The temperature dependence of the gaseous products of the nitrogen cycle

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Contents

Abstract

The nitrogen cycle is one of the key macronutrient cycles that controls the distribution of life on Earth. The nitrogen cycle is composed of a series of distinct microbially mediated processes which may be affected differently with warming. Climate change is likely to affect all components of the nitrogen cycle. However, the extent to which each component will be affected and how this will alter interactions in natural systems is unknown. Here we used laboratory and field experiments to investigate the effect of warming on nitrogen cycling. We used a combination of pure cultures, *in-situ* measurements and laboratory manipulations of environmental samples to explore responses in freshwater and marine systems. In pure cultures of denitrifying bacteria, denitrification rates increased by 117-164%, with a 4^{o}C temperature increase (11.5-15.5^{o}C). In freshwater mesocosms, long term warming rates of sediment denitrification increased by 247%, with no significant thermal response of sediment nitrification within these systems. Marine sediment rates of denitrification and anammox increased by 4.69-16.23% and 3.71-35.39% respectively, depending on N substrate. Whereas a 3^{o}C temperature increase in the water of the ETNP OMZ increased denitrification and anammox rates by 52.5% and 52.9% respectively, with no significant thermal response of nitrogen fixation in the OMZ surface waters. From this study, nitrogen removal processes increase with increasing temperature across systems but internal transformation and fixation of N show little to no thermal response. Further investigation into the causes of the observed variation in responses, such as substrate limitation and identification of microbes involved, will allow us to better understand and therefore better predict cross-system responses of the nitrogen cycle to global warming.

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Chapter 1: General introduction

Dinitrogen gas (N_2) is the most abundant gas in the atmosphere, comprising 79% of its total composition by volume (Francis et al., 2007). Once microbially fixed into a biologically available source, such as nitrate (NO_3^-), nitrite (NO_2^-) or ammonia (NH_3), it plays a crucial biological role as a key component in amino acids and therefore protein structure in all organisms (Canfield et al., 2010). Fixed nitrogen can be a limiting nutrient for primary production in the natural environment because it is crucial to make structures such as amino acids and nucleic acids (Falkowski, 1997; Wetzel, 1993). The nitrogen cycle involves a complex series of processes mediated predominately by a diverse array of bacteria and archaea which require both anoxic and oxic environments (Gruber & Galloway, 2008). The nitrogen cycle is extremely intricate with the transformation of nitrogen into a possible seven different oxidation states (Galloway et al., 2004), with this interspecies conversion achieved through respiration and fermentation and involves many different enzymes coded for by specific genes (Figure 1.1). The two key parts of the nitrogen cycle governing the availability of fixed nitrogen in the biosphere and the overall magnitude of primary production are ultimately nitrogen fixation and nitrogen removal to N_2 gas through both denitrification and anaerobic ammonium oxidation (Singh et al., 2011).

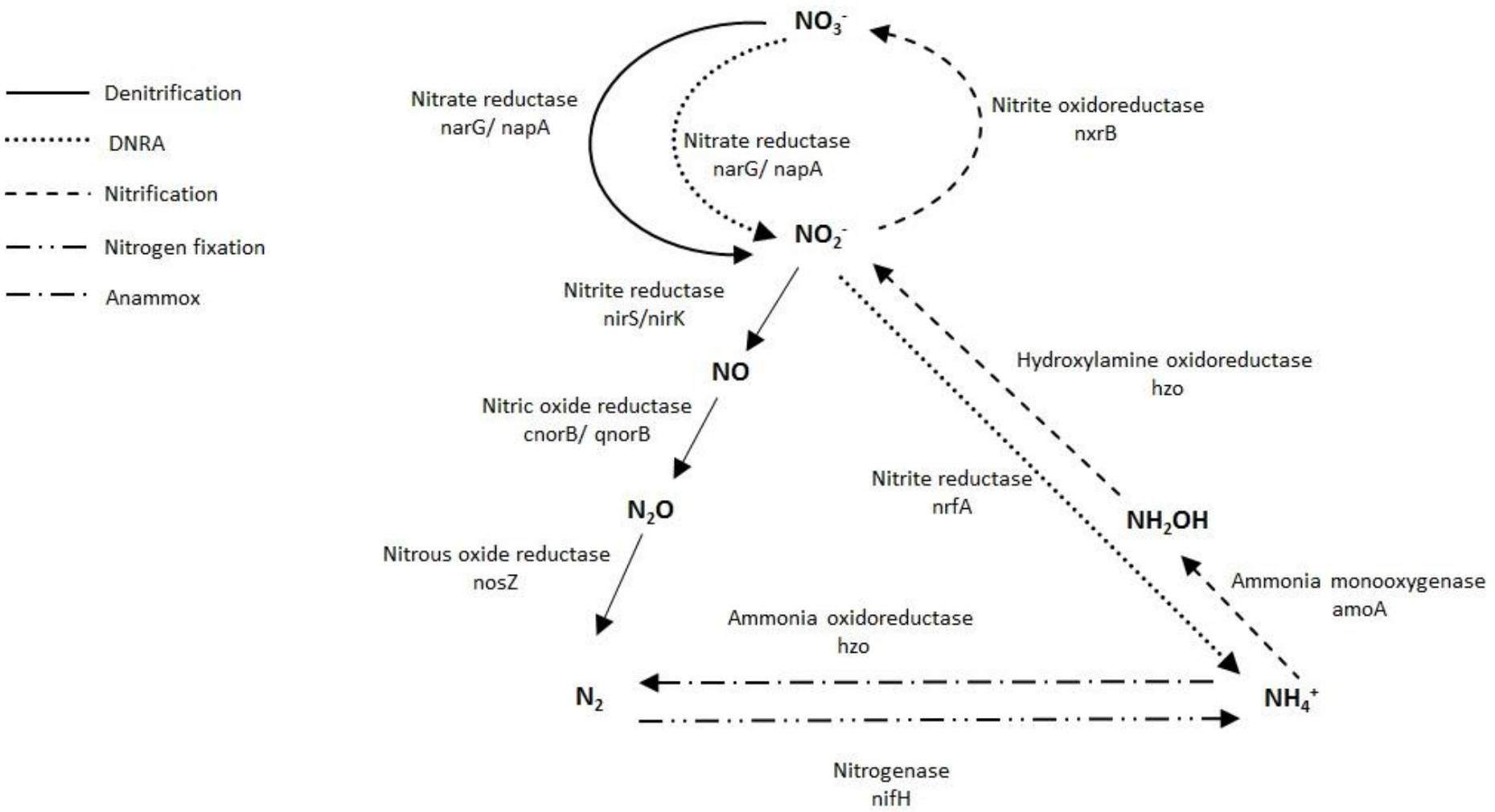

Figure 1.1 A simplified diagram of the nitrogen cycle including the different oxidation states, enzymes and genes involved with the different processes involved.

1.1 Denitrification

Denitrification is a dominant nitrogen removal process (Codispoti, 1995). Denitrifiers are a taxonomically diverse group of mainly heterotrophic, facultative aerobic bacteria (Knowles, 1982) which ultimately convert NO_3^- to N_2 gas (Falkowski, 1997) though a complex series of reactions involving numerous intermediates (Ferguson, 1994), some of which are sub substrates for others organisms. For example, the intermediate NO_2^- produced through NO_3^- reduction occurs within the bacterial cytoplasm and must travel to the cells periplasm for further reduction. It can therefore accumulate in the surrounding environment and be utilised by ammonium oxidising bacteria (Lam & Kuypers, 2011; Naqvi et al., 2000). The full denitrification pathway is as follows (Kalkowski & Conrad, 1991; Zumft, 1997):

$$NO_3^- \rightarrow NO_2^- \rightarrow NO + N_2O \rightarrow N_2$$

Canonical denitrification is complete reduction of either NO_3^- or NO_2^- through to N_2 gas (Codispoti, 2007) by a single organism (respiratory denitrification), though some bacteria have the ability to reduce NO_3^- and NO_2^- but do not produce N_2 gas as a final product (non-respiratory denitrification) (Tiedje, 1998) .

Denitrification requires depleted oxygen levels to undetectable which can be found in environments such as aquatic sediments and stratified water columns among others (Knowles, 1982; Francis et al., 2007). Denitrification is an important process to understand as it removes biologically available nitrogen from systems which may lead to reductions in primary productivity which would lead to reduction in carbon sequestration (Seitzinger, 1988). Global rates of nitrogen loss through denitrification from oceans have been estimated to be as great as 230 (± 60) Tg N y^{-1} (DeVries et al., 2012).

There are many interacting environmental factors that influence rates of denitrification, including NO_3^- concentration (Seitzinger, 1988; Teixeria et al, 2010), oxygen concentration (de Boi et al 2002), temperature (Maag and Vinther, 1996; Pina-Ochoa and Alvarez-Cobelas,

2006) and organic carbon availability (Nixon, 1981) which often have interacting effects. Of these factors, NO_3^- concentration appears to be the more dominant controlling factor recognised in the literature with increasing NO_3^- concentrations increasing the rate of denitrification (e.g. Seitzinger, 1988; Teixeria et al, 2010). However, if organic content of the sediment is low, additions of NO_3^- have shown to have a negligible effect on denitrification rates. Teixeria et al (2010) measured rates of denitrification in an estuary with a nutrient gradient with high NO_3^- concentrations in the upper reaches decreasing toward to mouth of the estuary. Rates of denitrification increased toward the lower estuary, where organic content of the sediments was greater due to pollution inputs, with denitrification rates positively correlating to NH_4^+ concentrations. High NH_4^+ concentrations could have led to production of NO_3^- through nitrification if enough oxygen was present, allowing these greater rates of denitrification to be observed (Magalhaes et al, 2005). Carbon content of sediments is also highly important for rates of denitrification. High carbon content can lead to higher rates of respiration (Duff et al, 2007) which will creating more favourable conditions of low oxygen (de Boi et al, 2002) and increase rate at which NO_3^- is used an electron acceptor (Stelzer et al, 2014). Seasonal variations of denitrification rates have also been observed, with warmer summer months stimulating denitrification. This has been observed in natural estuarine and coastal systems (Brin et al, 2014), as well as in experimental freshwater mesocosms where sediment denitrification doubled following a 3°C warming (Veraart et al, 2011). Though this effect of temperature is not in isolation. Increased temperatures also lead to increased oxygen consumption due to increased respiration (Gillooly et al, 2001; Perkins et al., 2012).

Denitrification is also one of the major contributors of nitrous oxide (N_2O) production, a potent greenhouse gas (GHG) with radiative forcing some 300 times greater than that of carbon dioxide (CO_2) (Ravishankara et al., 2009; Wright et al., 2012) and that can also damage the stratospheric ozone layer (Holtan-Hartwig et al., 2002; Knowles, 1982). N_2O affects

atmospheric chemistry and has been classified as an ozone depleting substance (ODS) with the highest weighted ozone depleting potential (ODP) and is predicted to remain this way for the rest of the 21st Century (Ravishankara et al., 2009).

1.2 Anaerobic ammonium oxidation (Anammox)

Anaerobic ammonium oxidation (Anammox) is an additional process of nitrogen removal and involves the anaerobic oxidation of ammonium (NH_4^+) using NO_2^- as an electron acceptor with a final product of N_2 gas (Dalsgaard et al., 2012) with the following transformations:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$$

The role anammox plays in removing nitrogen from ecosystems has gained a lot of interest in recent years. Anammox was first discovered in sewage treatment facility (Mulder et al., 1995) and has since been measured in estuarine sediments (Rich et al, 2008; Trimmer et al, 2003), coastal shelf sediments (Engstrom et al., 2005; Thamdrup & Dalsgaard, 2002b) and anoxic water bodies such as Oxygen Minimum Zones (OMZ's) (Beman et al., 2012; Hamersley et al., 2007; Dalsgaard et al., 2003; Kuypers et al., 2003). Anammox is carried out by autotrophic bacteria and is energetically more favourable than oxic nitrification (Jetten et al., 2001).

The contribution of anammox to N_2 production has been measured between 4-79% in coastal sediments (Engstrom et al., 2005) and 19-35% in anoxic water columns (Dalsgaard et al., 2003) with a recent mean average of 28% off the coast of Chile (Dalsgaard et al., 2012).

Anammox and denitrification often co-occur as they have similar metabolic requirements; the use of NO_{3-} as an electron acceptor in low oxygen environments (Brin et al, 2014). However, anammox organisms have a higher affinity for NO_{3-} compared to denitrifying organisms and therefore tend to dominate in conditions where NO_{3-} is limited (Gardner and McCarthy, 2009; Thamdrup and Dalsgaard, 2002). The anammox process also requires NH_4^+ to reduce NO_3^-, which becomes another controlling factor determining rates of this process (Brin et al, 2017).

Temperature has also been shown to have an influence on rates of anammox. Several studies have categorised anammox organisms as being slow growing and cold adapted and have noted a decrease in activity with increasing temperatures (Teixeira et al., 2012; Brin et al., 2014). However, a recent study has shown that rates of anammox activity responded in a similar manner to denitrification over a temperature range of 3-59^{o}C, with neither the thermal optimum nor activation energy changing with either process (Brin et al, 2017). This result led them to disregard previous studies classing anammox organisms as cold adapted.

1.3 Nitrification

Nitrification plays an important role coupling one recycling part of the nitrogen cycle, NH_4^+ from ammonification, to removal processes such as denitrification by providing NO_3^- and NO_2^- which will be reduced through several intermediates to N_2. Nitrification is an aerobic process oxidising NH_4^+ to NO_2^- and NO_3^- mediated by a series of enzymes (Carini & Joye, 2008) and is predominately an autotrophic process (Hovanec & DeLong, 1996) but it can also be carried out heterotrophically (Robertson et al., 1989). Until recently, nitrification was believed to be carried out in two separate processes; the oxidation of NH_4^+ to NO_2^- followed by further oxidation of NO_2^- to NO_3^- by phylogenetically distinct clades of heterotrophic and autotrophic nitrite-oxidising bacteria and archaea (van Kessel et al., 2015; Wuchter et al., 2006). The equations of the two separate steps of nitrification are as follows:

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^-$$

Recently this belief has come under scrutiny, with the discovery of complete nitrification occurring in a single organism, where the process of complete oxidation of NH_3 (comammox) to NO_3^- in a single organism was identified within two species of *Nitrospira* (van Kessel et al., 2015) carrying out the following conversion :

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+$$

Nitrification has been measured in a variety of systems such as freshwater lakes and streams (Small et al., 2013; Strauss & Lamberti, 2000) and oceans (Clark et al., 2008; Wuchter et al., 2006). In oligotrophic ocean surface waters, NH_4^+ regeneration rates of 10-160 nmol L^{-1} d^{-1} have been measured (Clark et al., 2008). It can also play a huge role in freshwater lakes, with estimates of 93-100% of the NO_3^- in Lake Superior produced through nitrification (Finlay et al., 2007). Nitrification also has the ability to produce the GHG, N_2O, through the oxidation of NH_4^+ under low oxygen conditions (Hynes & Knowles, 1984; Ritchie & Nicholas, 1972). Rates of N_2O production in the range of 1.68-7.94 nmol L^{-1} d^{-1} have been measured in euphotic oceans waters through nitrification (Dore & Karl, 1996).

