 *b*
	45	**0.40** ± 0.06 *c*	**0.64** ± 0.13 *a*	**0.57** ± 0.15 ***b***	**0.56** ± 0.04 *a*
7 – 10	**25**	**0.47** ± 0.12 *ab*	**0.13** ± 0.04 *b*	**0.37** ± 0.14 ***a***	**0.11** ± 0.02 *b*
	35	**0.93** ± 0.19 *a*	**0.25** ± 0.11 *b*	**0.69** ± 0.12 ***a***	**0.15** ± 0.04 *b*
	40	**0.74** ± 0.14 *a*	**0.31** ± 0.13 *b*	**0.55** ± 0.05 ***a***	**0.24** ± 0.03 *ab*
	45	**0.16** ± 0.02 *b*	**0.57** ± 0.12 *a*	**0.23** ± 0.02 ***b***	**0.38** ± 0.01 *a*
$HSD_{0.05}$		**0.48**	**0.22**	**0.51**	**0.16**

Different letters indicate significant difference among temperatures within each sampling time α =0.05.

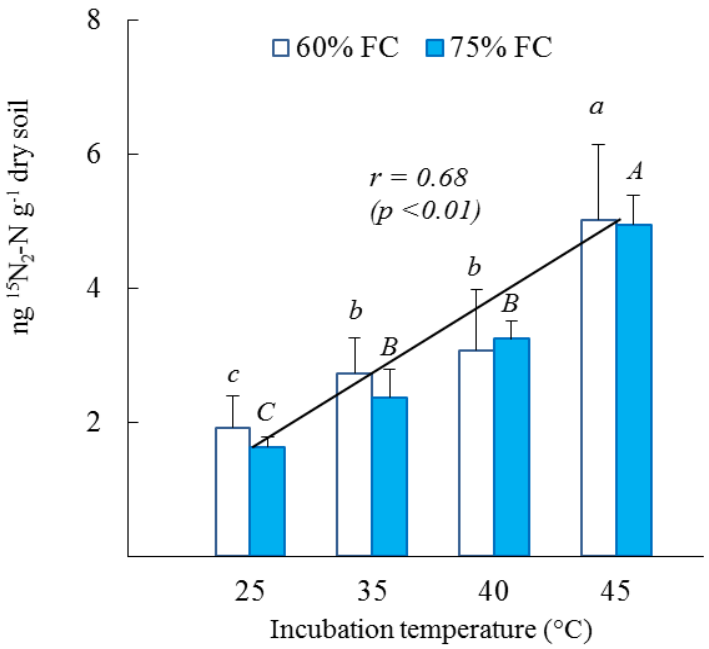

Figure 6.3. Cumulative ^{15}N-N_2 from denitrification over the 10 days of incubation at different temperatures and soil water contents following the addition of 100 µg N g^{-1} soil as $NH_4{}^{15}NO_3$. Different letters indicate significant differences among temperatures (lower letters for 60% FC and capital letters for 75% FC). There was no significant difference between the two field capacities at all temperatures. A trendline indictes the relationship between temperature and $^{15}N_2$ production. Vertical bars represent +1 SE (n = 4).

Table 6.2. The activation energy (E_a) for N_2O reduction during denitrification between 25 to 45 C in two water contents in the tested soil. Each value has a standard error of the mean followed it.

Time of incubation (days)	**60% FC**	**75% FC**	**Sig.**
0 – 3	**21.25** ± 12.2	**49.24** ± 12.5	*ns*
3 – 7	**29.41** ± 10.1	**35.94** ± 10.2	*ns*
3 – 10	**55.10** ± 13.4	**47.83** ± 16.5	*ns*

ns: non-significant difference (p<0.05)

6.3.4 Total denitrification

Cumulative denitrification rate ($^{15}N_2O$ + $^{15}N_2$) after 10 days of incubation varied from 6 to 16 ng ^{15}N g^{-1} soil with the treatments (Figure 6.4). Total denitrification rate was around 6 ng ^{15}N g^{-1} soil at 25 °C. Cumulative denitrification rate increased with rising temperature from 25 °C to 35 °C at the both FC tested. Denitrification responded differently to higher temperatures in soils, depending on moisture content. At 60% FC the rates decreased above 35 °C but at 75% FC the rates remained stable between 35 °C and 40 °C then decline at 45 °C ($p > 0.05$, Figure 6.4). Temperature interacted with soil moisture to control total denitrification rate ($p < 0.05$, Figure 6.4). The majority of N losses via denitrification was N_2O at all temperatures, except for 45 °C where N_2O production was almost equal to N_2 production.

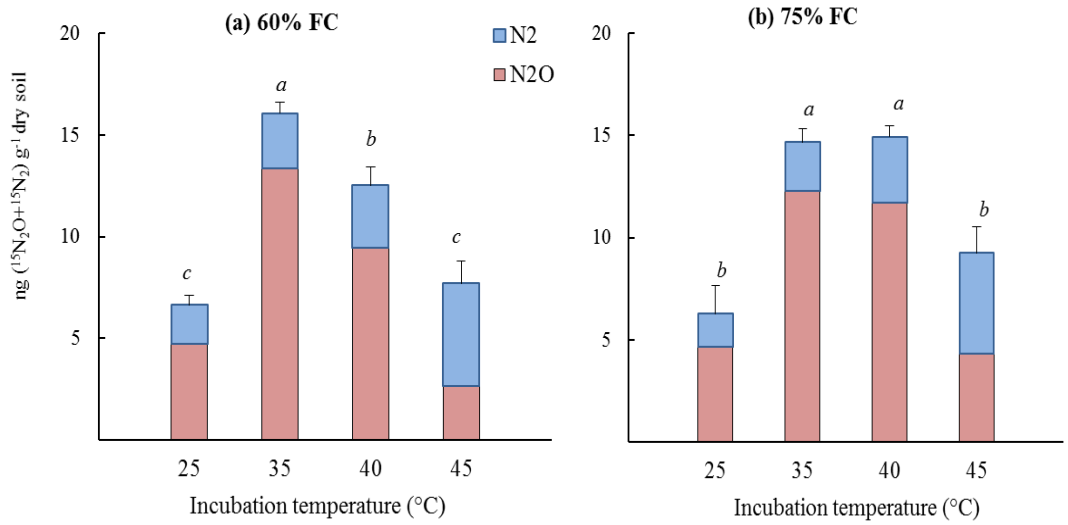

Figure 6.4. Cumulative ^{15}N-(N_2O+N_2) production from denitrification in soil incubated at different temperatures and soil moisture contents after 10 days following the application of 100 µg N g^{-1} soil as $NH_4{}^{15}NO_3$. Two-way ANOVA identified an interactive effect between temperature and soil moisture to control $^{15}N_2O$ production ($p < 0.05$). Different letters indicate significant differences in total N losses as $^{15}N_2O$ and $^{15}N_2$ among temperatures. Vertical bars indicate +1 SE (n = 4).

6.3.5 Soil respiration rate and O_2 availability

The rate of CO_2 production, a measure of soil respiration rate, in response to temperature and soil moisture, is shown in Figure 6.5. The soil respiration rates during the experiment were greatly influenced by temperature in both 60% and 75% FC treatments. The highest soil respiration rate was found at 45 °C, followed by 40 °C and 35 °C and the lowest CO_2 produciton was measured at 25 °C at all sampling times; the patterns were similar at both soil moisture contents. From days 7 – 10, soil moisture content impacted on the response of CO_2 production to temperature, as the rate of CO_2 production at 25 °C slighly decreased at 60 % FC while it increased under wetter soil (Figure 6.5a).

The concentration of O_2 availaibility decreased with increased CO_2 production (Figure 6.5c). As CO_2 production varied from 50 – 160 µg C g^{-1} soil, often occurred at 25 – 35 °C, the pressure of O_2 was calculated at 16 – 18 kPa. The concentration of O_2 decreased to 14 kPa since CO_2 was cumulative at 200 – 250 µg C g^{-1} soil, which was often measured between 35 °C and 40 °C. At the highest soil respiration rate, ~500 µg C g^{-1} soil at 45 °C and 75% FC, the partial pressure of O_2 concentration was 6 kPa (Figure 6.5c).

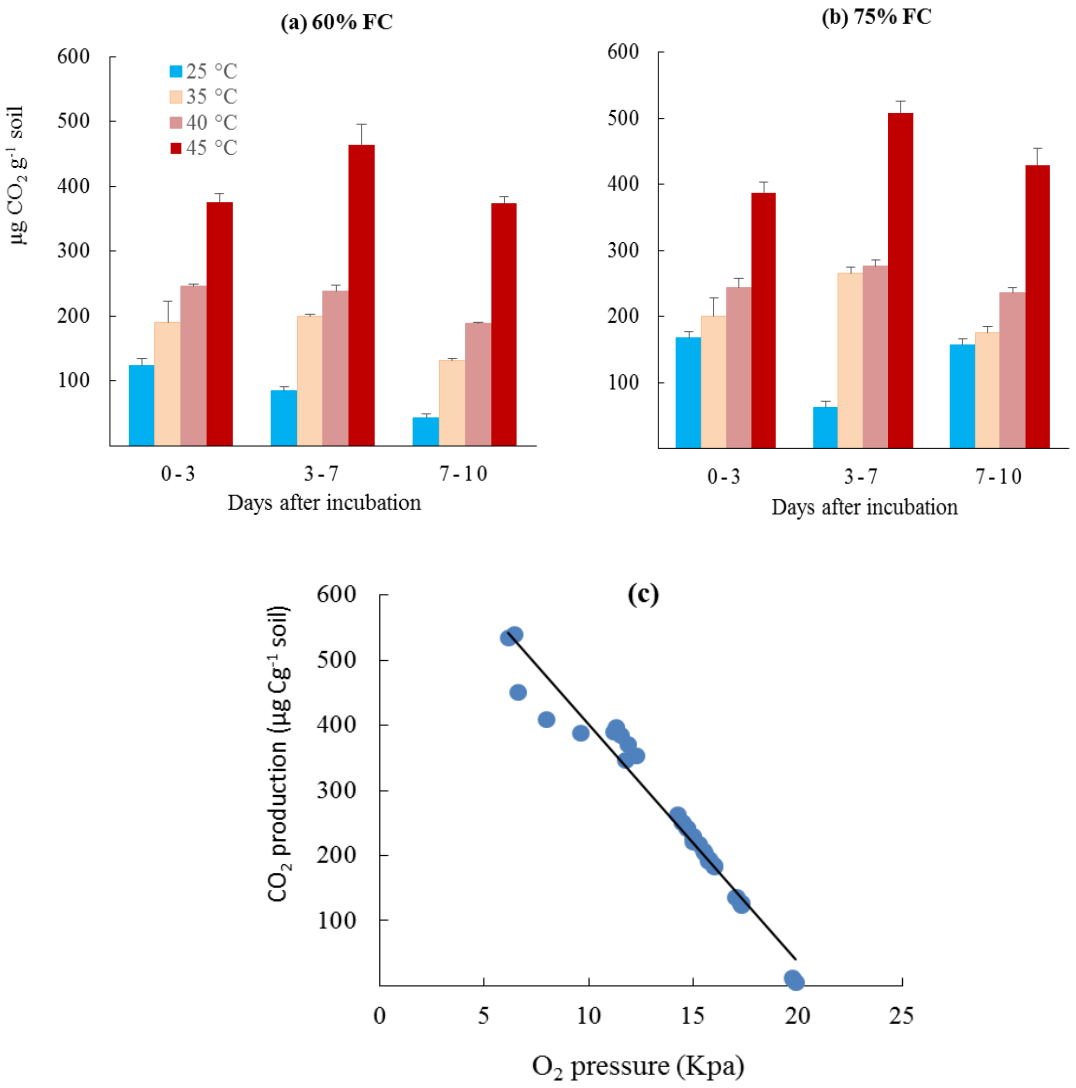

Figure 6.5. Cumulative CO_2 production in response to temperatures at different soil moisture contents (a & b) and estimated O_2 availability (c) during the experiment. Soil samples had addition of 100 μg N g^{-1} soil as $NH_4{}^{15}NO_3$. Error bars are +1 SE (n = 4).