Like all metabolic processes, there are environmental factors that affect the rates of nitrification. In addition to potential inhibition by sunlight (French et al., 2012; Merbt et al., 2012), ammonium oxidising organisms (AOO) have also affected by concentrations of NH_4^+. Whilst there have been studies to show increased rates of nitrification with increasing concentrations of NH_4^+ (Horak et al, 2013, Newell et al, 2013), other studies have found no response of increasing NH_4^+ concentrations (Shiozaki et al, 2016), suggestion other factors were at play such as low oxygen content of the soils.

The response of nitrification to temperature has contradicting results in the literature. Hansen et al (1981), measured highest rates of nitrification in the winter from Danish inshore sediments. This was due to a combination of reduced oxygen penetration into the sediments

(from 10 cm to only 2 cm) and potentially increased competition for NH_4^+ by more competitive autotrophic algae and heterotrophic bacteria. In winter, there is more oxygen present due to lower rates of respiration, less demand for NH_4^+ , therefore numbers of nitrifying organisms have an advantage and population numbers can increase, with the reverse observed with increasing temperatures (Hansen et a, 1981). Conversely, other studies have found strong correlations between temperature and nitrification rates. Berounsky and Nixon (1990) concluded rates of nitrification were positively correlated to temperature. They also determined temperature to be the dominant controlling factor, more so than NH_4^+ or oxygen content, with an r^2 of 0.90 and 0.96 for temperature alone, from sediments sampled opposite ends of the Narragansett Bay, USA.

The ammonium oxidising bacteria (AOB) and ammonium oxidising archaea (AOA) that carry out nitrification have different affinities for NH_4^+ (Martens-Habbena, 2009). AOA have often been found in high numbers in low NH_4^+ conditions, whilst AOB tend to have a metabolic advantage in higher NH_4^+ conditions (Hatzenpichler et al, 2008; Könneke et al, 2005). This adds complexity to trying to understand nitrification, as little is still known about the controlling factors on these organisms and their metabolic processes (Walker et al, 2010).

1.4 Nitrogen fixation

Nitrogen fixation is the biological conversion of N_2 gas into biologically available inorganic nitrogen compounds (Zumft, 1997). Ecosystems limited in N provide conditions ideal for organisms i.e. the diazotrophs that are able to convert unreactive N_2 into reactive forms that can be utilised by other biota in the system (Galloway et al., 1995). Over half the fixed N available in the ocean is estimated to be provided by microbial nitrogen fixation (Gruber & Sarmiento, 1997; Middleburg et al., 1996), with approximately 203 Tg N y^{-1} being biologically fixed globally (Fowler et al., 2013). Open oceans potentially play the most important role with

estimates of 60-200 Tg N y^{-1} (Duce et al., 2008), with many studies estimating an average of approximately 140 Tg N y^{-1}(Canfield et al., 2010; Galloway et al., 2004).

Nitrogen fixation is carried out by a diverse array of prokaryotes, which have the key enzyme, nitrogenase, that is necessary for nitrogen fixation (Fiore et al., 2010). Nitrogenase is inactivated by oxygen, so these organisms have different methods to prevent the inhibitory effect of oxygen. Cyanobacteria are photosynthetic-aquatic-nitrogen-fixing organisms that have daily cycles of photosynthesis during daylight hours, and nitrogen fixation in the dark, so that the oxygen created during photosynthesis does not inhibit the enzyme when it is active (Zehr, 2011). Most cyanobacteria are unicellular, but there are a few species (e.g. *Trichodesmium*) that are able to grow as multicellular colonies, with specialised sites for nitrogen fixation that are completely separated from the photosynthetic cells (e.g. Karl et al., 2002). These specialised sites for nitrogen fixation are called heterocyst's and allow the cyanobacteria to fix nitrogen in both light and dark conditions (Zehr, 2011).

Nitrogen fixation is an energetically costly reaction (Houlton et al, 2008; Howarth, 1988) with the enzyme that catalyses this reaction, nitrogenase, requiring a minimum of 16 molecules of ATP to fix one molecule of N_2 (Stam, Stouthamer, & van Verseveld, 1987). Even with concentrations as low as 0.14-0.16µM of nitrate or 1.4-12µM ammonium, N_2 fixation (diazotrophy) is inhibited (Horne et al, 1972).

The amount of reactive nitrogen in our biosphere has increased by approximately 210 Tg N y^{-1} (Fowler et al., 2013) predominately due to the Harber-Bosch process used to increase crop yields and the burning of fossil fuels (Galloway et al., 2008). Coastal areas and freshwater systems tend to have increased levels of reactive nitrogen from surface run off from the surrounding crop land that has had fertiliser additions. Due to this increase, many freshwater and coastal systems have excess nitrogen levels which will lead to eutrophication, increased rates of respiration and therefore reduce the ability of the system to sequester carbon. Increased

nutrient levels also reduces the competitive advantage that nitrogen fixing organisms have in low nutrient systems and they tend to be outcompeted by other biota and less new nitrogen is fixed into the system. Alternatively, those systems isolated from anthropogenic fixed nitrogen inputs will have more suitable conditions of low nitrogen concentrations. However, with increasing CO_2 levels and global warming, it is of concern that the rate of new nitrogen entering a system, away from anthropogenic sources of nitrogen inputs, will not be able to keep up and will ultimately become a limiting factor in primary production and therefore carbon sequestration (Vitousek et al., 2013).

1.5 Dissimilatory reduction of nitrate to ammonium (DNRA)

Dissimilatory nitrate reduction to ammonium (DNRA) is an anaerobic metabolic process carried out in a variety of systems including marine and estuarine sediments and water columns (Giblin et al, 2013, Hardison et al, 2015) by a variety of bacteria, archaea and eukarya (Kamp et al, 2011; Welsh et al, 2014) (Figure 1.1).

There are two types of DNRA, more common fermentation and DNRA linked to sulphur reduction (Burgin and Hamilton, 2006). The fermentation form of DNRA uses electrons from organic matter to reduce NO_3^- (Megonial et al, 2004; Teidje, 1988) and is carried out by a wide variety of microbes including species of *Pseudomonas* (Teidje, 1988). There is strong competition between DNRA and denitrification, as both are present in similar environmental conditions such as sediments with low oxygen with the presence of NO_3^-. Both processes have been documented as being controlled by NO_3^- concentrations and organic matter availability (Giblin et al, 2013, Christensen et al, 2000, Kraft et al, 2014), but the exact extent of their effects is not fully understood. DNRA has been observed to dominate over denitrification in anoxic sediments with high reactive organic carbon content (Hardison et al, 2015), and also where the ratio of organic carbon to NO_3^- is low (Algar and Vallino, 2014; Hardison et al,

2015). DNRA organisms have a higher affinity for NO_3^- than denitrifiers, so can out complete in low concentrations (Tiedje, 1988; Kelso et al, 1997). The second type of DNRA links oxidation of sulphur in reduced forms to the reduction of NO_3^- and is a chemolithoautrophic process (Brunet and Garcia-Gil, 1996). In the presence of high concentrations of free sulphides, DNRA may dominate over denitrification as the free sulphides have been observed to inhibit the final two steps of denitrification (Brunet and Garcia-Gil, 1996). The metabolic capability to link the oxidation of free sulphur to the reduction of NO3- has been observed across a wide range of genera including Thiobacillus, Thiomicospora and Thioploca (Otte et al, 1999; Jorgensen 1982; Kelly and Wood, 2000).

DNA has been measured in freshwater sediments, but this evidence is rare in the literature and is often conflicting (Burgin and Hamilton, 2007), with measurements often being below the limit of detection or negligible (Lansdown et al, 2012). In this later study, river sediments were incubated with excess peptone to provide a readily available organic carbon source and then had additions of an isotopic nitrogen tracer. Only 4% of the $^{15}NO_3^-$ converted was due to DNRA activity, which may have been down to dominance from denitrification in these incubations.

1.6 The role of climate change and anthropogenic influences

The nitrogen cycle is tightly coupled to the carbon cycle due to its strong regulation of primary production (Galloway et al., 2008; Tyrrell, 1999). Figure 1.2 shows a simplified version of the interlinking carbon and nitrogen cycles. If fixed nitrogen becomes limiting for plant growth, there will be a reduction in primary production which would lead to a decrease of CO_2 drawdown from the atmosphere (Karl et al., 2002). The nitrogen cycle can therefore play a key role in climate regulation and global warming (Falkowski, 1997) but the full complexity of this is still unknown.

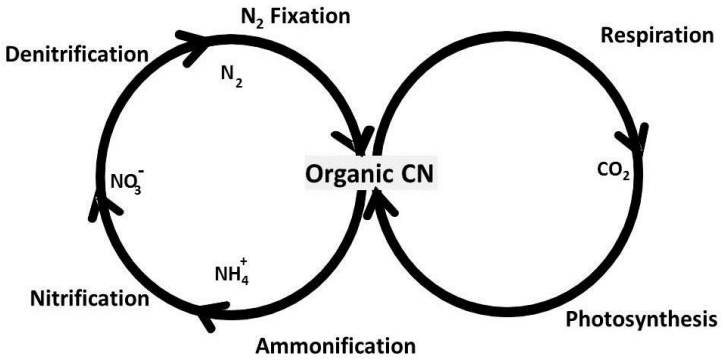

Figure 1.2 Simple diagram illustrating how the nitrogen and carbon cycles are closely interconnected. Note, for illustrative purposes not all pathways and intermediate e.g. anammox and nitrous oxide (N_2O) are included.

Climate change due to global warming is a well-accepted process that is occurring with warming predicted to continue increasing (IPCC, 2013). The effects of global warming include sea level rise, increased droughts, increased flooding, more extreme weather events and reduced biodiversity (Botkin et al., 2007; Loaiciga et al., 1996; Wigley, 2005) to mention a few. We must consider how fixed nitrogen will be balanced in ecosystems in response to increasing global temperatures. In an ideal situation, which is very difficult in reality, the rate at which nitrogen is lost from a system would be balanced by nitrogen fixation, so that primary production is not limited. At present we do not have the knowledge of how changes, created by rising temperatures, will alter the balance of the nitrogen cycle and to what extent (Falkowski, 1997). There is evidence of this delicate balance being disrupted in coastal sediments due to decreased primary production. Fulweiler et al. (2007) found estuarine sediments that were previously net sinks of nitrogen, reversed and became net sources of reactive nitrogen. Strong seasonal effects have been associated with potential rates of N_2 production through denitrification in estuarine and coastal sediments, with increasing rates with

in warmer months whereas the contribution of anammox showed no seasonal response (Brin et al., 2014). Brin et al. (2014) also found increased rates of denitrification significantly associated with Oxygen (O_2) consumption, which is an indicator of reactive sediments and organic matter availability.

We know warming has been show to increase the rates of photosynthesis and respiration in freshwater systems. Respiration has a greater thermal sensitivity (Gillooly et al., 2001; Perkins et al., 2012; Yvon-Durocher et al., 2012), therefore increases at a greater rate. Yvon-Durocher et al (2010) used experimental mesocosms to demonstrate the effect of a 4°C temperature increase (predicted global warming: IPCC, 2013) on the balance of respiration and primary production and found a 13% decrease in carbon sequestration. Respiration increased at a higher rate than photosynthesis with apparent activation energies of 0.62 eV and 0.43 eV for respiration and photosynthesis respectively. Photosynthesis has a much weaker temperature dependence than respiration (Allen et al, 2005; Dewar et al, 1999), creating an imbalance in net photosynthesis and net respiration.

In contrast to the carbon cycle, if analogous imbalances occur in the nitrogen cycle, this could affect the amount of reactive nitrogen available for plant productivity which would impact the carbon cycle by restricting the drawdown of CO_2 (Gruber and Galloway, 2008). This will be even more important in those systems away from anthropogenic nutrient loading with low nutrient levels. However, activation energies of BFN have been calculated as high as 1.06 eV (Houlton et al., 2008), which is greater than has been observed for denitrification (0.63 eV) (Canion et al., 2014) which would suggest both losses of reactive nitrogen and input through BNF could potentially increase at the same rate with warming.

On a long-term-global-scale, nitrogen fixation and nitrogen removal processes should keep the nitrogen cycle in balance and this has been seen in the past with ice-core analysis. However, as previous studies have determined, nutrient cycles can become unbalanced due to

anthropogenic inputs (through fertilizers and fossil fuel burning etc.). Over the past 40 years there has been a 120% increase in anthropogenic reactive nitrogen (Galloway et al., 2008) primarily through the Harber-Bosch process used for fertiliser production and burning of fossil fuels (Seitzinger et al., 2006). Most of the fixed nitrogen added to fields in the form of fertilisers is washed out from the soils and makes its way into freshwater systems (Paerl, 1997).

As previously mentioned, even with low concentrations of nutrients such as ammonia and nitrate, nitrogen fixation will no longer occur and the nitrogen fixers will be outcompeted by other photosynthetic organisms. The compounding effect of increased anthropogenic nutrient loading and increased global temperatures may have sever effects. If denitrification were to increase at a higher rate than nitrogen fixation, which has been suggested by previous studies looking into past glacial and interglacial periods (Altabet et al, 1995; Ganeshram et al, 1995), we could see a loss of photosynthetic productivity in aquatic systems (Codispoti, 1995). This would lead to reduced sequestration of CO_2 and create a positive feedback, causing a further increase in the Earth's surface temperature. This effect would be further exacerbated by N_2O production from increased rates of denitrification. The nitrogen cycle is a highly complex series of individual components closely linked to one another and we need to increase our knowledge to understand how they will be effected with our changing climate.

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Chapter 2: Effect of temperature on N_2 and N_2O production in pure cultures of denitrifying bacteria

2.1 Introduction

Denitrification is a major process for removing fixed nitrogen (Codispoti, 1995) and it is carried out by taxonomically diverse groups of, mainly heterotrophic, facultative anaerobic bacteria (Knowles, 1982) and, through a complex series of reactions involving numerous intermediates, ultimately reduces nitrate (NO_3^-) to di-nitrogen gas (N_2) via nitrous oxide (N_2O) (Falkowski, 1997). This process requires depleted oxygen (~ < 20-25 µM) down to those below detection which can be found in natural environments such as aquatic sediments, stratified water columns and water-logged soils (Francis et al., 2007; Knowles, 1982; Smith, 1997). Denitrification is an important process to understand as it removes biologically available, fixed nitrogen from ecosystems which may reduce primary productivity and ultimately carbon sequestration (Seitzinger, 1988) and is also a major source of N_2O. N_2O is a potent greenhouse gas (GHG) with radiative forcing effects some 300 times greater than that of carbon dioxide (CO_2) (Ravishankara et al., 2009; Wright et al., 2012) and causes damage to stratospheric ozone (Holtan-Hartwig et al., 2002; Knowles, 1982). N_2O has been classified as an ozone depleting substance (ODS) with the highest weighted ozone depleting potential (ODP) and is predicted to remain this way for the rest of the 21st Century (Ravishankara et al., 2009).