6.3.6 Soil nitrate contents

The average soil NO_3^- content after 10 days of incubation ranged from 23 to 46 µg N g^{-1} soil across all treatments (Figure 6.6). The concentration of NO_3^- increased with rising temperature from 25°C to 35 °C at 60% FC and from 25 °C to 40 °C at 75% FC. Thus, soil NO_3^- concentration was higher at 40 °C than at lower temperatures in soil at 75% FC while NO_3^- concentration did not differ between 35 °C and 40 °C at 60% FC. NO_3^- concentrations decreased above 40 °C both 60% FC and 75% FC (Figure 6.6).

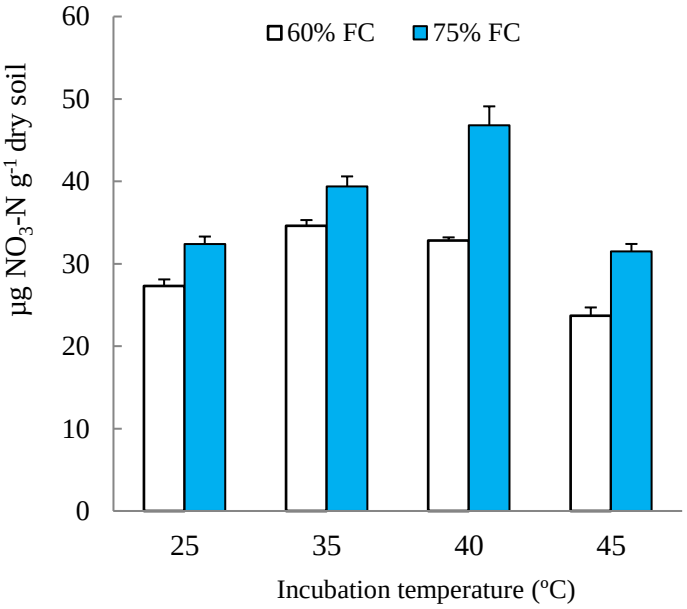

Figure 6.6. Nitrate availability (µg NO_3-N g^{-1} soil) exposed to different temperatures and soil moisture contents after 10 days of incubation. Soil NO_3^- content measured on day 0 was 25.7 ± 0.3 µg N g^{-1} soil.

6.4 Discussion

Our study identified that N_2O emissions were major N losses during denitrification. The results demonstrated that temperature interacted with soil moisture to control N_2O production from denitrification. The production of N_2O at 35 °C was similarly high as 40 °C in soil treated at 75%, while N_2O decreased above 35 °C at 60% FC, suggesting the influence of increased anaerobic zones on the rate of N_2O production. A greater anaerobic conditions at 75 % FC potentially allowed more NO_3^- to act as a termial electron acceptor and resulted in higher N_2O production, particularly at 35 to 40 °C, than at 60% FC, where higher aerobic microsites might have suppressed denitrification. Our results support the hypothesis of de Klein and Van Logtestijn (1996) that the highest denitrification rates likely occur under wet and warm conditions as a result of an interaction between soil moisture and temperature. During summertime under field conditions, often experienced temperatures between 30 °C to 45 °C (**Chapter 2**), high N losses as N_2O emission from denitrification may be expected once soil moisture is around 0.31 cm^3 cm^{-3} (or 75% FC). Soil water management by remaining at 0.25 cm^3 water cm^{-3} soil during the periods associated with high temperatures can reduce N loss as N_2O emisison from denitrification.

Temperature, rather than soil moisture, impacted on the reduction of N_2O to N_2 during denitrification in the present study. This N_2O reduction was previously estimated by using C_2H_2 (10% v/v) to inhibit N_2O reductase (N_2OR), in many studies (Keeney et al., 1979; Avalakki et al., 1995; Maag and Vinther, 1999; Rudaz et al., 1999; Dobbie and Smith, 2001). The thermal response of N_2 production is a challenging task in studies applied the C_2H_2 method because the incomplete activity of N_2OR may occur if there is uneven distribution of C_2H_2 in the whole soil. The results of this study, using an isotope-based approach, revealed $^{15}N_2$ production was positively correlated with increased temperature

up to 45 °C ($r = 0.68$, $p < 0.01$), regardless of soil moisture. This result suggested the thermal effect on N_2OR during denitrification by affecting the activation energy (E_a). Estimated E_a for N_2O reduction ranged from 47 to 74 kJ mol^{-1}, which is constent with previous values estimated by Holtan-Hartwig et al. (2002). Temperature may have an indirect effect on N_2OR by reducing oxygen availability in the soil microiste via soil respiration. Low O_2 pressure (< 2kPa) likely inhibits the activitiy of N_2OR, resulting in inhibition of N_2 production (Morley et al., 2008; Zhu et al., 2013). Our estimated O_2 pressures in the present study were much greater than 2 kPa, thus the activity of N_2OR was unaffacted by the deleption of O_2 concentration induced by soil respiration at high temperatures. A positive relationship between N_2 production and temperature implies that the proportion of N losses as N_2 would vary seasonally and a greater loss as N_2 would be associated with temperatures measured (range 35 ºC to 45 ºC) in summer (December to February) in the tested soil.

Large soil nitrogen losses as N_2O via denitrification occurred in the first 7 days, then slowed at days 7 – 10, suggesting that management of N_2O should be targeted in the first week after N fertiliser application. Our results reflect that effective land management that maintains low soil water during the period associated with high temperatures would reduce N_2O emissions. A decline in N_2O production from denitrification after day 7 could be related to the deleption of OC, as observed by CO_2 production. The increased N_2 production with rising temperature contributed to decreased N_2O emissions, which need further study in relation to other factors in soil such as copper (Cu), ferric ion (Fe^{2+}), or molybdate ion (Mo^{2+}). These ions may have a significant impact on the activitiy of N_2OR (Ferguson, 1998). Soil with insufficient Cu, which is used as co-factors of NosZ, would lead to incompete assembly of NosZ, inhibiting the reduction of N_2O (Richardson et al., 2009). An improved knowledge of the impact of these metals and temperature on N_2OR

may help in the development of strategies to reduce N losses, as N_2O emissions from agricultural systems. Further work aimed at understanding the expression of these enzymes may consider the presence of metal ions in conjunction with temperature. The optimal conditions for the reduction of N_2O to N_2 would contribute to reduce N_2O emisions from agricultural soil.

6.5 Conclusions

The results in our study demonstrated that temperature primarily controlled denitrification activity. The rate of denitrification increased with rising temperature from 25 °C to 35 °C, regardless of soil moisture, but denitrification responded differently to higher temperatures in soils, depending on moisture content. Denitrification rates decreased above 35 °C in soil treated at 60% FC but the rates remained stable between 35 °C and 40 °C then declined at 45 °C and 75% FC. The magnitudes of N_2O production at 75% FC exceeded those at 60% FC at all temperatures, due to greater anaerobic conditions. The reduction of N_2O to N_2 was enhanced by increasing temperature up to 45 °C while soil moisture beweeen 60% and 75% FC had little impact on N_2 production. The results suggest that the greatest denitrification likely occurrs under wet and warm conditions, where temperature may interact with soil moisture to promote the activity of denitrification, particularly N_2O emission. Soil water management by keeping low as 0.25 cm^3 water cm^{-3} soil during the periods exprienced with high temperatures may slow N loss as N_2O emisison via denitrification and reduce N losses from the system.

Statement of Authorship

Title of Paper	N_2O and N_2 production from denitrification respond differently to soil temperature and nitrate supply
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Principal Author

Name of Principal Author (Candidate)	Thang Lai
Contribution to the Paper	Conducted experiments, performed analysis on samples interpreted data, wrote manuscript and acted as corresponding author
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 10/9/2016

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Matthew Denton
Contribution to the Paper	Supervised development of work, helped in data interpretation and manuscrip evaluation.
Signature	Date 14/9/2016

Name of Co-Author	
Contribution to the Paper	
Signature	Date

Please cut and paste additional co-author panels here as required.

Chapter 7 N_2O and N_2 production from denitrification respond differently to soil temperature and nitrate supply*

7.1 Introduction

Denitrifiers use NO_3^- as a substrate during denitrification, which results in the depletion of NO_3^- around the microsite, where denitrification occurs. NO_3^- is highly mobile in soil and can move rapidly from the bulk soil to microsites in wet conditions. Nitrate can potentially act as a terminal electron acceptor (TEA) when O_2 becomes limiting (Cho et al., 1997a; Strong and Fillery, 2002). Total denitrification rate (N_2O+N_2) generally increases with increased the amounts of NO_3^- amendment (Luo et al., 1996a; Scholefield et al., 1997; Smith et al., 1998; Zhu et al., 2013). Changes in soil NO_3^- concentrations have significant impacts on the denitrification product ratios, i.e., N_2O/N_2 ratio. Increased soil NO_3^- often results in high N_2O production and increased N_2O/N_2 ratios (Gillam et al., 2008), due mainly to the inhibition of the activity of N_2O reductase (Blackmer and Bremner, 1978) and/or a preference for NO_3^- as a TEA in N_2O production (Cho et al., 1997a). Thus, soil NO_3^- concentration is commonly identified as one of the important factors in the regulation of total denitrification rate and partitioning of gaseous N losses from denitrification.

The impact of temperature on the partitioning of gaseous N losses as N_2O and N_2 during denitrification has been observed in some previous studies but the data are conflicted. Studies by Focht (1974) and Rudaz et al. (1999) reported no significant effects of temperature on denitrification products, while others (Keeney et al., 1979; Avalakki et

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al., 1995; Dobbie and Smith, 2001) have identified that low temperatures (< 15 °C) favoured N_2O formation, resulting in greater N_2O/N_2 ratios than at higher temperatures. A decrease in N_2O/N_2 ratios were found when temperatures increased above 15 °C (McKenney et al., 1984; Maag and Vinther, 1996). Temperature may have an impact on the N_2O/N_2 ratios by affecting the apparent activation energy, the energy required for a reaction to occur, for N_2O production and N_2O reduction to N_2 during denitrification (Holtan-Hartwig et al., 2002). Rising temperature can also enhance soil microbial respiration by simulating high O_2 consumption, resulting in O_2 deficiency at the denitrifying microsites, which potentially allows the supply of NO_3^- to act as a TEA and inhibits the reduction of N_2O to N_2. Soils in southern Australia experience temperatures above 35 °C during summertime (see **Chapters 3 & 4**), that could impact on the reduction of NO_3^- and N_2O during denitrification, particularly following NO_3^- fertiliser application, although there are currently no data on the total N denitrified and the partitioning of gaseous N losses to N_2O and N_2 at these temperatures.

Although the impact of soil NO_3^- concentration and temperature as individual factors on the activity of denitrification are generally recognised, little is known as to whether these factors interact to control N_2O and N_2O/N_2 ratios. Several studies (Malhi et al., 1990; Weier et al., 1993; White et al., 2002; Gillam et al., 2008) have tested the impact of NO_3^- on denitrification rate and the N_2O/N_2 ratio under constant temperatures. Others (Avalakki et al., 1995; Rudaz et al., 1999; Holtan-Hartwig et al., 2002) have evaluated the effect of a range of temperatures on denitrification at a single NO_3^- concentration. The combined effect of temperature and NO_3^- availability on the total denitrification rate and the N_2O/N_2 ratios therefore remains to be tested.

In the study reported in this chapter, a dermosol collected from a pasture was supplied with ^{15}N in the equivalent rates of 0 – 150 kg N ha^{-1} and incubated at temperatures ranging

from 25 °C to 45 °C in a short-term experiment. Denitrification gaseous products ($^{15}N_2O$ and $^{15}N_2$) were measured during the experiment to evaluate their thermal responses to different N availability.

7.2 Methods

7.2.1 Study sites and sample collection

The soil used in this study is a black dermosol (Isbell, 1996), collected from a dairy pasture farm in Victoria, Australia. Some characteristics of this soil are presented at Table 4.1 (**Chapter 4**). The annual variations in daily soil temperature and monthly water content from the field site are shown in Figure 4.1 (**Chapter 4**).

7.2.2 Laboratory incubation

7.2.2.1 Pre-incubation

Loose soil samples were wetted up to 60% $FC_{33\ kPa}$ (0.21 $cm^3\ cm^{-3}$ soil) then placed into a plastic bag with holes to allow gas exchange with the atmosphere. All soil samples were pre-incubated for 7 days at 25 °C to re-establish soil microbial activity. Soil moisture was checked every three days and water added if necessary to maintain the soil at 60% FC.

7.2.2.2 Incubation

Soil subsamples (50 g dry weight equivalent) were repacked in a core (ø37 mm × 42 mm; 10.75 cm^2 surface area) to a bulk density of 1.12 g cm^{-3}, equivalent to that measured in the field. Isotopically labelled nitrate solution ($^{14}NH_4{}^{15}NO_3$) was added slowly onto the top of upright cores to achieve the additions of 0, 100, 200 and 300 µg N g^{-1} soil (equivalent to 0, 50, 100, 150 kg N ha^{-1}). This solution increased soil moisture to 0.32 $cm^3\ cm^{-3}$ (or 75% FC), which reflects the average of soil moisture in the field during summer. Soil cores were then placed into 250ml jars equipped with a rubber septum

(Subaseals#25, Sigma-Aldrich, St Louis, MO, USA) in the cap. Each set of jars with various N treatments were incubated at 25, 35 or 45 ºC for 10 days. There were 12 treatment combinations (4 N × 3 temperature treatments) with 4 replications.

Two sets of soil incubations were established to monitor gas production and mineral nitrogen transformations during the experiment.

7.2.3 Sampling and analysis

In the first set of incubations, the headspace of 36 jars (three levels of NO_3^- × three temperatures × four replicates) was sampled on 0, 3, 7 and 10 days after addition of the N solution. A 20 ml sample of headspace gas was taken using a Hamilton 50 ml gas-tight syringe (Hamilton Company, Reno, NV, USA); and transferred into a pre-evacuated 12 ml Exetainer (Labco, Ceredigion, UK) for measurement of $^{14+15}N$-N_2O, ^{15}N-N_2O and ^{15}N-N_2. After gas sampling, the headspace of all jars was refreshed using a mini-fan for one minute. In the second set of incubations, soil samples were taken on day 0 and 10 to measure NH_4^+ and NO_3^- concentrations.

7.2.4 Gas measurement

The concentration of N_2O was analysed on a Varian 450 Gas Chromatograph (Agilent 7890, Bremen, Germany), as described in Section 3.2 (**Chapter 3**).

Trace gas isotope ratios ($\delta^{15}/\delta^{14}N$) were determined using a ThermoFinnigan GasBench and a Delta V^{TM} Isotope-ratio Mass Spectrometer (Thermo-Scientific, Bremen, Germany) (**Chapter 4**).

The estimation of nitrogen gases rates and O_2 availability are described in section 6.3 (**Chapter 6).**

7.2.5 Statistical analysis

Two-way ANOVA was used to determine the interaction between NO_3^- contents and temperature treatments. The Tukey ($HSD_{0.05}$) test was applied *post hoc* to identify any significant difference between treatments and to determine whether N_2O production, N_2O reduction and total denitrification varied significantly with NO_3^- content and temperature. Statistical tests on ratio such as N_2O/N_2 were based on log-transformed data. All statistical analyses were performed using Statistix 10 (Tallahassee, USA).

7.3 Results

7.3.1 Mineral nitrogen pools

The concentrations of NH_4^+ in soil after 10 days of incubation was influenced by temperature (Table 7.1). The amounts of NH_4^+ did not differ between 25 °C and 35 °C at all N supply. NH_4^+ concentration was much higher at 45 ºC than at 25 ºC to 35 ºC, regardless of N amendments. At 45 ºC, the net rates of NH_4^+ ranged from 27 to 35 µg N g^{-1} d^{-1} (Table 7.1). Unlike the response of NH_4^+, NO_3^- concentration increased at all temperatures in the control and supplied with 100 µg N g^{-1} soil (N_{100}). The greatest increase in NO_3^- was found at 35 °C in soil with N_{100}, followed by 25 °C and at least at 45 °C. In soils added with N_{200} and N_{300}, more NO_3^- accumulated in soil incubated at 25 °C compared to that at 35 ºC, but NO_3^- decreased at 45 ºC (Table 7.1).

7.3.2 Overall N_2O production

The addition of N supply to soil affected the temperature response of total N_2O production (from all processes) over the 10 days of incubation (Figure 7.1). In the soil without N application (control_N_0), N_2O production was relatively low and consistent at 25 °C throughout the experimental period but increased similarly with rising temperature at 35

°C and 45 °C between 3 and 7 days. The production of N_2O increased in the soil with N_{100} (equivalent to 50 kg N ha^{-1}) compared with N_0 (Figure 7.1). The average cumulative N_2O production after 10 days at 35 °C and 40 °C was ~1.0 µg N_2O–N g^{-1} soil in N_{100}. In the soils with 200 – 300 µg N g^{-1} soil added (equivalent to 100 – 150 kg N ha^{-1}, respectively), N_2O production responded similarly to temperature (Figure 7.1). The production of N_2O at 35 °C in N_{200} and N_{300} increased greatly with incubation time. N_2O production at 25 °C and 45 °C in N_{200} and N_{300} increased from 0 – 7 days, then attenuated from 7 – 10 days, and reached 0.8 µg N_2O–N g^{-1} dry soil on day 10. The average of cumulative N_2O production measured over the 10 days at 35 °C was 1.6 and 2.3 µg N_2O–N g^{-1} soil in N_{200} and N_{300} respectively (Figure 7.1).

Table 7.1. The concentrations of ammonium and nitrate at days 0 and 10 days after incubation at different temperatures and N applications to soil at 0 (N_0), 100 (N_{100}), 200 (N_{200}) and 300 µg N g^{-1} soil (N_{300}) as $NH_4{}^{15}NO_3$. Soil moisture was maintained at 75% field capacity.

N applied	N form	Day 0	Day 10					
			25 ºC		35 ºC		45 ºC	$HSD_{0.05}$
N_0	**Ammonium** (µg N g^{-1} dry soil)	**7.82** ± 0.8	**7.41** ± 2.4	*dB*	**8.36** ± 2.9	*dB*	**283.48** ± 12.4	*cA*	**212.32**
N_{100}		**99.32** ± 3.2	**52.38** ± 3.6	*cB*	**40.91**± 6.2	*cB*	**409.23** ± 19.7	*bA*	**51.34**
N_{200}		**169.84** ± 3.2	**151.83** ± 9.4	*bB*	**174.84** ± 12.9	*bB*	**510.99** ± 37.5	*bA*	**79.69**
N_{300}		**272.38** ± 5.4	**319.19** ± 13.3	*aB*	**288.14** ± 15.1	*aB*	**627.69** ± 31.7	*aA*	**92.64**
$HSD_{0.05}$			**42.37**		**28.65**		**105.54**		
N_0	**Nitrate** (µg N g^{-1} dry soil)	**12.37** ± 1.1	**15.16** ± 2.2	*cB*	**22.28** ± 1.9	*cA*	**18.17** ± 2.0	*cAB*	**5.54**
N_{100}		**26.80** ± 1.2	**39.44** ± 1.2	*bB*	**46.79** ± 2.3	*bA*	**31.53** ± 0.9	*bcC*	**4.85**
N_{200}		**48.84** ± 2.0	**53.58** ± 1.3	*aA*	**58.98** ± 2.1	*aA*	**41.92** ± 1.8	*abB*	**7.74**
N_{300}		**55.55** ± 2.2	**62.88** ± 2.9	*aA*	**63.01** ± 2.32	*aA*	**50.15** ± 4.6	*aA*	**15.84**
$HSD_{0.05}$			**10.04**		**11.05**		**13.82**		

Lower case letters indicate significant differences among N applied at each temperature, Upper case letters indicate significant differences among temperatures within each N supply (α <0.05)

7.3.3 Net rate of $^{15}N_2O$ production

Temperature interacted with N supply to control net rate of $^{15}N_2O$ production from denitrification throughout the experimental period ($p < 0.05$, Figure 7.2). Net rate of $^{15}N_2O$ was produced at 0.5 – 0.9 ng N g^{-1} d^{-1} in soil with N_{100} in the first three days of incubation and there was no significant difference among temperatures. The net rates of $^{15}N_2O$ were significantly higher at 35 °C than at 25 °C over the period 3 – 7 days ($p < 0.05$) but slowed at all temperatures in the last 3 days in soil with N_{100}. At higher N supply, the daily net rates of $^{15}N_2O$ varied from 0.5 to 2.5 ng N g^{-1} d^{-1} with temperatures, except for 45 °C, where $^{15}N_2O$ was relatively low at the period days 7 – 10. The rates of $^{15}N_2O$ production at 35 °C exceeded the amounts at 25 °C and 45 °C at all measurements in soils with N_{200} and N_{300}. The net rate of $^{15}N_2O$ at 35 °C in these treatments was 3 fold higher than at 25 °C or 45 °C (Figure 7.2).