Globally, nitrogen loss through denitrification from oceans alone has been estimated to be ~ 230 (± 60) Tg N y^{-1} (DeVries et al., 2012) with rates expected to increase with increasing global temperatures. Both direct and indirect effects of warming on denitrification have been observed in aquatic and terrestrial systems. Indirect effects include decreased oxygen concentrations through either reduced solubility of oxygen in aquatic systems, or increased respiration (e.g. Smith, 1997; Veraart et al., 2011).

Others have investigated the effects of temperature on N_2 and N_2O production through denitrification in a variety of environments. For example, strong seasonal effects, driven by temperature change, show greater potential for denitrification in estuarine and coastal sediments (Brin et al., 2014). Further, increased denitrification was significantly correlated with greater oxygen (O_2) consumption, which is an indicator of reactive sediments and organic matter availability which increase with warmer temperatures through increased respiration (Gillooly et al, 2001; Perkins et al., 2012). A clear direct effect of temperature was observed in freshwater experimental mesocosm where sediment denitrification doubled following a 3°C warming (Veraart et al, 2011). Calculated activation energies for N_2O production in soil samples exposed to a range of temperatures were 0.28-0.81 eV, revealing a strong, but variable temperature dependence (Holtan-Hartwig et al., 2002). However, reduction rates of N_2O to N_2 had similar activation energies within the same temperature range, suggesting no change in net flux of N_2O will be seen with increasing temperatures. In fact, lower temperatures actually showed a net flux of N_2O, due to reduction in enzymatic activity involved in N_2O reduction (Holtan-Hartwig et al., 2002). A hand full of studies have measured both N_2 and N_2O production, finding the ratio of N_2O/ N_2 increases with decreasing temperature (Avalakki et al., 1995; Bailey & Beauchamp, 1973; Keeney et al., 1979). In contrast, pond sediment slurries and grassland soils have shown a net increase in N_2O production with increasing temperature (Cantarel et al., 2012; Stadmark & Leonardson, 2007).

Whilst denitrifying bacteria have been extensively studied in pure cultures, most of the literature focuses on the response of N_2 and N_2O production with differing O_2 concentrations (e.g. Baumann et al., 1996; Kester et al, 1997) or measuring their ability to degrade different substrates (e.g Schocher et al., 1991). There are gaps in the literature of how pure cultures of these organisms will respond to the current pressure of increasing temperature in regards to production rates of N_2 and N_2O. It is important to understand the response of individual strains

of denitrifying bacteria to external variables such as increasing temperatures. Though in natural systems, these organisms are never in isolation, they will adapt differently to environmental factors and gaining knowledge of this will enable us to better predict future emissions of N_2 and N_2O.

This study investigated both the initial response of three strains of denitrifying bacteria (through the production of N_2 and N_2O) to warming and their thermal adaptation after short (~ 5 generations) periods of acclimatisation. Reported temperature dependencies for denitrification vary greatly which may be down to differences in community structure and the individual microbes present in those systems responding differently. Studying the response of pure strains will allow us to investigate the variability and responses within pure cultures, removing other confounding effects.

2.1.1 Aims and Hypothesise

The overall aim of this study was to investigate the thermal response of pure strains of denitrifying bacteria, by measuring metabolic activity through the production of both N_2 and N_2O gas, at a range of temperatures above, around and below their optimum range thermal. From this, the activation energies were calculated for each strain at each exposure temperature, with metabolic activity further investigated by calculating the ratio of N_2:N_2O produced. N_2O is a product of incomplete denitrification and is an indicator of the efficiency of the overall process. Two distinct experiments were carried out to investigate the thermal response of the pure cultures.

The first experiment investigated the metabolic activity of pure cultures of denitrifying bacteria that were incubated at different temperatures over several generations (10 ^{o}C, 22 ^{o}C, 27 ^{o}C and 37 ^{o}C). The aim of study one was to determine if the strains adapted metabolically to an extended period of exposure to these temperatures. Two main hypothesise were suggested for

this study; either there would be greater rates of gas production from those incubated in the warmer temperatures due to increased enzymatic activity, or similar gas production rates would be observed at all temperatures due to an adaptation of the enzymes controlling the different steps of the denitrification pathway.

The second experiment investigated the effect of short term temperature exposure on the strains denitrifying bacteria. The aim of this experiment was to measure the change in metabolic activity to a range of temperature when incubated at a single, optimum temperature and how the different strains responded to these temperature changes. The main hypothesis for this study was each strain would increase its production of N_2 and N_2O to a maximum at a temperature close to its optimum. Either side of this temperature, metabolic activity would decrease due to reduced enzymatic activity. The second hypothesis would be the three strains would respond in a similar manner. These strains were isolated from similar ecological niches and therefore have the same environmental requirements and could be expected to have similar metabolic responses to changes in temperature.

2.2 Methods

2.2.1 Strains of denitrifying bacteria

Three strains of denitrifying bacteria were selected for experimental analysis of the thermal sensitivity of denitrification in pure cultures. The selected strains were *Paracoccus denitrificans* (PD 1222), *Pseudomonas marginalis* (DMS 13124) and *Pseudomonas brenneri* (DMS 15294), all of which are mesophilic chemoorganoheterotrophic bacteria. The strains were chosen as they culture easily and on the same media which reduced variability within experiments. Pure cultures of *P. denitrificans* were obtained internally within the department and both *P. marginalis* and *P. brenneri* were obtained as freeze dried cultures from a culture

collection (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Germany) which were rehydrated on delivery.

2.2.2 Preparation of pure cultures

The three strains of denitrifying bacteria were grown in Tryptic soya broth media (Sigma Aldrich) with additions of 0.1 M ammonium chloride (NH_4Cl), 1M sodium nitrate ($NaNO_3^-$) and 1 M potassium dihydrogen phosphate (KH_2PO_4). Autoclaved media (5 mL) was dispensed into gas-tight vials (12 mL Exetainer, Labco, UK) and purged with oxygen free nitrogen (OFN, 99.998%, British Oxygen Company) to be completely anoxic (Tschech and Fuchs., 1987). OFN was gently bubbled for 10 minutes through the media within the gas-tight vials using a syringe filter attached to a long sterile needle, with an additional sterile needle as a valve through the septa. The sterile oxygen free media was left for 24 hours before inoculation to ensure it was not visibly contaminated.

To inoculate the growth media, 50 µL of one of the three strains from the starting cultures were injected through the septa of the vials using aseptic techniques. Inoculated vials for additional starting cultures were then placed on an orbital shaker at 65 rpm at room temperature (22^{o}C) to ensure constant but gentle mixing, this reduced the chances of clumping and maximised growth. After each inoculation, a loop spread of each culture was added to growth plates containing the same growth medium, with the addition of agar (20g L^{-1}). This was done to ensure the cultures were still uncontaminated by other organisms by observing the growth of single, pure cultures on the agar plates. Growth of any a different formation or colour would indicate contamination by another microorganism. This had to be done frequently as the growth medium used was a broad growth medium suitable for many other heterotrophic microorganisms.

From the growth curves, specific growth rate (h^{-1}) could then be calculated using the following equation:

$$\mu = \frac{\ln OD_2 - \ln OD_1}{t_2 - t_1}$$

Where μ is the specific growth rate (h^{-1}), $\ln OD_2 - \ln OD_1$ is the difference in natural log optical density between the start of the exponential phase (OD_1) and the end of the exponential phase (OD_2). T_2 is the time point of OD_2 and T_1 is the time point of OD_1 (h^{-1}) (Widdel, 2007).

The generation time (or doubling time) was calculated by dividing by the time it took for the OD to double by the length of the exponential growth phase.

2.2.3 Measuring thermal sensitivity of denitrification with prior temperature adaptation

The first investigation into the thermal response of pure cultures of denitrifying bacteria considered the effect of N_2 production rates after adaptation to temperatures. Using the same preparation methods above, sterile gas-tight vials (12 mL) containing media (5 mL, headspace 7 mL) were inoculated with starting cultures of bacteria (50 µL) which were stored at room temperature (22 ^{o}C). The inoculated vials were placed into different temperature controlled rooms on an orbital shaker. When the cultures began to double within the exponential phase, these were subsampled into new vials to ensure there was no limitation of growth and to keep the cultures in exponential phase. This was repeated five times before the experiment began allowing five generations to have grown at the specific temperature. The generation time was calculated by growth rate calculations carried out in preliminary experiments, measuring increase in turbidity when the pure strains were grown at the experimental temperatures, as mentioned above. This experiment was to investigate short-term adaptation to temperature of N_2 and N_2O production rates by pure cultures.

The experiment was initiated by adding a subsample (50 µL) of the thermal adapted cultures into gas-tight oxygen free vials (12 mL) containing growth media (5 mL). The media differed with the addition of $Na^{15}NO_3^-$ instead of $Na^{14}NO_3^-$ as the nitrate source to allow the production of N_2 to be traced by mass spectrometry. Time series sampling was carried out with replicates at each time point, at 4 different temperatures (Table 2.1). Figure 2.1 shows the basic experimental design which was repeated for all three strains of denitrifying bacteria. The experiment was stopped with an injection of zinc chloride (100 µL, 50% (w/v), Sigma Aldrich) through the septa. Samples for background reference amounts of N_2 (^{15}N natural abundance) and N_2O (background concentration) were prepared in the same way as above but injected with zinc chloride prior to inoculation. Production rates of N_2 and N_2O were measured as excess above reference samples. Additional vials were prepared as controls with the same media batch and left un-amended to ensure media was not contaminated.

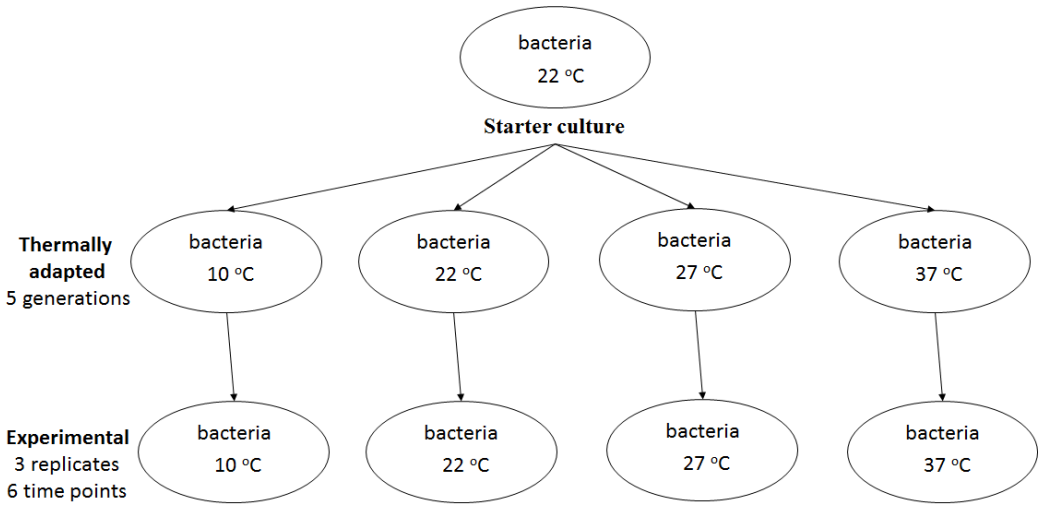

Figure 2.1 A schematic diagram for the experiment to investigate the thermal sensitivity of denitrification in pure cultures of denitrifying bacteria with prior thermal adaptation.

Table 2.1 Experimental design for temperature adaption of denitrification with pure cultures of denitrifying bacteria.

Strains	Temperatures	Time points (hours)	Replicates at each time point
P. denitrificans	10 °C, 22 °C, 27 °C & 37 °C	0, 2.5, 5.5, 22, 26, 31	3
P. brenneri		0, 1.5, 4.5, 7.5, 23, 27	3
P. marginalis		0, 0.5, 3, 7, 22.5, 27	3

2.2.4 Measuring thermal sensitivity of denitrification with no prior temperature adaptation

Cultures for these short-term temperature exposure experiments on the rates of N_2 and N_2O production were prepared in the same way as for the temperature adaptation experiment. The method differs in that only the starting cultures, which were kept at 22°C, were used for inoculations. Once vials were inoculated with the starting culture of denitrifying bacteria, they were exposed for a short period of time (12 h) to a range of temperatures using a thermal gradient bar (Figure 2.2). The thermal gradient was created using an aluminium thermal gradient block containing aluminium racks into which the 12 mL vials were fitted. A final temperature range between 11°C and 37.5°C was obtained. The thermal gradient within the block was created using a heated water bath (Grant TC120) at one end and a chilling unit (Grant RC350G) at the other. To obtain the widest temperature range possible the thermal gradient had to be completed in three separate ranges, 11-16°C (chiller: 2°C, heated water bath: 20°C), 16-36°C (chiller: 2°C, heated water bath: 80°C), 31-37.5°C (chiller: 25°C, heated water bath: 60°C). As with the previous method, reference samples were prepared in the same way to measure background concentration of both N_2 and N_2O. The rates of N_2 and N_2O were measured as excess above the reference samples. Two strains were investigated in this experiment: *P. denitrificans* and *P. brenneri*, with measurements of production of N_2 and N_2O

at a single time point as preliminary experiments allowed us to determine the length of the exponential phase to ensure that the experiment was carried out in this phase of linear production. Further experimental details can be seen in Table 2.2 and a schematic diagram of the basic experiment which was repeated for two strains of denitrifying bacteria can be seen in Figure 2.3.