7.3.4 Rate of $^{15}N_2O$ reduction

Temperature was more important factor than N supply controlling the reduction of $^{15}N_2O$ to $^{15}N_2$ during denitrification (Figure 7.3). With N supply, the rate of $^{15}N_2O$ reduction increased with rising temperature in the first 7 days of incubation. The highest rate of $^{15}N_2O$ reduction was measured at 45 °C, average on 0.50 – 0.65 ng N g^{-1} d^{-1}, followed by 35 °C at 0.2 – 0.40 ng N g^{-1} d^{-1} and the lowest rate occurred at 25 °C, producing at 0.10 – 0.18 ng $^{15}N_2$–N g^{-1} d^{-1}. From days 7 – 10, the production of $^{15}N_2$ slowed at all temperatures (Figure 7.3). Thermal response of $^{15}N_2O$ reduction was unaffected by the addition of N, except for the control, where the rate of $^{15}N_2O$ reduction was below 0.05 ng N g^{-1} d^{-1} at all temperatures.

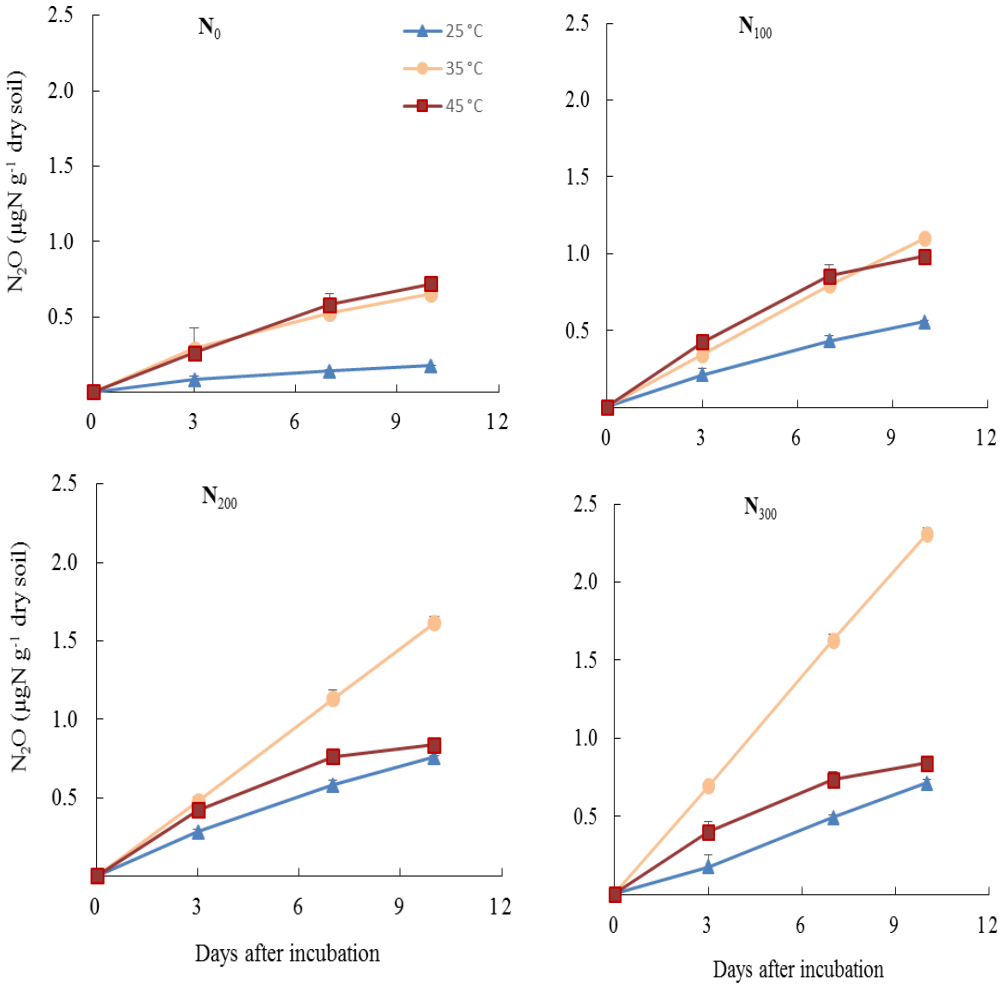

Figure 7.1 The cumulative production of N_2O over time in response to temperature following amendments of N fertiliser to soil at 0 (N_0), 100 (N_{100}), 200 (N_{200}) and 300 μg N g^{-1} soil (N_{300}) as $NH_4{}^{15}NO_3$. Soil moisture was maintained at 75 % field capacity. Error bars indicate +1 *SE* (n = 4).

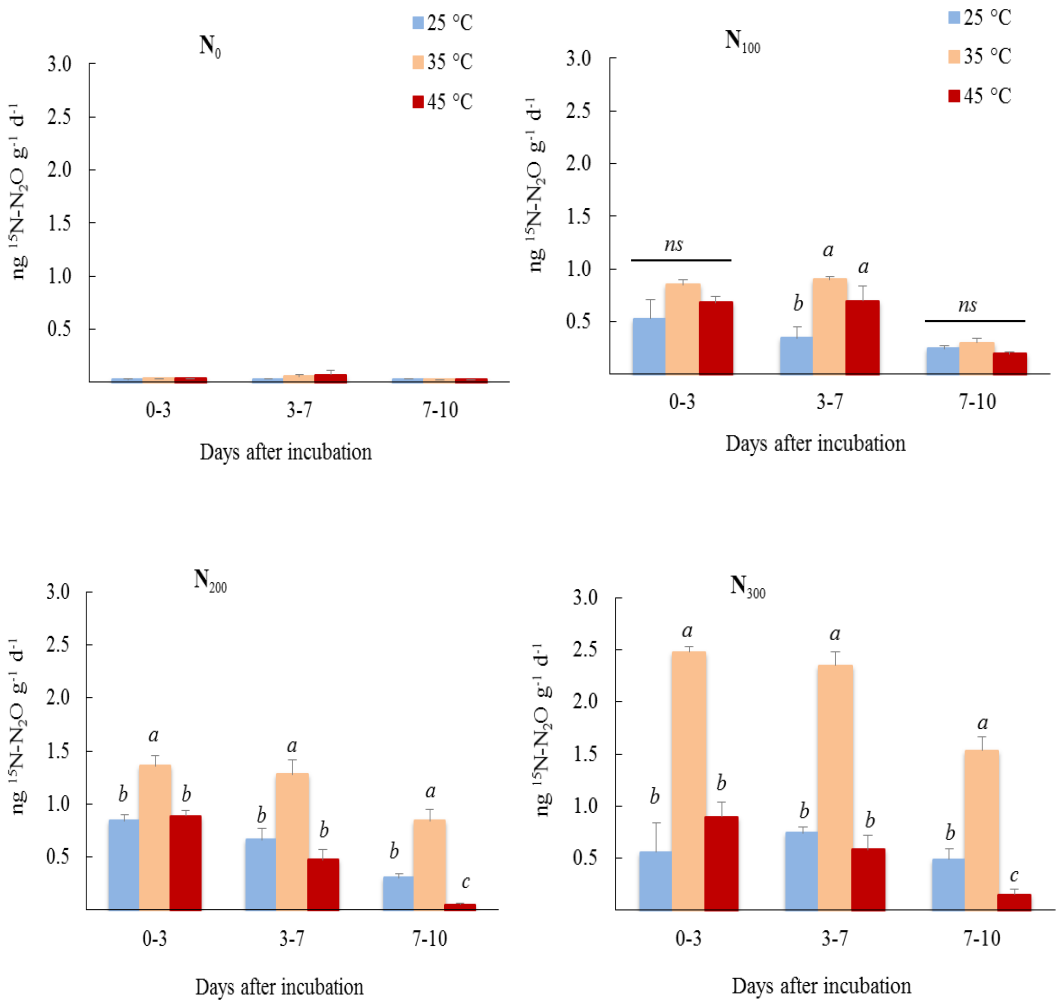

Figure 7.2 Daily rates of ^{15}N-N_2O over time in response to temperature following amendments of N fertiliser to soil at 0 (N_0), 100 (N_{100}), 200 (N_{200}) and 300 µg N g^{-1} soil (N_{300}) as $NH_4{}^{15}NO_3$. Soil moisture was maintained at 75 % field capacity. Error bars indicate +1 *SE* (n = 4). *ns*: non-significant difference; different letters indicate significant difference among temperatures within each N supply.

7.3.5 Total dentirification and N_2O/N_2 ratios

Total denitrification (N_2O + N_2) rates at all temperature were low in the control but increased between 0.45 to 2.66 ng N g^{-1} d^{-1} in the soils with N supply (Table 7.2). High $N_2O/(N_2O + N_2)$ ratios between 25 °C to 35 °C indicated that the major N losses via denitrification was N_2O emission, particularly with N_{300}. The proportion of N_2O to total denitrification at these treatments was around 0.8 (or accounting for 80% of total denitrification rate). The greatest denitrification rate was found at 35 °C in the soil with N_{200} and N_{300} (Table 7.2).

The changes in the N_2O/N_2 ratio were regulated by both N_2O production and reduction. The N_2O/N_2 ratios between 25 °C and 35 ºC were 3.2 to 7.8 due to high N_2O and low N_2 production. A decrease in N_2O production and increased N_2O reduction resulted in a low N_2O/N_2 ratio at 45 °C. $^{15}N_2$ production was nearly equal to $^{15}N_2O$ production (ratio 1.0 – 1.2) at 45 °C (Table 7.2).

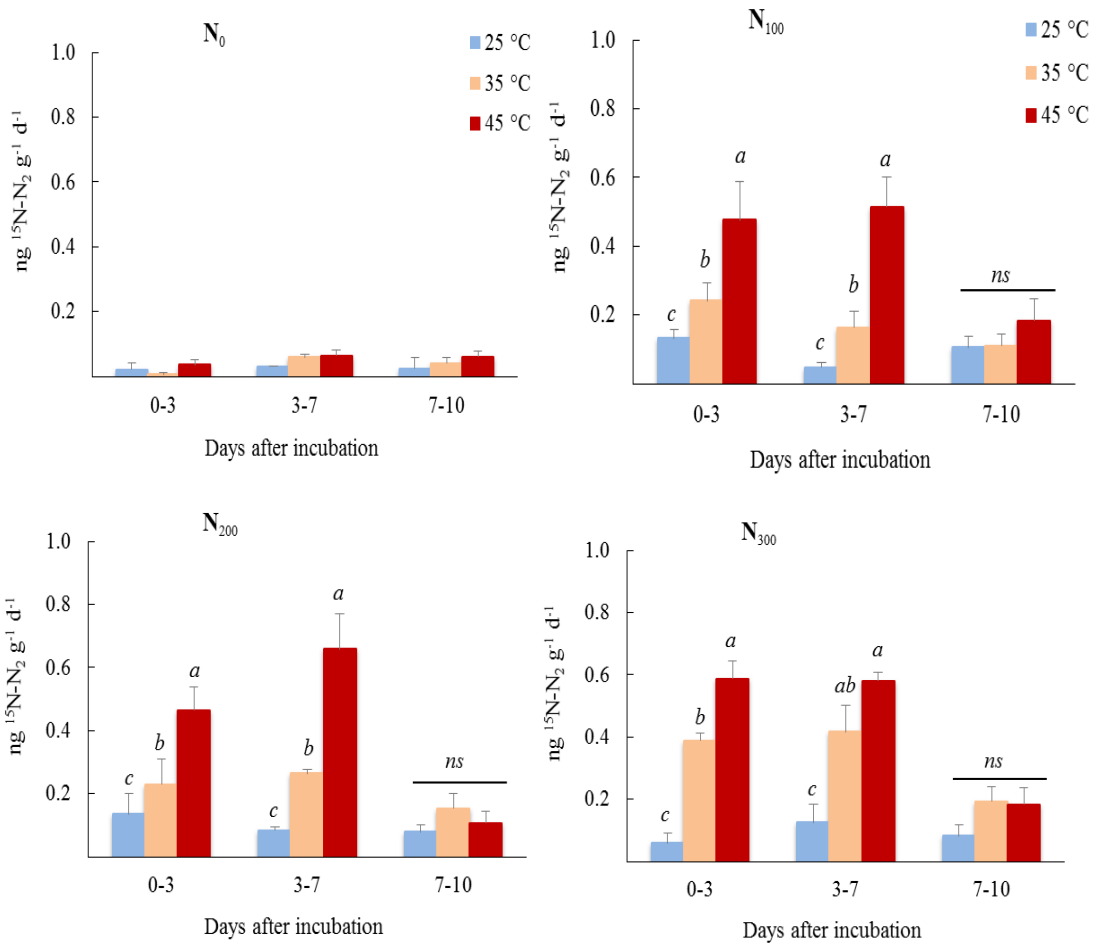

Figure 7.3 Daily rates of ^{15}N-N_2 over time in response to temperature following amendments of N fertiliser to soil at 0 (N_0), 100 (N_{100}), 200 (N_{200}) and 300 µg N g^{-1} soil (N_{300}) as $NH_4{}^{15}NO_3$. Soil moisture was maintained at 75 % field capacity. Error bars indicate +1 *SE* (n = 4). *ns*: non-significant difference; different letters indicate significant difference among temperatures within each N supply.

Table 7.2. Average of rates of ^{15}N-N_2O production, ^{15}N-N_2 production, the ratios of $N_2O/(N_2O+N_2)$ and N_2O/N_2 from denitrification in the first 7 days of incubation at different temperatures and N additions to soil at t 0 (N_0), 100 (N_{100}), 200 (N_{200}) and 300 µg N g^{-1} soil (N_{300}) as $NH_4{}^{15}NO_3$. Soil moisture was maintained at 75% field capacity (− 33 kPa).

Factors		**$^{15}N_2O$ production**	**$^{15}N_2$ production**	**$^{15}N_2O + {}^{15}N_2$**	**$N_2O/(N_2O+N_2)$**	**N_2O/N_2**
		ng N g^{-1} d^{-1}	*ng N g^{-1} d^{-1}*	*ng N g^{-1} d^{-1}*		
N applied	Temperature (°C)					
	25	**0.03** ± 0.002 *a*	**0.04** ± 0.02 *a*	**0.08** ± 0.03 *a*	**0.49** *a*	**1.2** *a*
N_0	**35**	**0.04** ± 0.007 *a*	**0.06** ± 0.01 *a*	**0.10** ± 0.02 *a*	**0.45** *a*	**0.8** *a*
	45	**0.05** ± 0.002 *a*	**0.06** ± 0.02 *a*	**0.11** ± 0.04 *a*	**0.33** *a*	**0.8** *a*
	25	**0.35** ± 0.10 *b*	**0.10** ± 0.02 *b*	**0.45** ± 0.10 *a*	**0.76** *a*	**3.6** *a*
N_{100}	**35**	**0.93** ± 0.04 *a*	**0.18** ± 0.04 *b*	**1.11** ± 0.07 *a*	**0.83** *a*	**5.2** *a*
	45	**0.44** ± 0.08 *b*	**0.42** ± 0.06 *a*	**0.86** ± 0.13 *a*	**0.50** *b*	**1.0** *b*
	25	**0.64** ± 0.07 *b*	**0.11** ± 0.03 *b*	**0.75** ± 0.08 *b*	**0.86** *a*	**7.8** *a*
N_{200}	**35**	**1.26** ± 0.12 *a*	**0.24** ± 0.05 *b*	**1.49** ± 0.15 *b*	**0.84** *a*	**5.8** *a*
	45	**0.48** ± 0.04 *b*	**0.45** ± 0.03 *a*	**0.92** ± 0.05 *a*	**0.51** *b*	**1.1** *b*
	25	**0.66** ± 0.06 *b*	**0.10** ± 0.02 *b*	**0.76** ± 0.05 *c*	**0.87** *a*	**7.6** *a*
N_{300}	**35**	**2.30** ± 0.09 *a*	**0.36** ± 0.07 *a*	**2.66** ± 0.15 *a*	**0.87** *a*	**7.0** *a*
	45	**0.56** ± 0.07 *b*	**0.48** ± 0.08 *a*	**1.05** ± 0.14 *b*	**0.55** *b*	**1.2** *b*
$P_{(T \times N)}$		< 0.01	0.07	< 0.01	0.45	0.08
$HSD_{0.05}$		**0.38**	**0.22**	**0.54**	**(4.21)**	**(2.45)**

Different letters in the column indicate significant difference among temperatures within each N supply.. Values in parentheses were based on Arcsine transformed data

7.3.6 The activation energy

Estimated activition energy (E_a) required for the N_2O reductase (N_2OR) during denitrification varied from 20 to 98 kJ mol^{-1} with N supply (Table 7.3). There was no significantly difference in E_a for N_2OR among N amendements at all sampling time ($p > 0.05$, Table 7.3), suggesting that *Nos*Z was unaffected by N availability during the experiment.

7.3.7 Soil respiration and O_2 availability

The rates of soil microbial respiration were affected by incubation temperature but insensitive to N concentration throughout the experiment (Figure 7.4a). Soil respiration rate increased with increasing temperature, regardless of N content. The highest cummulative CO_2 production was found at 45 °C, followed by 35 °C; the lowest CO_2 produciton was measured at 25 °C (Figure 7.4a).

As CO_2 production varied from 120 – 140 µg C g^{-1} soil, often occurred at 25 °C, the pressure of O_2 was calculated at around 17 – 18 kPa (Figure 7.4b).. The concentration of O_2 decreased to 15 kPa since CO_2 increased to 200 – 250 µg C g^{-1} soil, which was measured at 35 °C. Higher soil respiration rate, ~250 – 350 µg C g^{-1} soil, at 45 °C resulted in lower O_2 concentrations (Figure 7.4b).

7.3.8 Inter-relationships

The relationships among temperature, N supply, O_2 availability and denitrification products are shown at Table 7.4. N_2 production was positively correlated with temperature ($r = 0.83$, $p < 0.01$) but unaffected by N amendments ($p > 0.05$, Table 7.4). The rate of denitrification was largely influenced by N supply ($r = 0.50$, $p < 0.01$). The N_2O/N_2 ratio and O_2 content were negatively correlated with temperature, indicating the impact of temperature on N_2O/N_2 by regulating O_2 availability.

Table 7.3. The activation energy (E_a) for N_2 production from denitrification between 25 ºC to 45 ºC at N applied to tested soil (with 100 (N_{100}), 200 (N_{200}) and 300 µg N g^{-1} soil as $^{14}NH_4{}^{15}NO_3$ (N_{300})).

Time of incubation (days)	**N_{100}**	**N_{200}**	**N_{300}**	**Sig.**
0-3	**48.84** ± 12.9	**71.65** ± 25.7	**91.30** ± 34.5	*ns*
3-7	**98.89** ± 9.8	**80.17** ± 3.6	**61.01** ± 11.1	*ns*
7-10	**20.93** ± 7.3	**42.94** ± 17.5	**54.49** ± 21.8	*ns*

ns: non significant difference ($p > 0.05$)

Table 7.4. Correlation coefficients (r values) among temperature, nitrogen applied (N), N_2O, N_2, denitrification ($N_2O + N_2$), N_2O/N_2 ratio and O_2 (n = 36) in the first 7 days of incubation.