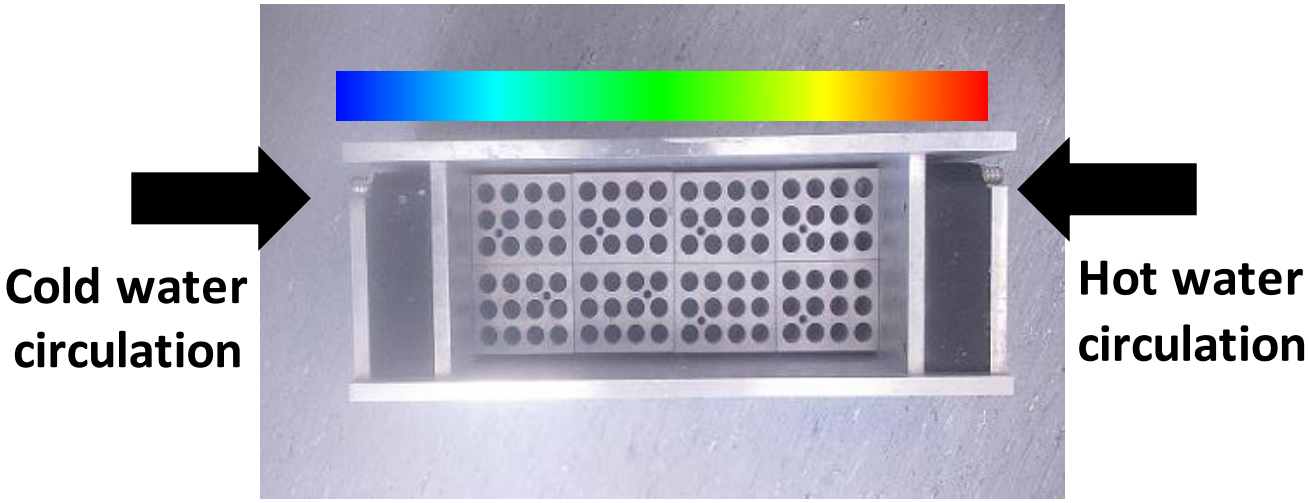

Figure 2.2 Solid aluminium thermal gradient bar used for short term temperature experiments. Heated water circulation at one end and cooled water circulation at the opposite end created a thermal gradient along the bar. Gradient block is shown open but was fitted with insulation and an aluminium lid during the incubations.

Table 2.2 Experimental design for rates of denitrification with pure cultures of denitrifying bacteria with no-prior temperature exposure. Only one time point was necessary as previous analysis determined the phase of constant N_2 production (Trimmer et al., 2006).

Strains	Temperatures (°C)	Time point (hours)	Replicates at each time point
P. denitrificans	11.5, 12, 12.5, 13, 14, 14.5, 15.5, 16.5, 22, 22.5, 28, 28.5,35, 36.7	12	3
P. brenneri		12	3

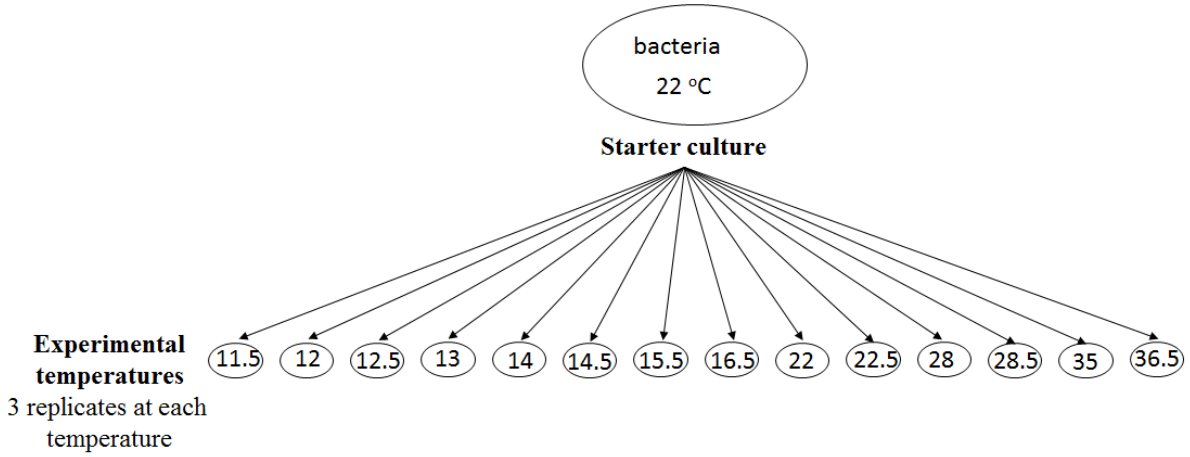

Figure 2.3 A schematic diagram for the experiment to investigate the thermal sensitivity of denitrification in pure cultures of denitrifying bacteria with no prior thermal adaptation across a wide temperature range.

2.2.5 Gas analysis for N_2 and N_2O

Samples for both the prior temperature exposure and non-prior temperature exposure thermal sensitivity of denitrification experiments were sampled for N_2O concentration using a gas chromatograph fitted with a micro-electron capture detector (GC/μECD, Agilent Technologies UK Ltd., South Queensferry, U.K.; Nicholls et al., 2007). Headspace N_2O concentrations were calculated from peak areas using a known standard concentration (Scientific and Technical Gases) and the total amount in the vial (headspace plus media) was corrected for temperature, pressure and solubility (Weiss & Price, 1980).

Production of $^{15}N\text{-}N_2$ ($^{29}N_2 + {}^{30}N_2$) gas from the denitrification experiments was measured in the headspace of each vial (culture) by continuous-flow isotope ratio mass spectrometry (Thermo-Finnigan, Delta Matt Plus) previously described in Trimmer and Nicholls (2009). Gas production rates were normalised to cell density by dividing by their respective OD_{680} values. As mentioned above, all measurements were taken whilst the cultures were in the linear growth phase (exponential) (Widdel, 2007).

2.2.6 Deriving apparent activation energies

The rates of the measured processes were log transformed and the incubation temperatures were converted to $1/kT$, where k is the Boltzmann's Constant (8.62×10^{-5} eV k^{-1} (T)) and T is the absolute temperature in Kelvin. The natural log rates were plotted against centered temperature ($1/kT$-$1/kT$c, where $1/kT$c is the average $1/kT$ for the thermal range included), on an Arrhenius plot where the negative slope of the regression line gives an estimate of the apparent activation energy in electron volts (eV) where 1eV is equivalent to 96.49 kJ mol^{-1}. Plotting in this manner centres the inverse temperature around zero so that the intercept (normalisation constant) is equivalent to the mean rate of activity (Perkins et al, 2012).

2.2.7 Statistical analysis

To determine whether there were any significant differences between the strains of pure denitrifyers, both parametric t-test and non-parametric Kruskal-Wallis one-way analysis of variance was carried out and either t-value or H value, respectively, was reported. To determine whether there were any significant effect of temperature on the rates of N_2 and N_2O production, one-way ANOVA was carried out on the slope of the rates and the p values recorded.

2.3 Results

2.3.1 Thermal sensitivity of denitrification with prior temperature adaptation

The rate of cell specific N_2 production increased with temperature for all 3 strains of denitrifying bacteria, with rates corrected for by absorbance (Figure 2.4). For *P. denitrificans*, the average rate of non-log-transformed N_2 production ranged from 128.9 (± 4.9) nmol N OD_{680}^{-1} h^{-1} at 10°C to an optima of 1683376 (± 192641) nmol N OD_{680}^{-1} h^{-1} at 27°C. For *P. brenneri*, the average rate of non-transformed N_2 production ranged from 705.5 nmol N OD_{680}^{-1} h^{-1} (± 43.7) at 10°C to an optima of 3657.3 (± 298.1) nmol N OD_{680}^{-1} h^{-1} at 27°C. *P. marginalis* produced a minimum average rate of non-transformed N_2 169.9 (± 13.5) nmol N OD_{680}^{-1} h^{-1} at

10°C to an optima rate of 1973.7 (± 77.6) nmol N OD_{680}^{-1} h^{-1} at 27°C, with deactivation observed at 37°C.

Due to deactivation at 37°C, activation energies were only calculated up to 27°C, and all statistics were carried out using data up to 27°C for all 3 strains. Similar and low apparent activation energies were observed for all three strains of pure cultures for the production rates of natural log N_2 (Table 2.3). A significant effect of temperature on N_2 production was observed for all 3 pure culture strains (Table 2.6). *P. marginalis* had the lowest apparent activation energy but the relationship between N_2 and temperature was most significant for this strain ($p < 0.0001$). No significant difference was observed between the three strains for natural log N_2 production rates ($p > 0.05$). Therefore, the N_2 production rates were pooled together for the three strains (Figure 2.4D) and the effect of temperature on the slope of N_2 production rates was highly significant (ANOVA, $f = 18.83$, $df = 26$, $p < 0.001$), suggesting as temperature increases, so does the cell specific rate of N_2 production, normalised to turbidity.

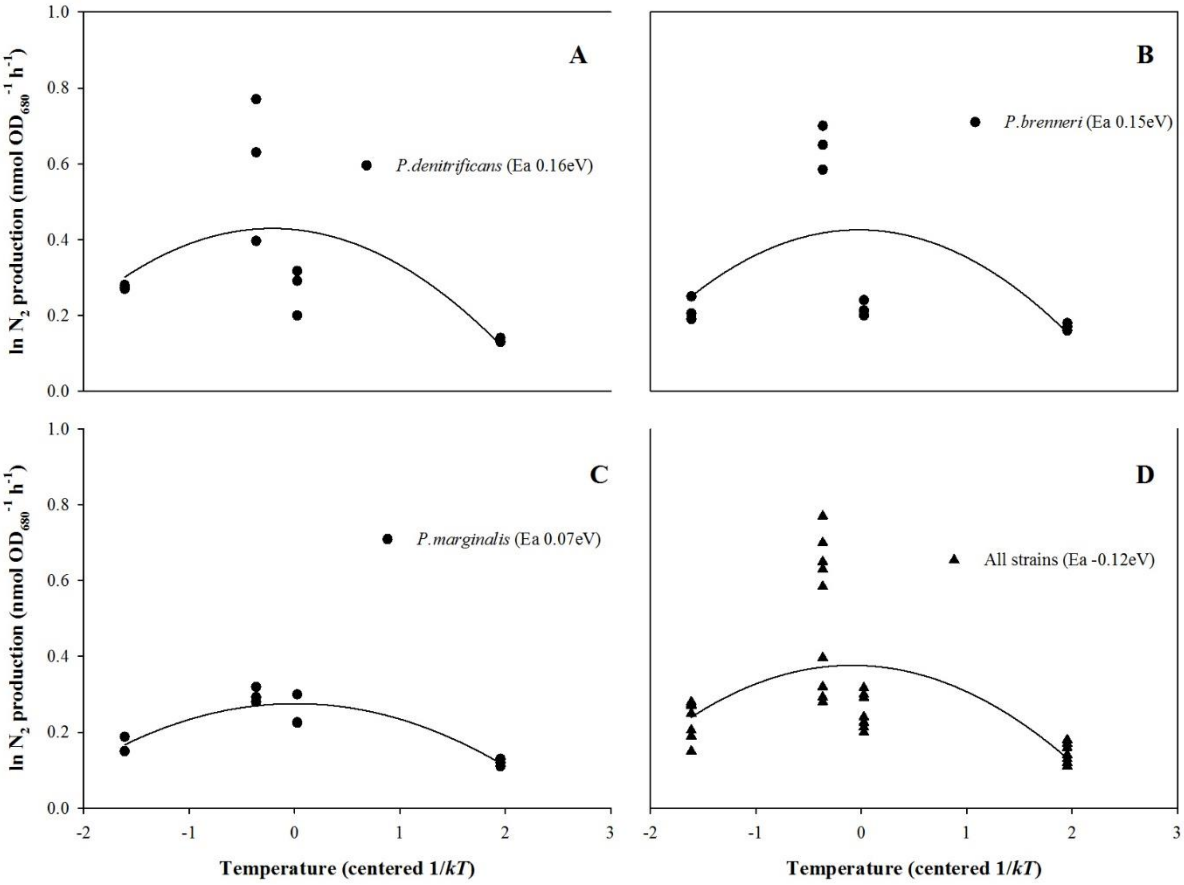

Figure 2.4 Arrhenius plot for the thermal sensitivity of $^{15}N_2$ production rates (natural log nmol OD_{680}^{-1} h^{-1}) for 3 strains of denitrifying bacteria *P. denitrificans*, *P. brenneri*, *P. marginalis* and all 3 strains rates pooled together against incubation temperature in pure cultures (centered1/*kT*, where *k* = Boltzmann's constant and *T* is in Kelvin) with prior temperature exposure. Temperature increases from right to left.

Table 2.3. Apparent activation energies (eV) of natural log of cell specific N_2 production for 3 strains of denitrifying bacteria grown in pure cultures calculated on the linear section of the data (10 – 27°C) with prior temperature exposure.

Species	*Ea (eV)*	r^2
P. denitrificans	0.16	0.55
P. brenneri	0.15	0.48
P. marginalis	0.07	0.91
Pooled data for all 3 strains	0.12	0.45

For each of the three strains, a non-significant effect of temperature on the growth-specific rate of N_2O production was observed ($p > 0.05$) (Table 2.6, Figure 2.5). Production was linear up to 27°C, therefore activation energies and statistical analysis was carried out on N_2O production rates up to 27°C. For *P. denitrificans*, the average rate of N_2O production ranged from 7.5 (± 1.1) nmol N_2O OD_{680}^{-1} h^{-1} at 10°C to an optima of 62.1 (± 15.67) nmol N_2O OD_{680}^{-1} h^{-1} at 27°C. For *P. brenneri*, the average rate of N_2O production ranged from 41.9 (± 7.43) nmol N_2O OD_{680}^{-1} h^{-1} at 37°C to 129.4 (± 15.6) nmol N_2O OD_{680}^{-1} h^{-1} at 27°C. *P. marginalis* produced a minimum average rate of N_2O 102.8 (± 34.1) nmol at 10°C and a maximum rate of 342.0 (± 45.8) nmol N_2O OD_{680}^{-1} h^{-1} at 27°C, with deactivation observed at 37°C. Apparent activation energies were calculated, suggesting a non-significant effect of temperature for all 3 strains (Table 2.6). *P. denitrificans* had a slightly greater thermal response than the other two strains (Table 2.4). *P. brenneri* had significantly higher rates of N_2O production than *P. marginalis* ($H = 15.1$, $df = 2$, $p < 0.001$). No other statistical difference of N_2O production rates were observed between strains. When the N_2O production rates from all 3 strains were pooled together, to get an average estimate of the process of N_2O production, a non-significant effect of temperature is still observed (ANOVA, $p > 0.05$).