	Temperature	N	N_2O	N_2	$N_2O + N_2$	N_2O/N_2
N_2O	-0.04	0.49**				
N_2	0.83**	0.18	0.17			
$N_2O + N_2$	0.18	0.50**	0.97**	0.42*		
N_2O/N_2	-0.62**	0.31	0.41*	-0.60**	0.22	
Oxygen	-0.72**	-0.22	0.19	-0.63**	0.02	0.44*

* significant at $p < 0.05$

** significant at $p < 0.01$

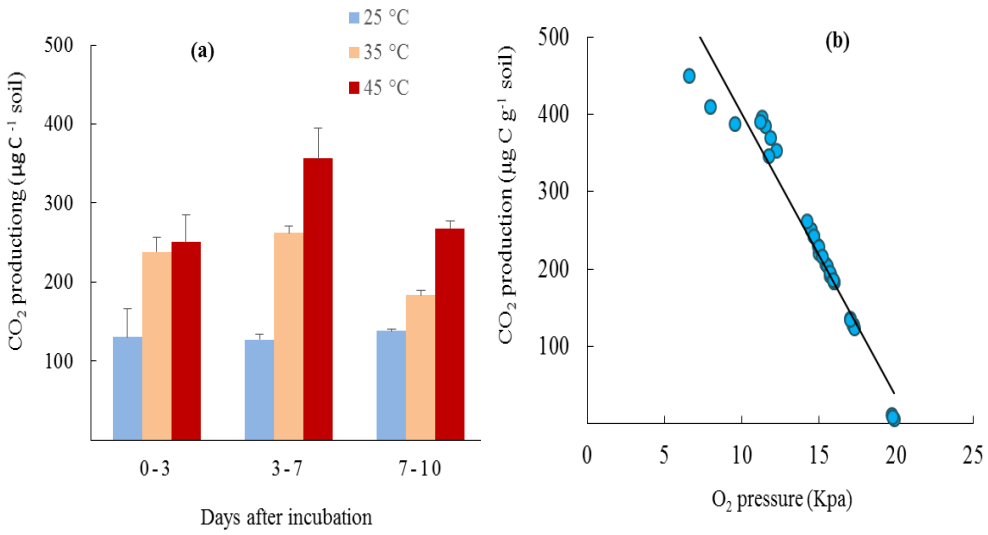

Figure 7.4 Cumulative CO_2 production vs time during incubation at different temperatures, regardless of N application (a) and the relationship between CO_2 production and O_2 availability (b). Soil moisture was maintained at 75% field capacity (33kPa). Error bars are +1 SE (n = 12).

7.4 Discussion

Our results demonstrated that N_2O production and total denitrification rate were controlled by an interactive effect between N supply and temperature, which have been often neglected in previous studies (Malhi et al., 1990; Weier et al., 1993; Holtan-Hartwig et al., 2002; White et al., 2002; Gillam et al., 2008). Irrespective of temperature, N_2O and $N_2O/(N_2O+N_2)$ were positively correlated with increased NO_3^- additions to soil, which supports other studies (Luo et al., 1996b; Scholefield et al., 1997; Gillam et al., 2008). However, NO_3^- concentration alone accounted for less than 50% of total N_2O emission in the present study, since NO_3^- interacted with temperature to influence N_2O production and denitrification rate. The rate of N_2O production was highest at 35 °C when either 200 or 300 µg N g^{-1} dry soil was added (equivalent to 100 or 150 kg N ha^{-1}, respectively) while N_2O was less affected by temperature at lower soil N (50 kg N ha^{-1}). This result supports the previous finding by Blagodatskaya et al. (2014b) that the thermal response of N_2O emissions in soil can be influenced by soil nitrogen and oxygen availability. Rising temperature in our study increased soil respiration rates and resulted in the depletion of O_2 in soil microsites, which could affect whether NO_3^- or N_2O is used as a terminal electron acceptor (TEA) during denitrification (Cho et al., 1997b; Strong and Fillery, 2002; Gillam et al., 2008). The preference for TEAs in the denitrifying pathway is typically in the order $O_2 > NO_3^- > N_2O$ (Firestone and Davidson, 1989). Once O_2 was limited following high respiration rates and NO_3^- was depleted around the microsites due to the reduction of nitrate, the increasing demand for TEAs would promote the movement of NO_3^- to the microsites, resulting in high N_2O production at 35 °C. A reduction in N_2O from denitrification at 45 °C could be both related to an optimal temperature controlling the reduction of nitrate and an enhancement of the reduction of N_2O to N_2. The results suggest that high potential N losses as N_2O from denitrification would be expected during

summertime in similar soil types following N fertiliser application and high soil moisture content (0.32 cm^3 cm^{-3}). An effective management of N in the field would be one that was monitored to meet crop N demand, particularly during summertime, to reduced N losses as N_2O from denitrification.

Temperature was more important than NO_3^- availability in regulating the reduction of N_2O to N_2 from denitrification, with lower N_2O/N_2 ratios above 35 °C. The influence of temperature on the N_2O/N_2 ratio has been unclear in previous studies. It is typically known that low temperatures (< 15 °C) favoured N_2O formation, leading to greater N_2O/N_2 ratios than at higher temperatures (Keeney et al., 1979; Avalakki et al., 1995; Dobbie and Smith, 2001) but some exceptions exist. The results from (Focht (1974) and Rudaz et al. (1999) found no significant effects of temperature on denitrification products. Our results demonstrate that the N_2O/N_2 ratio increased between 25 °C and 35 °C then decreased between 35 °C and 45 °C due to a decline in N_2O production and an increase in N_2 production above 35 °C. A positive effect of temperature on N_2 production at all NO_3^- treatments ($r = 0.83$, $p < 0.01$) indicates a significant role of temperature in regulating N_2O reductase. Reduction of N_2O in soils has been attributed exclusively to denitrifiers possessing *nos* operons with *nosZ* encoding N_2OR, the enzyme system catalysing N_2O to N_2 reduction (Kolb and Horn, 2012; Zheng and Doskey, 2015). However, it is now recognised that *nosZ* is harboured by a much broader range of denitrifier and non-dentrifier microorganisms, including archaeal, bacterial and fungal phyla, than previously thought (Sanford et al., 2012; Graf et al., 2014). Although some studies (Knowles, 1982; Weier et al., 1993; Ruser et al., 2006) have reported the inhibition of N_2OR at high soil NO_3^- concentration (between 200 – 300 µg N g^{-1} soil), this impact was unlikely to have had an effect on the activity of N_2OR since the NO_3^- contents (15.1 – 63.0 µg N g^{-1} soil) were much lower than the reported critical amounts. The activation energy (E_a) required

for N_2OR is consistent with previous findings (Holtan-Hartwig et al., 2002), who estimated the Ea for N_2OR of 60 kJ mol^{-1}. Increased N_2 production above 35 °C reflects high potential N loss as N_2 (~ 50% of N losses via denitrification), which is rarely measured in the field due to the high atmospheric background of N_2. These rates would be typical of summertime irrigated conditions where soil NO_3^- is not limiting denitrification in wet soil (0.31 cm^3 cm^{-3}).

High soil NH_4^+ contents observed at 45 ºC at the end of experiment suggests an increasing impact of high temperature on the regulation of NH_4^+ availability. Soil NH_4^+ content is a result from the balance between NH_4^+ production processes (N mineralisation, dissimilatory nitrate reduction to ammonium (DNRA) and consumption processes (nitrification, N immobilisation). Cumulative NH_4^+ content at 45 ºC resulted from high rates of N mineralisation and/or the activity of DNRA. Nitrogen mineralisation rate is generally described to increase with rising temperature (Sierra and Marban, 2000; Zaman and Chang, 2004; Thangarajan et al., 2015) and gross N mineralisation rates are 3 – 4 fold higher at 40 – 50 ºC than at 20 – 30 ºC (Zaman and Chang, 2004), which supports the higher NH_4^+ contents at 45 ºC in the present study. It was argued that high NH_4^+ from N mineralisation is related to increased substrate decomposition at temperatures above 40 ºC (Sierra and Marban, 2000; Zaman and Chang, 2004). DNRA, a biological process to convert nitrate to ammonium, however, is not well understood (Schmidt et al., 2011) and few studies have attempted to examine the rate of DNRA under anaerobic conditions. It was reported by Silver et al. (2001) that DNRA rate exceeded the amounts of N losses as N_2O and N_2 from nitrification and denitrification and accounted for 75% turnover of NO_3^- pool in the ultisols. However, the dependence of DNRA on soil temperature has not been assessed, although temperature explain 35% of the variation in DNRA rates in river sediments (Tomaszek and Rokosz, 2007). The rapid conversion of NO_3^- to NH_4^+ via

DNRA in soil has the potential implications for ecosystem N conservation by reducing soil NO_3^- availability, potentially preventing the N loss via leaching and denitrification. Increased DNRA rate also contributes to prevent nitrate toxicity in grazing animals under high N fertiliser application (> 200 kg N ha^{-1}) (Bolan and Kemp, 2003). More research will be needed to evaluate the thermal response of DNRA, particularly at high temperatures (35 – 50 ºC) to understand the source of high NH_4^+ availability observed at 45 ºC in the present study.

7.5 Conclusions

Temperature and nitrogen supply interacted to affect the total N losses as N_2O emission, which accounted for 50 – 80% of total denitrification rate. The reduction of N_2O to N_2 was influenced by incubation temperature but was unaffected by soil N supply. The production of N_2 increased up to 45 °C, but N_2O production declined, resulting in the low N_2O/N_2 ratios at high temperatures. The results suggest that high potential N losses as N_2O would be expected following N fertiliser application and high soil moisture content in the tested soil exposed to around 35 °C. Temperatures above this would decrease the N losses as N_2O because the reduction of N_2O to N_2 was promoted, possibly due to denitrifying nitrous oxide reductase. The results demonstrate how temperature and NO_3^- availability regulate the magnitude of N_2O and N_2 production from denitrification. The finding explained why temperature or NO_3^- concentration alone is a poor predictor of N_2O emission, since temperature interacts with NO_3^- influencing the total N denitrified and associated N_2O production. High soil NH_4^+ availability observed at 45 ºC suggests an increasing impact of high temperature on the regulation of NH_4^+ availability via N mineralisation and the potential for dissimilatory nitrate reduction to ammonium.

Chapter 8 General discussion

This chapter provides an overview of the research conducted as part of this thesis. The key results from various laboratory experiments are summarised and some potential implications are discussed. Future research is proposed to address some remaining challenging questions.