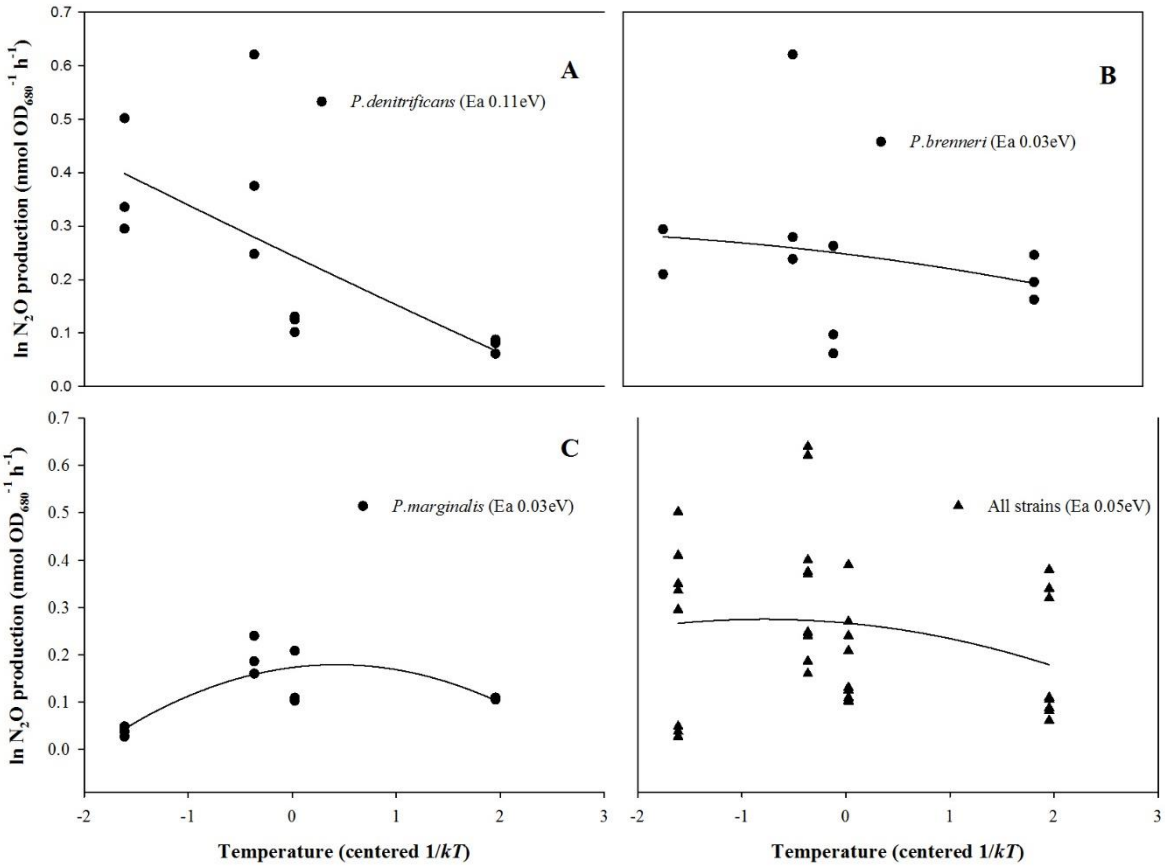

Figure 2.5 Arrhenius plots for the thermal sensitivity of N_2O production rates (natural log nmol OD_{680}^{-1} h^{-1}) for 3 strains of denitrifying bacteria pure cultures. *P. denitrificans*, *P. brenneri*, *P. marginalis* and all 3 strains rates pooled together against incubation temperature (centered $1/kT$, where k = Boltzmann's constant and T is in Kelvin) with prior temperature exposure. Temperature increases from right to left.

Table 2.4. Apparent activation energies (eV) of natural log N_2O production for 3 strains of denitrifying bacteria grown in pure cultures with prior temperature exposure (10–27°C).

Species	***Ea (eV)***	r^2
P. denitrificans	0.11	0.38
P. brenneri	0.03	0.08
P. marginalis	0.03	0.35
Pooled data for all 3 strains	0.05	0.11

The ratio of N_2O/N_2 evolution was calculated to determine the number of N_2O molecules produced for every molecule of N_2, with a positive ratio indicating more molecules of N_2O are produced for every molecule of N_2. N_2O is a genuine intermediate in the biochemical pathway of denitrification and allows us to investigate the effect of temperature on the efficiency of complete denitrification. All 3 strains of denitrifying bacteria showed a decrease in the ratio of N_2O/N_2 with increasing temperature as the rate of N_2O is unaffected by temperature, whereas N_2 increases, with deactivation energies suggesting a thermal response (Table 2.5, Figure 2.6).

P. denitrificans had a maximum average ratio of 0.059 (± 0.01) at 10°C and a minimum average ratio of 0.026 (± 0.007) at 24°C with a non-significant effect of temperature (Table 2.6). *P. brenneri* had a maximum average ratio of 0.11 (± 0.01) at 10°C and a minimum average ratio of 0.004 (± 0.0005) at 24°C with a significant effect of temperature (Table 2.6). *P. marginalis* had the greatest maximum average ratio of 0.65 (± 0.27) at 10°C and a minimum average ratio of 0.14 (± 0.003) at 24°C with a significant effect of temperature (Table 2.6). For all three strains, as temperature increases, N_2 increases and N_2O production rates stay relatively constant. The ratio of N_2O/ N_2 is significantly greater in *P. marginalis* than *P. denitrificans* ($H = 7.65$, $df = 2$, $p = 0.01$), with no other statistical differences observed. Again, the ratios from all 3 strains was pooled together to get an estimate of the overall ratio at each temperature. Increasing temperature had a significant effect on the pooed data with the ratio increasing with decreasing temperatures (ANOVA, $f = 4.33$, $df = 26$, $p < 0.05$).

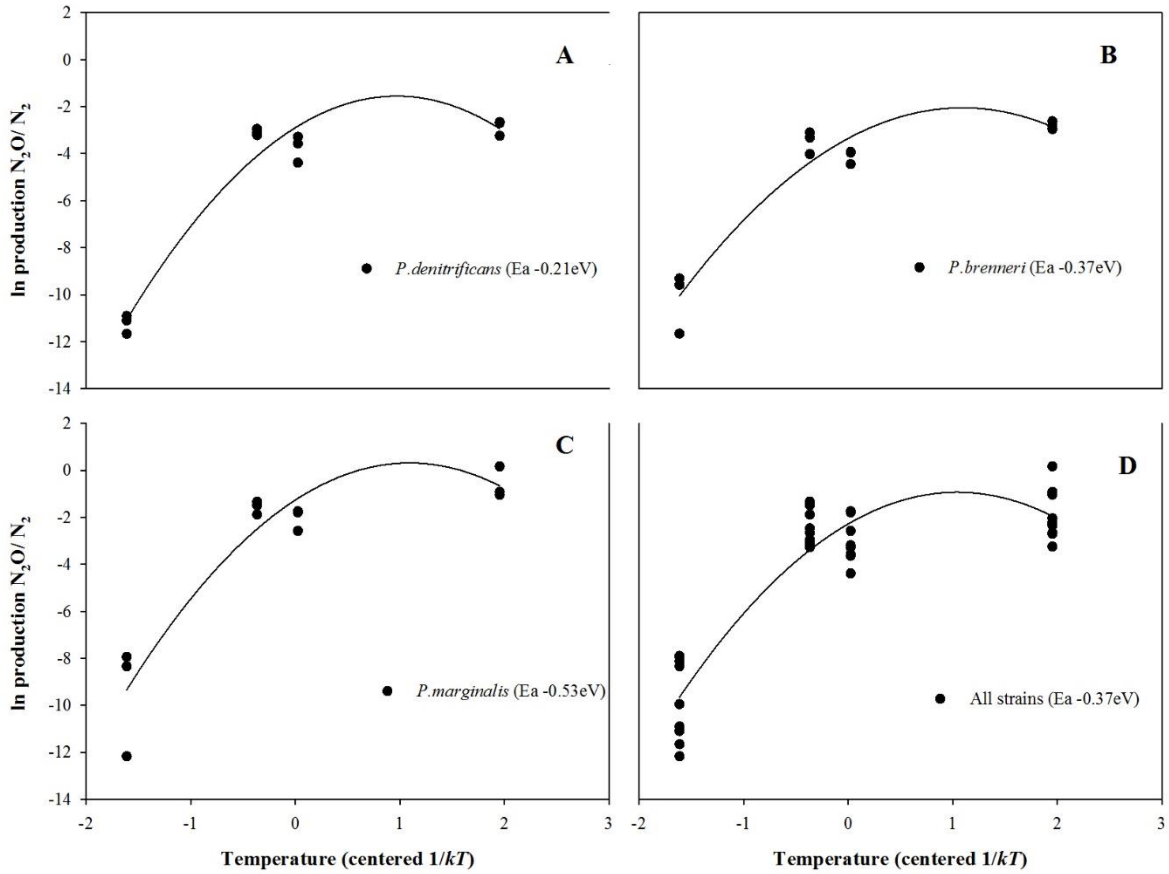

Figure 2.6 Arrhenius plots for the thermal sensitivity of natural log N_2O/N_2 production for 3 strains of denitrifying bacteria; *P. denitrificans*, *P. brenneri*, *P. marginalis* and all 3 strains rates pooled together against incubation temperature (centered $1/kT$, where k = Boltzmann's constant and T is in Kelvin) with prior temperature exposure. Temperature increases from right to left.

Table 2.5 Apparent deactivation energies (eV) of natural log N_2O/N_2 production for 3 strains of denitrifying bacteria grown in pure cultures calculated on the linear section of the data with prior temperature exposure.

Species	*Ea (eV)*	r^2
P. denitrificans	-0.21	0.19
P. brenneri	-0.37	0.50
P. marginalis	-0.52	0.54
Pooled data for all 3 strains	-0.36	0.15

Table 2.6 Statistical significance for measured processes with one way ANOVA testing for the effect of temperature on N_2 and N_2O with for 3 pure strains of denitrifying bacteria with prior temperature exposure.

Test	Strain	variable	*f*	*Df*	*p*
ANOVA	*P. denitrificans*	ln N_2	8.66	8	< 0.03
ANOVA	*P. brenneri*	ln N_2	6.41	8	< 0.05
ANOVA	*P. marginalis*	ln N_2	67.4	8	< 0.0001
ANOVA	*P. denitrificans*	ln N_2O	4.23	8	> 0.05
ANOVA	*P. brenneri*	ln N_2O	0.54	8	> 0.05
ANOVA	*P. marginalis*	ln N_2O	3.28	8	> 0.05
ANOVA	*P. denitrificans*	ln N_2O/ N_2	1.68	8	> 0.05
ANOVA	*P. brenneri*	ln N_2O/ N_2	6.92	8	< 0.05
ANOVA	*P. marginalis*	ln N_2O/ N_2	8.14	8	< 0.03

2.3.2 Thermal sensitivity of denitrification with no prior temperature adaptation

The rates of N_2 production across the entire thermal gradient were calculated and plotted on an Arrhenius plot (Figure 2.7) to identify the thermal response of the two strains. To calculate apparent activation energies, the temperature range was split into 11.5-16^{o}C (range one, Figure 2.8A) and 16.5^{o}C to 36^{o}C (range two, Figure 2.8B) for both *P. denitrificans* and *P. brenneri* as the response is non-linear, with optimal temperatures in the middle range. The average rates at the individual temperatures was also plotted against temperature (^{o}C) to show the thermal response in a more familiar format (Figure 2.8C and 2.8D). The two strains have different optimal temperatures for N_2 production (denitrification). *P. denitrificans* shows an increase across the entire temperature range, but with the rate of production decreasing after 16^{o}C. Conversely, *P. brenneri* shows an increase in production with temperature up to 16^{o}C, with lower N_2 production rates above 16.5^{o}C.

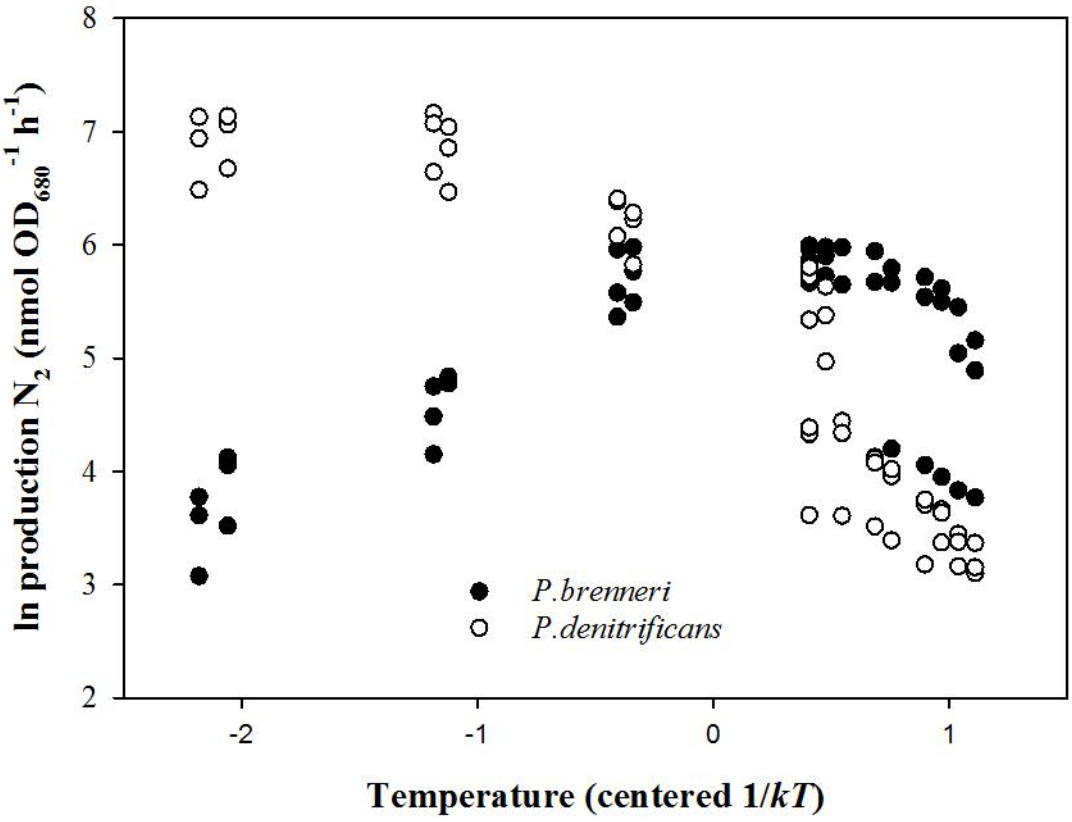

Figure 2.7 Arrhenius plots for the thermal sensitivity of N_2 production rates (natural log nmol OD_{680}^{-1} h^{-1}) for two pure strains of denitrifying bacteria (*P. denitrificans and P. brenneri*) grown in pure cultures, with no prior temperature exposure, against incubation temperature (centered 1/*kT*, where *k* = Boltzmann's constant and *T* is in Kelvin) across the entire thermal gradient (11.5-36.5°C).

The rate of N_2 for *P. denitrificans* reached a maximum average of 1077 (± 159) nmol N OD_{680}^{-1} h^{-1} at 28.5°C. A highly significant effect of temperature was observed for N_2 production rates for *P. denitrificans* ($p < 0.001$, Table 2.8) for both ranges. A greater apparent activation energy was calculated from the lower temperature range with a steeper slope (Table 2.7). The rate of N_2 production for *P. brenneri* had an optimum temperature of 22°C with a maximum average N_2 production rate of 319 (± 44) nmol N OD_{680}^{-1} h^{-1}. A significant effect of temperature was

observed for N_2 for *P. brenneri* ($p < 0.01$, Table 2.8) for temperature range two (16.5-36.5°C). A deactivation in the rates of N_2 production was calculated in the higher temperature range and a strong activation energy calculated in the lower temperature range for *P. brenneri* (Table 2.7). *P. brenneri* produced significantly more N_2 than *P. denitrificans* within temperature range one ($t = -7.46$, $df = 14$, $p < 0.0001$) and significantly less than *P. denitrificans* within temperature range two ($t = 4.11$, $df = 12$, $p < 0.01$). As the production rates were normalised for turbidity, a proxy for cell density, the specific growth rate of N_2 production is greater for *P. brenneri* than *P. denitrificans*, with a much higher intercept in the lower temperature (Figure 2.8A), and this relationship is reversed in the higher temperature range (Figure 2.8B). *P. denitrificans* appears to have a wider thermal niche than *P. brenneri*.

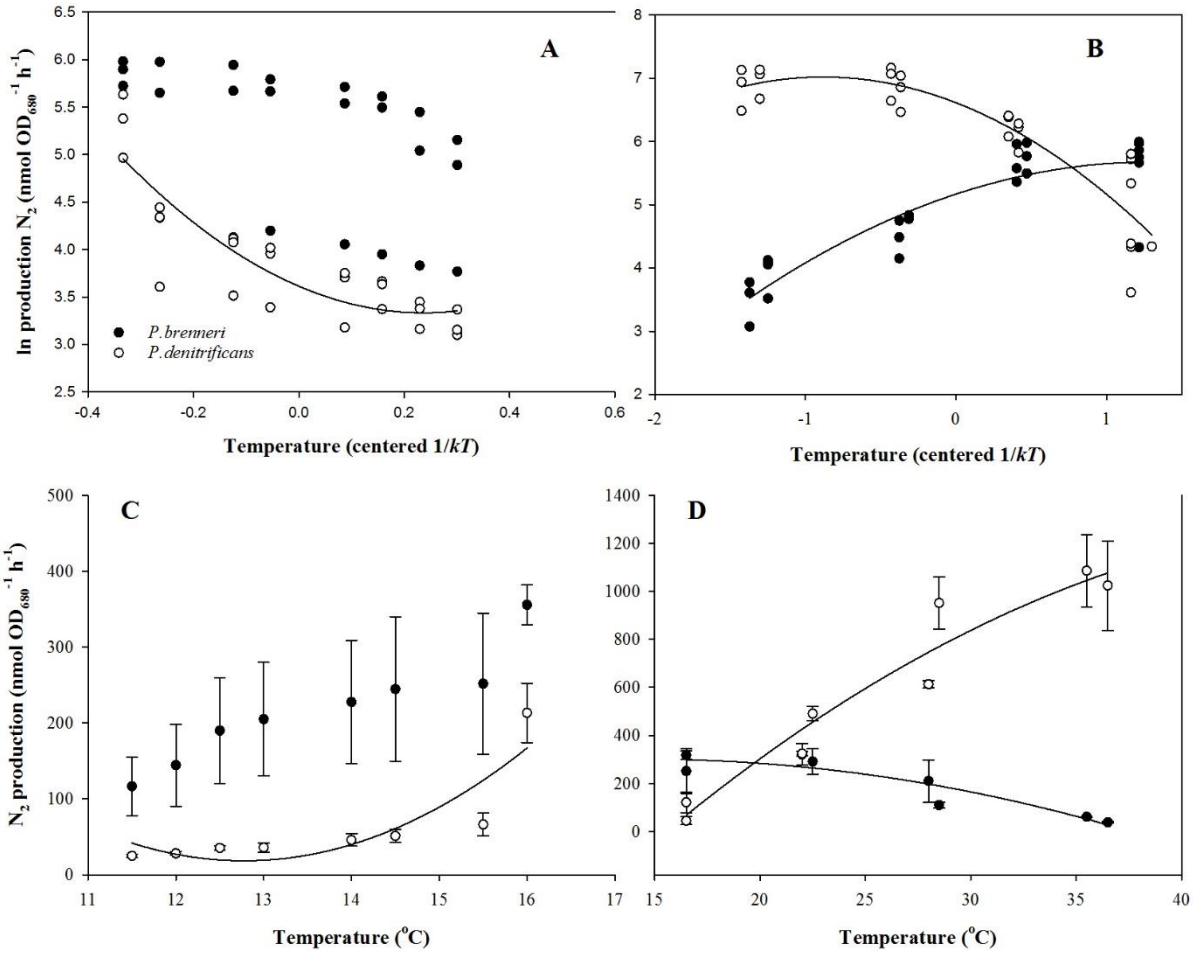

Figure 2.8 Arrhenius plots for the thermal sensitivity of N_2 production rates (natural log nmol OD_{680}^{-1} h^{-1}) for two pure strains of denitrifying bacteria (*P. denitrificans and P. brenneri*) grown in pure cultures, with no prior temperature exposure, against incubation temperature (centered $1/kT$, where k = Boltzmann's constant and T is in Kelvin). A) Temperature range one (11.5-16°C), B) Temperature range two (16.5-36.5°C). Temperature increases from right to left. Average rates of N_2 production (± *se*), C) Temperature range one (11.5-16°C), D) temperature range two (16.5-36.7°C) against incubation temperature (°C). Filled circles are for *P. brenneri* and open circles are for *P. denitrificans*.

Both *P. denitrificans* and *P. brenneri* show very little response in production of N_2O in response to increasing temperature between 11.5 to 16°C, with rates increasing slightly after 16.5°C for *P. denitrificans* and decreasing for *P. brenneri* (Figure 2.9), so again the data were

divided into two temperature ranges (range one 11.5-16°C and range two 16.5-36.5°C, Figure 2.10), to allow accurate calculations of apparent activation energies (Table 2.7). The rate of N_2O for *P. denitrificans* reached a maximum average of 152 (± 58) nmol N_2O OD_{680}^{-1} h^{-1} at 28.5°C, the optimal temperature.

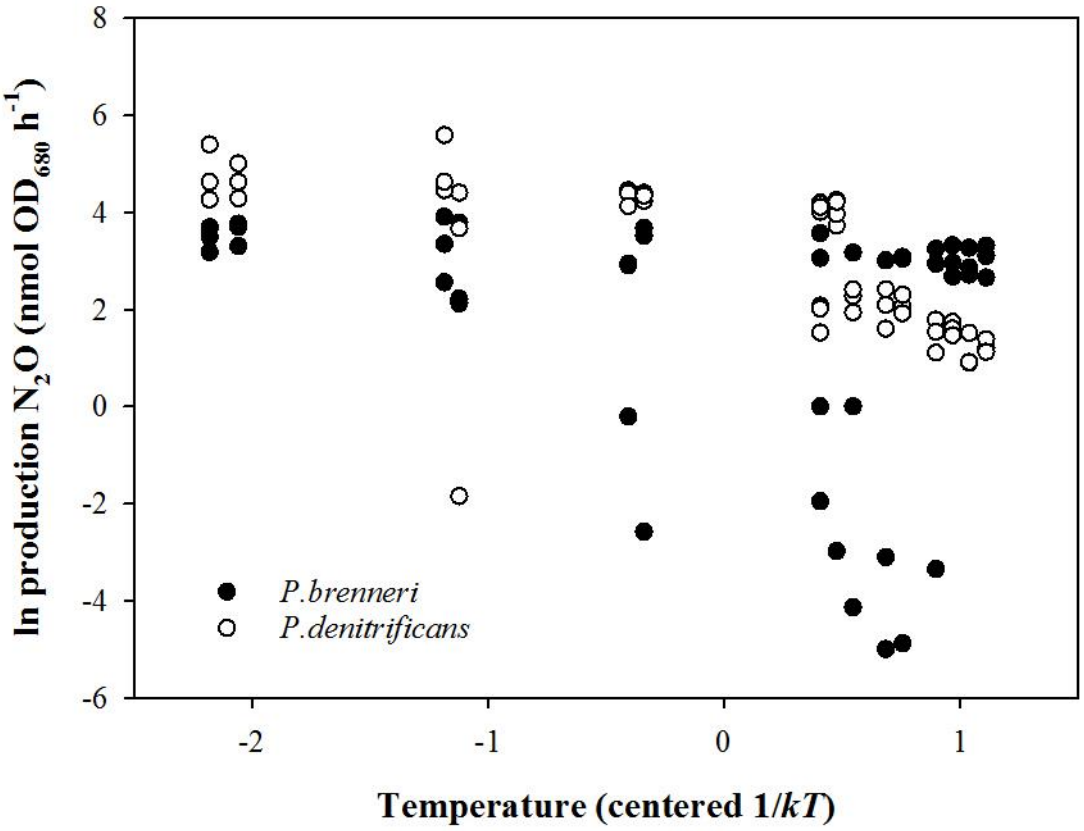

Figure 2.9 Arrhenius plot for the thermal sensitivity of N_2O production rates (natural log nmol OD_{680}^{-1} h^{-1}) for two pure strains of denitrifying bacteria (*P. denitrificans and P. brenneri*) grown in pure cultures, with no prior temperature exposure, against incubation temperature (centered $1/kT$, where k = Boltzmann's constant and T is in Kelvin) across the entire thermal gradient (11.5-36.5°C).

A highly significant effect of temperature was observed for rates of N_2O production for *P. denitrificans* within temperature range one ($p < 0.001$) and temperature range two ($p < 0.05$, Table 2.8), with significantly greater production rates observed in the higher temperature range

between 16.5 and 36°C (t = -14.09, df = 14, p <0.0001). The rate of N_2O for *P. brenneri* reached a maximum average of 37 (± 3) nmol N_2O OD_{680}^{-1} h^{-1} at 22°C. No significant effect of temperature was observed for N_2O for *P. brenneri* with either temperature range (Table 2.8). N_2O production rates are significantly greater within temperature range one for *P. brenneri* (U = 0.00, t = 36, df = 14, p = 0.001) than *P. denitrificans*. However, the two strains swap positions when temperatures increase in the second range of 16.5-36.5°C, with *P. denitrificans* N_2O production rates are significantly greater than *P. brenneri* (t = 4.39, df = 12, p <0.001). Results indicate that the two strains have different responses to temperature for production rates of N_2O.

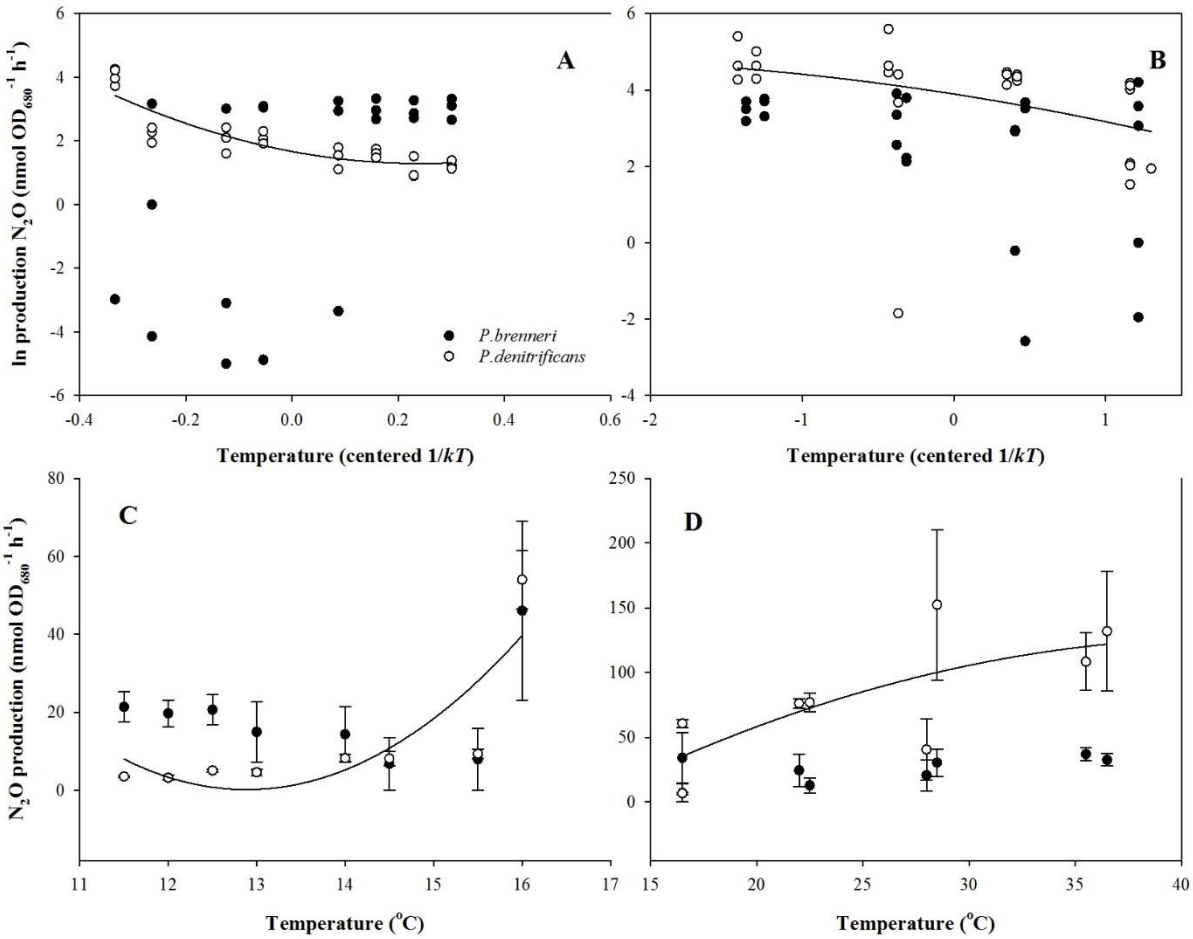

Figure 2.10 Arrhenius plots for the thermal sensitivity of N_2O production (nmol OD_{680}^{-1} h^{-1}) for two strains of denitrifying bacteria (*P. denitrificans* and *P. brenneri*) grown in pure cultures, with no prior temperature exposure, against incubation temperature (centered $1/kT$, where k = Boltzmann's constant and T is in Kelvin). A) Range one (11.5-16°C), B) Range two (16.5-36.5°C). Temperature increases from right to left. Average rates of N_2O production (± *se*) C) Temperature range one (11.5-16°C) D) temperature range two (16.5-36.7°C) against incubation temperature. Filled circles are for *P. brenneri* and open circles are for *P. denitrificans*.

The ratio of N_2O/ N_2 production shows very little thermal response for either strain across the entire temperature range (Figure 2.11). Within the temperature range of 11.5-16°C, both *P. denitrificans* and *P. brenneri* show almost no response to increasing temperature (Figure 2.12

A). Within temperature range two, more of a thermal response can be observed (Figure 2.12 B). The average rates at the individual temperatures was also plotted against temperature (°C) to show the thermal response in a more familiar format (Figures 2.12 C and 2.12 D).