8.1 Concept of research

The effects of temperature on N_2O production from soil are very complicated as temperature influences the growth and activity of soil microorganisms and the activation energy for a number of processes that are responsible for the production of N_2O. Temperature may also change soil OC, N availability, soil moisture, O_2 concentration and pH, which affect biological N_2O production. The overall aim of this thesis is to investigate the effects of high temperatures (> 30 °C) on biologically produced N_2O and N_2 from Australian soils. Acetylene inhibition (Yoshinari et al., 1977) and ^{15}N labelling techniques (Bateman and Baggs, 2005) were applied to determine nitrogenous gases (N_2O and N_2) from nitrification and denitrification processes in soil, which account for 60% of globally anthropogenic N_2O production (Butterbach-Bahl et al., 2013). The thermal responses of nitrification and denitrification are typically described below 30 °C, while Australian agricultural soils experience particularly high diurnal and seasonal temperature fluctuations, often 30 – 45 °C during the summer months, December to February (Meteorology, 2015). The response of N_2O production, particularly from heterotrophic nitrification, has rarely been explored at high temperatures. The direct interactions among temperature, soil moisture and NO_3^- on N_2O and N_2 production during nitrification and denitrification processes are difficult to quantify under field conditions (Knowles, 1990; Groffman et al., 2006), while the laboratory studies) have been often neglected these

interactive effects (Chapter 2. Therefore, these interactive effects need further investigation under relevant conditions experienced in Australian soils.

8.2 Summary of results

8.2.1 The effects of fluctuating temperature and NH_4^+ content on nitrification and N_2O

Laboratory experiments in Chapter 3 investigated if soil NH_4^+ availability affected nitrification rate (NO_3^-) and associated N_2O production. By comparing nitrification rates at different NH_4^+ contents, it was demonstrated that NO_3^- and N_2O production from nitrification were limited when initial soil NH_4^+ was below 4 µg N g^{-1} soil at 20 ºC. The addition of N (between 50 to 150 µg N g^{-1} dry soil) to soil resulted in higher net rates of nitrification and associated N_2O production than in the control (without addition of N). The rates were unaffected by an increase in NH_4^+ between 50 and 150 µg N g^{-1} soil. The results confirm that soil NH_4^+ is vital for the activity of autotrophic nitrification at the temperatures tested, and that the magnitudes of NO_3^- and N_2O production from this process would be regulated by factors other than NH_4^+. Once the threshold of soil NH_4^+ concentration was reached for autotrophic nitrification, temperature and oxygen availability would be important factors in regulation of nitrification and associated N_2O production from soils.

Diurnal fluctuating temperature, which occurs naturally under field conditions, has been neglected in most studies that have assessed the thermal response of nitrification and N_2O production. When fluctuating temperature regimes induce different rates of nitrification and N_2O emissions compared with those under a daily constant temperature, the rates of N_2O and nitrification estimated from soils using the daily constant temperature would be problematic. Through an examination of nitrification and N_2O production under

fluctuating temperature protocol ranging from 15 °C to 25 °C compared with a constant temperature (CT, 20 °C – 24h) over 30 days, our results revealed that nitrification and associated N_2O emission did not differ between a CTP and the FTPs with the same mean temperature under the experimental conditions. The growth responses of ammonia oxidising bacteria (AOB) populations were the same when fluctuating temperature patterns applied or when a constant temperature was maintained throughout the experimental period. The consistent rate of nitrification or N_2O production could be explained by a lack of difference in AOB populations between a CT and FTPs, due to the thermal adaption of AOB to local climate. N_2O production from heterotrophic nitrification and/or denitrification was higher under FTP2 (15 °C – 12h and 25 °C – 12h) than under a CTP in the second 14 days of incubation but this should be treated with caution due to high variability of N_2O under FTP2. In the first 14 days of incubation, total N_2O production and nitrification rate were unaffected by fluctuating temperature patterns. This result simplified the assessment of thermal responses of nitrification and N_2O production by using daily constant temperature in further experiments.

8.2.2 The influence of temperature on nitrification and associated N_2O

By assessing the production of N_2O from nitrification at a range of constant temperatures from 10 °C to 45 °C, which are encountered in Australian soils, the thermal responses of autotrophic and heterotrophic nitrification as the pathway of N_2O production were investigated (Chapter 4). The results suggested that temperature was more important than soil type in controlling N_2O from nitrification. The production of N_2O from nitrification increased with rising temperature and peaked between 35 °C and 40 °C, where nitrification accounted for 55 % of total N_2O emissions. The result demonstrates an important role of nitrification as the source of N_2O in dry conditions (~ 0.25 g water cm^{-3}) and supports a temperature optimum function (T_{opt}) for N_2O production from nitrification. This result is

indicated as a part of the conceptual diagram integrating key results from the thesis (Figure 8.1). T_{opt} for N_2O production from nitrification was higher than the values reported in previous studies. Below 35 °C, autotrophic nitrification was the main source of N_2O, and was positively correlated with the growth of the AOB population. Between 35 °C and 40 °C, heterotrophic nitrification was the primary source of N_2O, principally due to decreased O_2 availability in the soil microsites, which was induced by higher microbial respiration rates at these temperatures. The finding indicates a significant N_2O production from heterotrophic nitrification in soils exposed to high temperatures, typically between December to February in Australia (Meteorology, 2015). Heterotrophic nitrifiers used NH_4^+ as the prime substrate for nitrification, particularly at 35 °C and 40 °C, as evidenced by an increase in $^{15}N_2O$ production in the presence of C_2H_2. The substrate selection of heterotrophic nitrifiers, namely the oxidation of NH_4^+, may be influenced by the addition of 100 µg NH_4-N g^{-1} soil in our studies, although the use of organic N as the preferred substrate has been previously identified (Schimel et al., 1984; Islam et al., 2007). When soil NH_4^+ was not limiting, autotrophic and heterotrophic nitrification responded dissimilarly to temperatures above 35 °C.

In a study comparing N_2O production in soils from two contrasting climates, Chapter 5 showed that the tropical soil produced higher N_2O emissions than the temperate soil did, at a range of temperatures from 20 °C to 40 °C. T_{opt} for nitrification also varied between climatic regions, at 20 °C to 30 °C in the temperate and at 30 °C to 35 °C in the tropical soil. The differences in the thermal response of nitrification could be related to the growth rates of the AOB populations. Estimated AOB populations in the tropical soil exceeded those in the temperate soil between 25 °C and 35 °C, suggesting that soil microorganisms mediating N in contrasting climates responded differently to temperature. The distinct growth rate of AOB in each climate was considered to be a result of the physiological

adaptation of microbial communities to local temperature regimes. Autotrophic nitrification had greater N_2O emissions than heterotrophic nitrification and/or denitrification below 35 °C, regardless of the climate from which soils were tested. At higher temperatures, a decrease in nitrification rate resulted in increased the proportion of N_2O to NO_3^- (P_n), regardless of climatic regions. P_n increased with rising temperature and was above 1.0% at 35 °C (Figure 8.1). The results suggest that an effective function describing the dependence of P_n on temperature may allow a quick estimate of N_2O through measurement of NO_3^- produced during nitrification across regions.

8.2.3 The influence of temperature, soil moisture and nitrate on denitrification

By examining the production of N_2O at different temperatures and soil moisture contents (Chapter 6), an interactive effect between temperature and soil moisture was identified to control the production of N_2O from denitrification. At 60% FC, N_2O production declined above 35 °C while, at 75% FC, N_2O production from soil incubated at 35 °C was similar to that at 40 °C. Temperature, rather than soil moisture, impacted on the conversion of N_2O to N_2 during denitrification. N_2O reduction has been attributed mainly to denitrifying-*nosZ*, although there is new evidence that *nosZ* is harboured by denitrifier and non-dentrifier microorganisms (Kolb and Horn, 2012; Zheng and Doskey, 2015). Our results revealed that $^{15}N_2$ production was positively correlated with increased temperature up to 45 °C ($r = 0.68$, $p < 0.01$), suggesting the possible impact of temperature on *nosZ*. The maxium denitrification rate ($N_2O + N_2$) measured between 35 °C and 40 °C indicates that greater amount of N losses as N_2O and N_2 may be expected once soil moisture is around 0.31 cm^3 cm^{-3} (or 75% FC) in summer than in winter or spring under field conditions. Denitrification rate occurred faster in the first 7 days of incubation, suggesting that key management to mitigate N_2O should be targeted in the first week after N fertiliser

application. Thus, the management soil water, partiularly the retention of soil moisture at 0.25 cm^3 water cm^{-3} soil during the periods associated with high temperatures can reduce N loss as N_2O from denitrification.

By evaluating the response of N_2O production at different temperatures and NO_3^- contents (Chapter 7), it was identified that temperature interacted with NO_3^- to control N_2O production from denitrification. Temperature or NO_3^- concentration alone was a poor predictor of N_2O emission and total denitrification rate in our study. The rate of N_2O production was greatest at 35 °C when either 200 or 300 µg N g^{-1} dry soil was added (equivalent to 100 or 150 kg N ha^{-1}, respectively) while N_2O was less affected by temperature at lower N addition (~ 50 kg N ha^{-1}). Increasing temperature enhanced soil microbial respiration rate, resulting in decreased O_2 availability in the soil microsites. The deficiency of O_2 presumably increased the use of NO_3^- as a preferred terminal electron acceptor (TEA) during denitrification, resulting in high N_2O released from denitrification at 35 °C (Figure 8.1). The reduction of N_2O to N_2 during denitrification increased up to 45 °C, regardless of NO_3^- concentrations (Figure 8.1), resulting in decreased N_2O/N_2 ratios between 40 °C and 45 °C.

Interestingly, cumulative NH_4^+ contents in the soils at 45 °C were greater than at lower temperatures (Chapter 4, Chapter 6, Chapter 7, Figure 8.1). This finding suggests a significant impact of high temperatures on biological processes controlling NH_4^+ availability. Cumulative NH_4^+ in the tested soils (chromosol and dermosol) at 45 °C reflects that the production of NH_4^+ from N mineralisation and/or dissimilatory nitrate reduction to ammonium (DNRA) exceeded the consumption of NH_4^+, including nitrification and N immobilisation. Mineralisation of N is known to increase with rising temperature up to 45 °C (Sierra and Marban, 2000; Zaman and Chang, 2004). DNRA is a biological process of conversion of NO_3^- to NH_4^+ with the lack of O_2 availability by

organisms that have fermentative rather than oxidative metabolism (Tiedje, 1988). Temperature was identified as one of key factors determining the rate of DNRA in the water of rivers (Tomaszek and Rokosz, 2007) while the thermal response of DNRA in soil is poorly understood (Schmidt et al., 2011). In our studies, DNRA could be a potential mechanism that might explain the changes in NH_4^+ and NO_3^- pools at 45 °C. Increased soil DNRA rate has implications for ecosystem N conservation, as the reduction of NO_3^- to NH_4^+ would potentially prevent NO_3^- toxicity in grazing animals under high N fertiliser application (Bolan and Kemp, 2003) and slow the rates of leaching and denitrification.