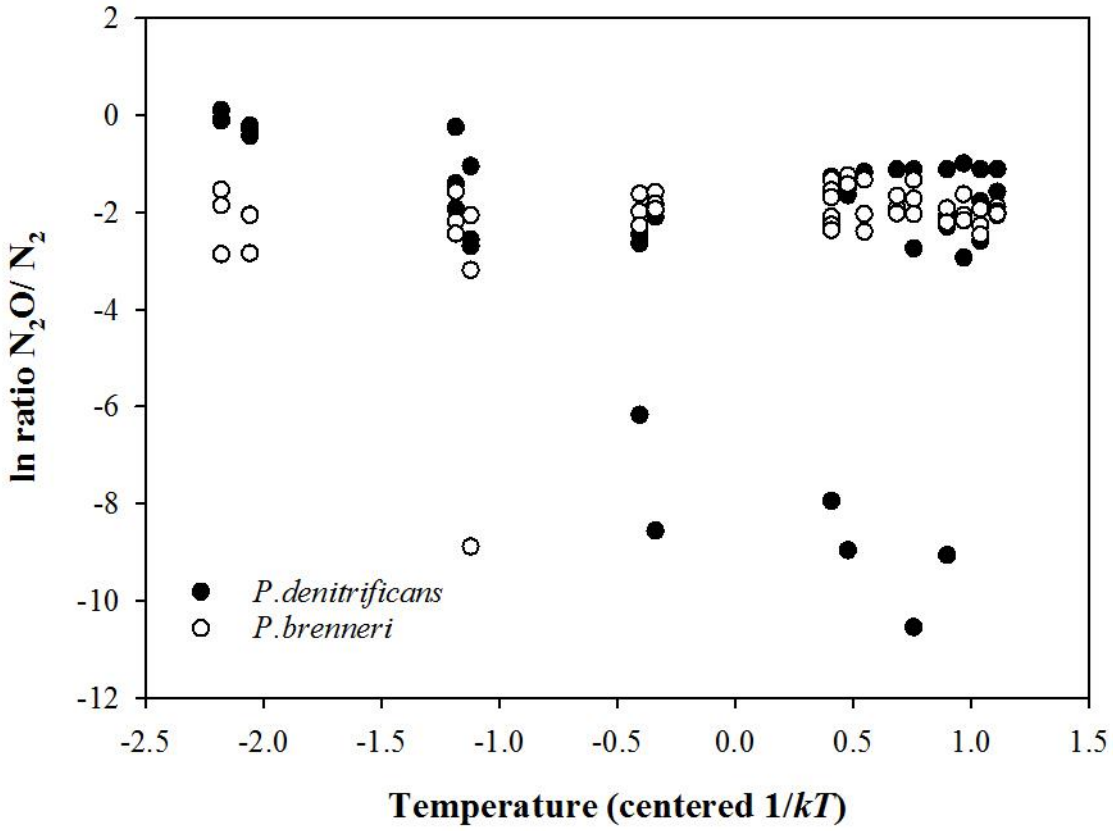

Figure 2.11 Arrhenius plots for the thermal sensitivity of N_2O/N_2 production rates (natural log nmol OD_{680}^{-1} h^{-1}) for two pure strains of denitrifying bacteria (*P. denitrificans* and *P. brenneri*) grown in pure cultures, with no prior temperature exposure, against incubation temperature (centered $1/kT$, where k = Boltzmann's constant and T is in Kelvin) across the entire thermal gradient (11.5-36.5°C). Temperature increases from right to left.

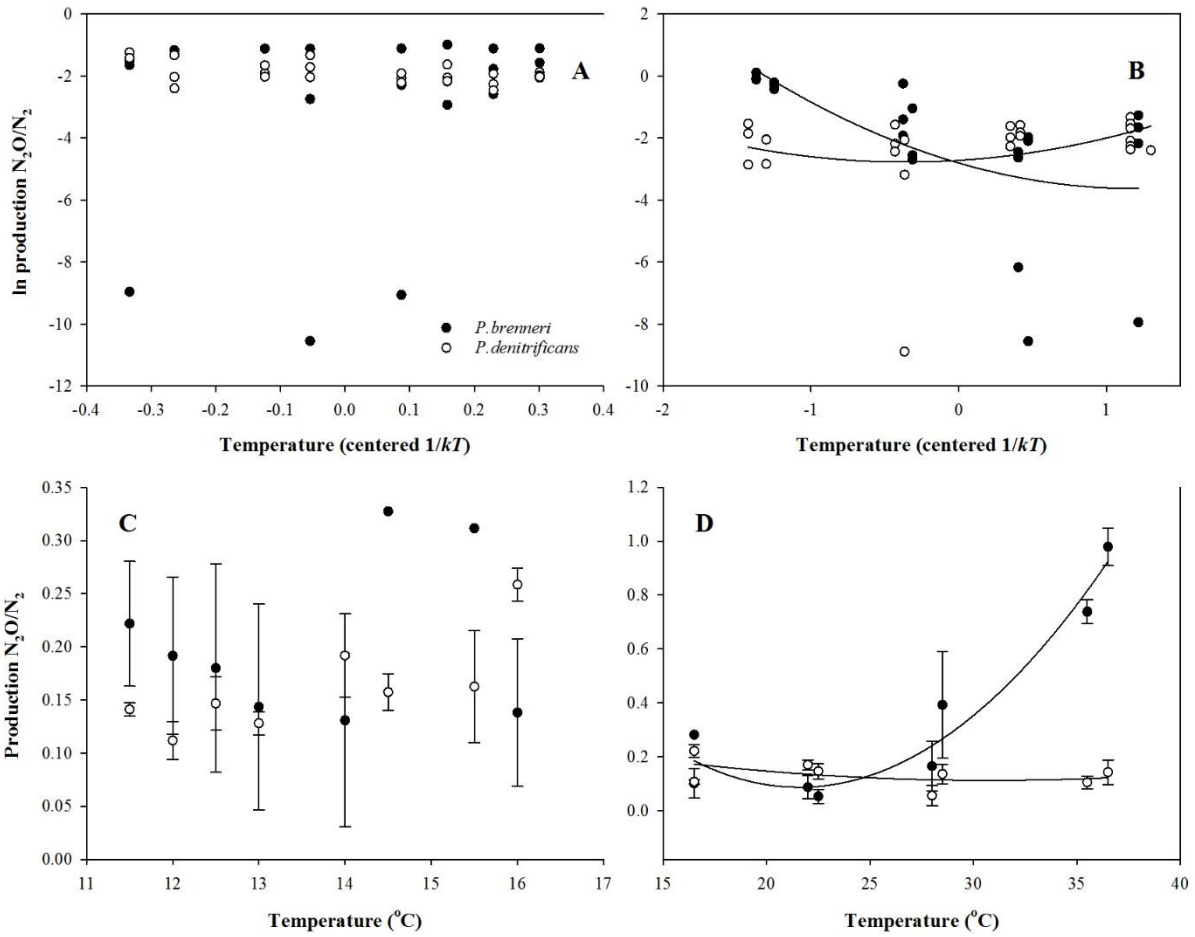

Figure 2.12 Arrhenius plots for thermal sensitivity of N_2O/N_2 ratio for two strains of denitrifying bacteria (*P. denitrificans and P. brenneri*) grown in pure cultures, with no prior temperature exposure, against incubation temperature (corrected $1/kT$, where k = Boltzmann's constant and T is in Kelvin). A) Temperature range one (11.5-16°C), B) Temperature range two (16.5-36.5°C). Temperature increases from right to left. Average rates of N_2O/N_2 production (± *se*) C) Temperature range one (11.5-16°C) D) temperature range two (16.5-36.7°C) against incubation temperature. Filled circles are for *P. brenneri* and open circles are for *P. denitrificans*.

The ratio of N_2O/N_2 for *P. denitrificans* reached a maximum average of 0.26 (± 0.015) at 16°C. The effect of temperature was non-significant within temperature range one ($p > 0.05$), but significant within range two for *P. denitrificans* ($p < 0.05$, Table 2.6). The ratio in temperature

range one shows a positive effect of temperature with a positive activation energy, with the reverse observed with the higher temperatures (Table 2.7), though both are very minor responses to temperature.

The ratio of N_2O/N_2 for *P. brenneri* had an optimum temperature of 36.5°C with a maximum average ratio of 0.98 (± 0.07). No significant effect of temperature was observed for temperature range one for *P. brenneri* ($p > 0.05$) but a significant effect was observed within temperature range two ($p < 0.05$, Table 2.8).

The ratio of N_2O/N_2 was significantly greater in *P. brenneri* than *P. denitrificans* within the lower temperature range ($t = -4.375$, $df = 14$, $p < 0.001$), with no significant difference in the ratio in the higher temperature range of 16.5-36°C ($p > 0.05$). Higher ratio's in *P. brenneri* is confirmed by the lower production rates of N_2O compared to production rates of N_2O by *P .denitrificans*.

Table 2.7 Apparent activation (Ea) energies (eV) of measured variables *P. denitrificans* and *P. brenneri* grown in pure cultures, with no prior temperature exposure, calculated on the linear section of the data.

Strain	Variable	Temperature range	*Ea (eV)*	*r2*
P. denitrificans	ln N_2	11.5-16°C	1.38	0.59
P. denitrificans	ln N_2O	11.5-16°C	1.40	0.52
P. denitrificans	ln ratio N_2O/N_2	11.5-16°C	0.02	0.0003
P. brenneri	ln N_2	11.5-16°C	1.10	0.68
P. brenneri	ln N_2O	11.5-16°C	0.21	0.02
P. brenneri	ln ratio N_2O/N_2	11.5-16°C	-0.25	0.43
P. denitrificans	ln N_2	16-36.5°C	0.58	0.74
P. denitrificans	ln N_2O	16-36.5°C	0.26	0.32
P. denitrificans	ln ratio N_2O/N_2	16-36.5°C	- 0.31	0.35
P. brenneri	ln N_2	16-36.5°C	- 0.90	0.87
P. brenneri	ln N_2O	16-36.5°C	-0.18	0.08
P. brenneri	ln ratio N_2O/N_2	16-36.5°C	0.69	0.45

Table 2.8 Statistical significance for measured processes testing the sensitivity of temperature on the slopes (* indicate non-significant results). Kruskal Wallis Rank Sum test was used for non-normal data (indicated by ×) and linear mixed effect model and ANOVA for statistically normal data for cultures with no prior temperature exposure.

Strain	Temperature Range	Response Variable	Random Variable	X^2	*df*	*p*
P. denitrificans	11.5-16°C	ln N_2	Replicate	39.98	4	<0.001
P. denitrificans	11.5-16°C	ln N_2O	Replicate	15.6	4	<0.001
P. denitrificans	11.5-16°C	ln ratio N_2O/N_2	Replicate		4	>0.05*
P. denitrificans	16.5-36.5°C	ln N_2	Replicate	37.12	4	<0.001
P. denitrificans	16.5-36.5°C	ln N_2O	/	15.39	7	<0.05
P. denitrificans	16.5-36.5°C	ln ratio N_2O/N_2	/	14.15	7	<0.05×
P. brenneri	11.5-16°C	ln N_2	/	5.53	7	>0.05*×
P. brenneri	11.5-16°C	ln N_2O	/	4.77	7	>0.05*×
P. brenneri	11.5-16°C	ln ratio N_2O/N_2	/	2.52	7	>0.05*×
P. brenneri	16.5-36.5°C	ln N_2	/	20.29	7	<0.01 ×
P. brenneri	16.5-36.5°C	ln N_2O	/	3.82	7	>0.05*×
P. brenneri	16.5-36.5°C	ln ratio N_2O/N_2	/	15.99	7	<0.05 ×

2.4 Discussion

The data from the current study indicates N_2 production has a stronger thermal sensitivity than N_2O production in pure strains of denitrifying bacteria. Furthermore, prior temperature exposure causes adaptive responses, reducing their thermal sensitivity and optimal temperatures.

With no prior temperature exposure, the activation energies of N_2 production from *P. denitrificans* and *P. brenneri* were greater within the lower temperature range (11.5-16°C) (1.38 eV and 1.10 eV respectively) than the higher temperature range (16.5-37.5°C) (0.58 eV and -0.90 eV respectively). These activation energies are similar to than those in the literature

from environmental samples in riverbed sediments (0.84 eV), phototrophic river biofilms (1.42 eV), temperate marine sediments (0.40-0.63 eV) and estuarine sediments (0.37-0.55) (e.g. Boulêtreau et al., 2012; Brin et al., 2016; Sheibley et al., 2003). Our values of N_2 production and activation energies have the potential to be higher than those recorded in environmental samples as there are no other factors confounding the potential rates, i.e. the cultures were not substrate limited. *P. denitrificans* shows only a slight decrease in activation energy of N_2 production between the lower and higher temperature ranges, suggesting this strain has a broader thermal niche than *P. brenneri* which shows deactivation in the higher temperature range. This is likely due to denaturing of the enzymes which can happen when exposed to temperatures above their normal range. The higher intercept indicates *P. brenneri* is more competitive than *P. denitrificans* below 16^{o}C and the reverse is evident above 16.5^{o}C (Figure 2.8).

The activation energies of N_2 production with prior exposure for the three strains (*P. denitrificans, P. brenneri* and *P. marginalis*) were all very similar and therefore were pooled together to get an overall activation of the process which was much lower (0.12 eV) than those calculated with no prior exposure. The lower temperature dependency in samples which experienced prior temperature exposure suggested that these cultures have adapted and decreased their thermal sensitivity. *P. denitrificans* and *P. brenneri* have very similar activation energies and intercepts and optimal temperature with prior exposure. These findings suggest, with prior temperature exposure, *P. denitrificans* and *P. brenneri* have become more similar in their physiological responses in regards to their capacity to produce N_2 and the thermal sensitivity of the process.

The activation energies for N_2O production from *P. denitrificans* and *P. brenneri*, with no prior temperature exposure, are again greater in the lower temperature range (1.4 eV and 0.21 respectively) than in the higher temperature range (0.26 eV and -0.18 respectively). These

responses to warming observed in N_2O production are comparable with those reported in arable (0.29-0.79 eV) and desert soils (0.42 eV) (Holtan-Hartwig et al., 2002; Peterjohn, 1991). The rates of N_2O production from the two strains of denitrifying bacteria (*P. denitrificans and P. brenneri*) with no prior temperature exposure had differing thermal responses. *P. denitrificans* had a significant increase in production of N_2O with increasing temperature, whereas *P. brenneri* had no significant thermal response. Slightly higher production of N_2O was observed from *P. brenneri* in the lower temperature, indicated by a higher intercept. Whereas in the higher temperatures, significantly greater N_2O production was measured from *P. denitrificans*, again indicated by a slightly greater intercept than *P. brenneri*. The response of N_2O for these two strains follows the trend observed with the production of N_2 prior temperature exposure. However, the data is more variable than that of N_2.