8.3 Conclusions

This work has improved the knowledge of biological N_2O and N_2 production at high temperatures, typical of environments of southern Australia, which have had relatively little research focus regarding N_2O emissions. High temperatures (> 30 °C) could potentially increase the losses of N applied as N_2O and N_2 from nitrification and denitrification. Temperature appeared to be more important than soil type in controlling N_2O from nitrification, which slowed at 10 °C to 25 °C and peaked at 35 °C to 40 °C. These results suggest a higher optimum temperature for the N_2O from nitrification than previous studies. Autotrophic nitrification produced N_2O predominantly below 35 °C, while heterotrophic nitrification, which used NH_4^+ for nitrifying, released N_2O principally between 35 °C and 40 °C. Climatic conditions affected the magnitudes of total N_2O emissions in soils. N_2O emissions in the tropical soil exceeded those in the temperate soil. T_{opt} for nitrification was higher in the tropical than temperate climates although the ration of N_2O/NO_3^- from nitrification at different temperatures was independent of climatic region. The ratio of N_2O/NO_3^- was positively correlated with increased temperature, and was above 1.0% at 35 °C, regardless of climate. The rates of nitrification and associated

N_2O production were closely related to the growth of AOB population, as influenced by temperature.

Temperature interacted with soil moisture and NO_3^- supply to regulate N_2O from denitrification, while the reduction of N_2O to N_2 was affected mainly by temperature. The highest denitrification rate ($N_2O + N_2$) was found at 35 °C in the soils maintained at 75% FC and treated with N additions between 100 – 150 kg N ha^{-1}. Decreased N_2O/N_2 ratios at 40 °C to 45 °C was due to both the decreased N_2O production and increased N_2O reduction at these high temperatures, suggesting a significant NO_3^- loss as N_2 during summer, particularly in soils that are wet during that period. Increased temperature encouraged the use NO_3^- as TEA in soil microsites, since O_2 became limiting, thereby increasing N_2O production. Temperature likely influenced the regulation of denitrifying enzyme expressions. The reduction of N_2O to N_2 during denitrification was independent of NO_3^- availability in our controlled experiments. Interestingly, the study indicated that NH_4^+ contents increased rapidly at 45 °C after 10 days of incubation in both chrososol and dermosol soils. This result was hypothesised to be a result of high rates of mineralisation N and/or the action of DNRA at this temperature (Figure 8.1).

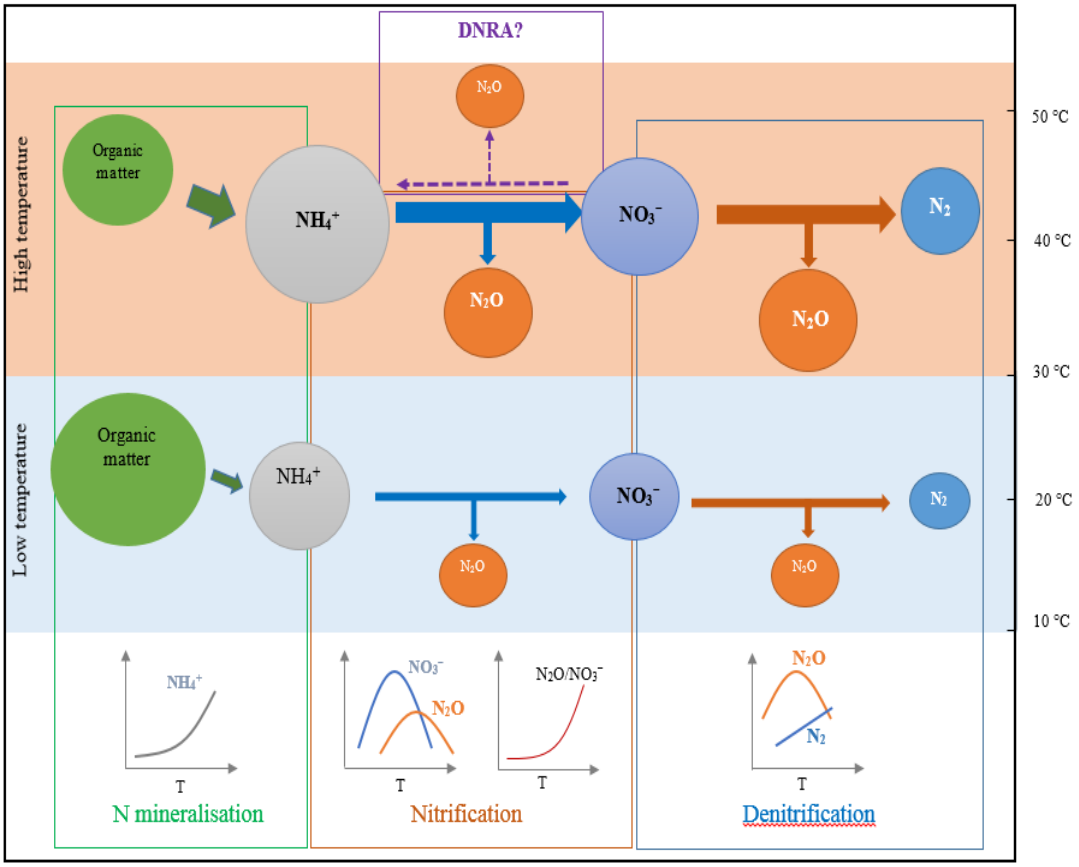

Figure 8.1. A conceptual model of the effect of temperature on soil biological processes responsible for N_2O and N_2 production, constructed by integrating results obtained from experimental chapters. The size of the circles and arrows indicates the relative impact of temperature on size of the pools of each nitrogen form. The diagrams in the lower portion of the figure indicate some key results of the study in relation to temperature optima for N_2O production and the thermal effect on NH_4^+.

8.4 Implications

The production of N_2O from soils has been measured across climatic regions that varied markedly with temperature. Our results indicate that an optimum function should be used to describe the effect of temperature on biological N_2O production from soils. The temperature optima for N_2O production is likely to vary between climatic regions. The comparison of N_2O production at a range of temperatures between two contrasting climates demonstrated that the optimum temperature for N_2O production is lower in the temperate than in the tropical climate. The magnitudes N_2O emission and nitrification are also likely to be greater in the tropical than the temperate climates, based on our current data set. These results reflect that soil microorganisms from different climates respond dissimilarly to temperature. Therefore, ecosystem models predicting N_2O should consider an optimum function of temperature for a better prediction of N_2O emission from soils across regions.

Temperature interacted with soil moisture or NO_3^- availability to regulate N losses as N_2O emission from denitrification. These interactions are likely to occur under field conditions to influence the production of N_2O. One practical recommendation to farmers based on our work would be to manage soil moisture and N availability carefully in the field, taking into account the crop demand for N throughout the season. Managing the system to reduce excesses of soil water and NO_3^- will reduce N_2O emissions from denitrification under field conditions, particularly during summer time, which often experience high temperatures.

Nitrogen mineralisation and DNRA might be important processes regulating soil NH_4^+ availability at high temperatures. It is possible to take advantage of the hottest months

(December to February) to promote the rates of N mineralisation and/or DNRA under natural conditions, which help to conserve soil N.

8.5 Future research

The research work in this thesis suggests a number of areas that require further investigation, including:

Ammonium oxidising archaea (AOA), which may be involved in the nitrification process (Di et al., 2010), were not measured by the most probable number (MPN) technique in our studies. Although the growth and activity of AOA were presumably negligible in the soils with the additions above 50 μg NH_4-N g^{-1} in the experiments presented in this thesis, the thermal response of AOA needs to be understood, particularly in low fertilised or un-fertilised soils. The abundance of Archaeal *amo*A genes can be quantified using primers crenamoA23F and crenamoA616R (Tourna et al., 2008) for amplification in QuantiFastTM qPCR master mix (Qiagen). Amplification efficiency for archaeal *amo*A qPCR helps improve the knowledge of the role of AOA in N cycle and the thermal response of AOA relative to N_2O production. Ammonium oxidising bacteria populations estimated by MPN are often variable in response and time-consuming. Amplification of bacterial *amo*A genes can be performed similarly to archaeal *amo*A qPCR, except for the primers. The primers for bacterial *amo*A can be A-1R and amoA-2R (Rotthauwe et al., 1997). Future research should apply these qPCR techniques to precisely evaluate the growth and activity of bacterial *amo*A genes and archaeal *amo*A genes in response to high temperatures.

The isolation of nitrifying and denitrifying strains responsible for N_2O and N_2 production in tested soils is needed to confirm the mechanisms that control these nitrogenous gases. The growth of nitrifying and denitrifying strains in cultures allows better evaluation of the

effects of temperature on the growth and gene expression, relative to N_2O and N_2 production, particularly *Nos*Z. This would provide a clear understanding of the mechanisms of high temperature in controlling N_2O and N_2 production from nitrification and denitrification.

The production of N_2O from other processes such as nitrifier-denitrification and chemical reactions could be an interesting subject for future work. Nitrifier-denitrification, a reduction of NO_2^- following the oxidation of NH_4^+ in nitrification, could contribute to significant N_2O production under low soil pH, since O_2 becomes limiting (Wrage et al., 2001; Zhu et al., 2013), suggesting an important role for nitrifier-denitrification as the source of N_2O in acidic soils. Chemo-denitrification can also occur under low soil pH and in the presence of Fe^{2+} (Van Cleemput, 1998). In this process, nitrite (NO_2^-) produced during denitrification can rapidly react with Fe^{2+} in soil to release N_2O. Another abiotic pathway of N_2O is the oxidation of hydroxylamine (NH_2OH), which is formed during nitrification as a mediated product (Bremner, 1997; Heil et al., 2015). Although these reactions may contribute relatively small quantities of N_2O, further research will be needed to understand these processes as the sources of N_2O and N_2 from soils.

The thermal response of DNRA could be an interesting area to explore in future work. This biological process, however, is one of the least understood in the N cycle (Schmidt et al., 2011). Few studies have examined the rates of DNRA from soils under anaerobic conditions. DNRA rate was responsible for 75% turnover of the NO_3^- pool in the ultisols (Silver et al., 2001). In that study, the conversion of NO_3^- to NH_4^+ exceeded the total N gas losses ($N_2O + N_2$) from nitrification and denitrification. The evidence that temperature is a key factor controlling the variation in DNRA rates in rivers was reported by (Tomaszek and Rokosz, 2007). However, the dependence of DNRA on soil temperature has not been documented. Increased DNRA in soil would potentially benefit N

conservation because a high rate of DNRA reduces NO_3^- concentration in soil, slowing N losses as N_2O from denitrification or leaching. More research will be needed to evaluate the thermal response of DNRA, particularly at high temperatures (35 – 45 °C), which are typically experienced in Australian soils during summer.

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