The activation energies of N_2O production with prior exposure for the three strains (*P. denitrificans, P. brenneri* and *P. marginalis*) was pooled together to get an overall activation of the process which was much lower (0.05 eV, r^2 0.11) than those calculated with no prior exposure and those from the literature. However, this has followed the same pattern of N_2 production and again suggests an adaptation has occurred, making this process less sensitive to temperature after prior exposure, with the three strains behaving very similarly to one another.

Prior warming has reduced the thermal sensitivity by reducing the activation energies of N_2 and N_2O production in these pure cultures of denitrifying bacteria, as well as reducing the production rates of N_2 and N_2O at similar measurement temperatures compared to with no prior exposure. This could be down to 'thermal adaptation' of the bacteria which is a term used to encompass a variety of physiological changes, such as instantaneous response to temperature to mutations being selected for to increase fitness in a changing environment (Bradford et al., 2008). Instantaneous responses of denitrification to warming has been observed in the present

study as well as previous publications, with increases in production of N_2 and N_2O when exposed to warmer temperatures but very few studies have considered the long term response of denitrification. Long term, as well as short term (i.e. that shown in this study), increase in N_2 and N_2O production in response to warming is expected (e.g. Brin et al., 2016; Holtan-Hartwig et al., 2002; Smith, 1997; Veraart et al., 2011). However, it has been shown that long term warming can actually cause physiological thermal adaptations and after an initial increase in rates, the rates return to pre warming values. Bradford et al. (2008) carried out long term warming (>15 years) experiments measuring respiration rates (as CO_2 production) from temperate soils and found after the initial increase of respiration, rates returned to those in the un-warmed control soils due to thermal adaptations of the bacterial community. Similar findings have been found in e.g. boreal forest soils (Jarvis & Linder, 2000), Arctic ecosystems (Oechel et al., 2000) and tall grass prairie systems (Luo et al., 2001). This thermal adaptation of communities to increased warming may have wider implications, weakening the response of heterotrophic respiration to global warming, and over estimating the increase of CO_2 release with current global warming predictions (Bradford et al., 2008; Luo et al., 2001). As denitrification is a form of heterotrophic respiration, we may see similar responses, with no long term effects on the release of N_2 and N_2O, as bacteria can change their physiology to maximise fitness in changing environments as seen with previously mentioned studies (e.g. Bradford et al., 2008; Jarvis & Linder, 2000; Luo et al., 2001; Oechel et al., 2000).

The ratio of N_2O/ N_2 produced gives us an indication of the efficiency of denitrification. The decreasing of the ratio with increasing temperature with prior exposure was significant in all three strains and again the data was pooled for all three strains to give us an overall activation energy of the ratio (-0.36 eV, r^2 0.15). This ratio was driven by the increase in N_2 production being greater than that of N_2O, suggesting increased efficiency by a greater occurrence of complete denitrification, which has been frequently found in environmental samples (e.g.

Avalakki et al., 1995; Bailey & Beauchamp, 1973; Cavigelli & Robertson, 2000; Holtan-Hartwig et al., 2002; Keeney et al., 1979). Though the causes for these observed ratios differ between studies. Holtan-Hartwig et al (2002) found temperate soils had greater ratios of N_2O/ N_2 in colder months but when these soils were exposed to a range of temperatures (5-20^oC), the activation energies of the enzymes involved in N_2O production and N_2O reduction (N_2 production) were very similar meaning no net flux of N_2O was observed. The net flux of N_2O and therefore greater ratio of N_2O/ N_2 was observed at temperatures around 0^oC. This suggested the activity of enzyme involved in N_2O reduction decreased more strongly than the enzyme involved with N_2O production.

2.5 Conclusions

In this study, prior warming affected the capacity of denitrifying bacteria to produce N_2 and N_2O when exposed to a range of temperature compared to no prior exposure. Thermal sensitivity is observed in both treatments, but much greater with those that have not acclimatised to the exposure temperatures. This is an indication of potential thermal adaptation. This muddies the waters when predicting the response of denitrification with increased warming. The initial response of temperature with no-prior temperature exposure is much greater, however, evidence of the similar metabolic process of heterotrophic respiration, and suggests this response may not be long lived. Bacterial communities are able to thermally adapt their physiology and reduce initial increased rates to those of pre-warmed conditions whilst still at the increased temperatures. To better understand these complex metabolic process, more long-term studies need to be carried out considering both individual and community adaptations and combining pure culture and environmental samples will provide the most robust datasets.

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Chapter 3: The effect of moderate long-term warming (4°C) on sediment denitrification and nitrification activity in experimental mesocosms

3.1 Introduction

With the increasing use of fossil fuels and in invention of the Harber-Bosch process, the amount of reactive nitrogen (N) entering the biosphere has doubled the amount of reactive nitrogen that is naturally fixed by biological nitrogen fixation (Galloway et al., 2008; Seitzinger et al., 2006). A large proportion of the fixed N makes its way into freshwater systems where it can cause eutrophication (Paerl, 1997).

Denitrification is a nitrogen removal process reducing nitrate (NO_3^-) and nitrite (NO_2^-), converting it to nitrous oxide (N_2O) and di-nitrogen gas (N_2) via a series of intermediates (Groffman et al., 2006). It is a form of respiration and important for removing reactive nitrogen out of systems that have excess concentrations due to anthropogenic inputs, specifically from fertiliser use (Tiedje, 1998).

Denitrification also produces N_2O as an intermediate under anaerobic conditions (Ritchie & Nicholas, 1972). N_2O is a potent greenhouse gas (GHG) with radiative forces some 300 times greater than that of carbon dioxide (CO_2), (Ravishankara et al., 2009; Wright et al., 2012) and can also damage the stratospheric ozone layer (Holtan-Hartwig et al., 2002; Knowles, 1982) so production of this GHG will lead to further warming. Emissions of N_2O from rivers, estuaries and coastal systems are estimated to be 1.9 Tg N_2O y^{-1}, which is approximately 35% of the total global emissions (Seitzinger et al., 2000), with the majority of those emissions from rivers (1.1 Tg N_2O y^{-1}) (Seitzinger and Kroeze, 1998).

A second important nitrogen removal process from aquatic systems is anaerobic ammonium oxidation (anammox). Anammox microorganisms convert ammonium (NH_4^+), using nitrite (NO_2^-) as an electron acceptor, to produce N_2 (Dalsgaard et al., 2012; Strous et al, 1998; Van Hulle et al, 2010). Anammox has been measured in a variety of different aquatic systems

including oceanic oxygen minimum zones (Dalsgaard et al, 2012), estuaries and shelf sediments (Brin et al, 2014; Canion et al 2014) and freshwater lakes and rivers (Schubert et al, 2006; Zhang et al, 2007). One of the first measurements of significant rates of anammox activity in freshwater was measured in Lake Tanganyika, with anammox rates of up to 10 nM N_2 h^{-1} within the tropical deep suboxic waters (100-110 m) (Schubert et al, 2006). Lesser rates were also detected in a permanently stratified temperate lake, Lake Rassnitzer, Germany, up to 504 nmol N_2 L^{-1} d^{-1} (Hammersley et al, 2009). Rates of anammox would be expected to be lower in a temperature region compared to a tropical region as they are generally slow growing (Strous et al, 1998), and have high optimum temperatures around 30-40^{o}C (Lotti et al, 2015; Zhang et al, 2017). What is interesting with Hammersley et al (2009) study, is that rates of anammox were greater in the colder months of January and October (maximum of 504 nmol N_2 L^{-1} d^{-1}), with rates as low as 16 nmol N_2 L^{-1} d^{-1} when temperatures were higher in May. Similarly, Brin et al (2014) measured anammox rates in a New England estuary and also found no correlation with season. Rates of up to 8.7 nmol N_2 h^{-1} mL^{-1} sediment were measured in slurries along the estuary which positively correlated to organic matter content and NO_3^- of the pore water with no correlation to seasonal differences in temperature. Conversely, other studies have found temporal variation of anammox rates. Zhao et al (2013) measured seasonal rates of anammox in the Taihu River region, China. The anammox rates ranged from 0.11 to 6.79 µmol N_2 m^{-2} h^{-1}, with the greatest rates observed at the end of summer to early autumn, positively correlating to water temperature and NO_3^- concentration.

This suggests more factors are at play and temperature is not the only dominant driver of anammox activity. The knowledge of factors controlling anammox in freshwaters is very limited but many studies have come to their own conclusions of the dominant controlling factors in other aquatic, more saline, environments. Different dominant controls in estuarine and coastal systems, for example, include organic matter content of the sediment (Dalsgaard

and Thamdrup, 2002; Dalsgaard et al, 2005), NO_3^- concentration (Rich et al, 2008; Dong et al, 2009; Teixeira et al, 2012), NO_2^- concentration in the sediments (Meyer et al, 2005) and temperature (Dalsgaard and Thamdrup et al, 2002).

Nitrification is another important part of the N cycle that couples the mineralisation of organic matter (ammonification) to the oxidised species of NO_3^- and NO_2^- that can, in turn, be denitrified. Nitrification can also produce N_2O as a bi-product through NH_2OH metabolism in ammonium oxidising bacteria and also through the Archaea.

Nitrification has been measured in a variety of systems such as freshwater lakes and streams (Small et al., 2013; Strauss & Lamberti, 2000) and oceans (Clark et al., 2008; Wuchter et al., 2006). It can also play a large role in freshwater lakes, with estimates of 93-100% of the NO_3^- in Lake Superior being produced through nitrification (Finlay et al., 2007).

There is clear evidence of climate change affecting ecosystems across the Earth through increasing temperatures , rising sea levels and changing weather patterns, to mention a few (IPCC, 2013). Since the industrial revolution, average global temperatures have been increasing due to the burning of fossil fuels increasing production rates and amplifying the natural effect of harmful GHG such as CO_2, methane (CH_4) and N_2O into the atmosphere (Jana et al., 2013). With the continual burning of fossil fuels and use of fertilisers it is imperative that we understand how the biogeochemical cycling of the bio-elements that both produces and consumes these GHG, and how they are going to respond to further warming.

Warming has been shown to enhance both photosynthesis and respiration in freshwater systems, but to different extents; respiration has a greater thermal sensitivity than photosynthesis (Gillooly et al., 2001; Perkins et al., 2012; Yvon-Durocher et al., 2012), therefore CO_2 tends to be is produced at a greater rate than it is consumed within a warmed ecosystem relative to an un-warmed control. Yvon-Durocher et al (2010) used experimental mesocosms to demonstrate the effect of a 4°C temperature increase (predicted global warming:

IPCC, 2013) on the balance of respiration and primary production and found a 13% decrease in carbon sequestration within these systems. Respiration increased at faster than photosynthesis with apparent activation energies of 0.62 eV and 0.43 eV respectively, altering the balance of the carbon cycle. Other have reached similar conclusion with modelling based on environmental data (Allen et al, 2005; Dewar et al, 1999), These studies suggest a positive feedback mechanism where warming may lead to further increases of CO_2 emissions and therefore further warming through the greenhouse effect.

The nitrogen cycle is tightly coupled to the carbon cycle due to its strong regulation of primary production (Galloway et al., 2008; Tyrrell, 1999). If fixed nitrogen becomes limiting for plant growth, there will be a reduction in primary production which would lead to a decrease of CO_2 drawdown from the atmosphere (Karl et al., 2002). The nitrogen cycle can therefore play a key role in climate regulation and global warming (Falkowski, 1997) but the full complexity including interactions and response to temperature is still unknown. Following the metabolic theory of ecology, enzymatic activity increases with increasing temperatures (Brown et al., 2004) and therefore we should expect to see an increase in rates of denitrification as global temperatures increase. We have much evidence of how the carbon cycle may be affected with warming but little research has been done into the long term effects of increasing temperatures on the nitrogen cycle in freshwaters. One study using a short term freshwater experimental mesocosm set up measured an increase in denitrification by 24-28% with only a 1^{o}C increase (Veraart et al, 2011).

The use of experimental mesocosms has come under criticism in the past (Carpenter, 2016), in that they are disconnected from natural systems. However, many mesocosm studies have proved successful in that they have increased our understanding of processes of e.g. warming effects on community structure (Yvon-Durocher et al., 2011) and primary productivity (Yvon-Durocher et al., 2015) and so can be used as models to mimic natural systems (Drenner &

Mazumder, 1999). The use of experimental mesocosms is especially useful in temperature manipulation experiments, allowing us to consider the specific effects of warming on biogeochemical processes in a semi-natural environment.

3.1.1 Aims and Hypothesise

The main aim of the present study is to investigate the effect of long term warming on denitrification and nitrification with the use of freshwater mesocosms. This will be carried out with two different experiments:

1) *In situ* seasonal measurements of potential rates of denitrification and nitrification will be measured using slurries at mesocosm *in-situ* temperatures in the field to investigate the long term treatment effect of moderate warming (3-5^{o}C over ~10 years). The aim of this study is to determine if the rates of denitrification and nitrification are significantly different between sediments from the heated and ambient mesocosms and whether this is observed across seasons. We hypothesised for this seasonal study is that rates of both denitrification will be significantly greater in the heated mesocosms than the ambient due to increased metabolic activity with warmer temperature. But this may very seasonal due to other limiting factors such as carbon and nutrient availability.
2) Seasonal measurements of potential rates of denitrification and nitrification will also be measured after exposure to a range of temperatures using a thermal gradient bar in the laboratory, allow us to investigate the short term thermal response in sediments from the heated and ambient experimental mesocosms. The aim of the second experiment is to determine if the long term warming has altered the capacity of denitrification and nitrification compared to the ambient treatment. This will be investigated by exposing sediment slurries from the ambient and heated mesocosms to the same temperatures in the laboratory and measuring rates of denitrification and nitrification. We hypothesised, for

this second experiment that sediments from the warmed treatment will have a greater capacity for both denitrification and nitrification at the warmer temperatures compared to the ambient treatment at the same exposure temperatures. This may be due to the bacteria responsible for these processes increasing their efficiency after exposure to long term warmer temperatures.

3.2 Methods

3.2.1 Experimental design

Experiments were carried out using freshwater mesocosms specifically designed for ecosystem scale temperature manipulation. They are installed at the Freshwater Biological Association River Laboratory, East Stoke, Dorset, UK (2° 10'W, 50° 13'N). There are 20 mesocosms, each holding 1m^3 of water. This size of mesocosms enables them to act as shallow pond systems recently highlighted to play a disproportionality large role in emissions of GHGs (Holgerson & Raymond, 2016) and in organism community structure and nutrient cycling which has been shown in several previous studies (Jones et al, 2002; Liboriussen et al, 2005; Yvon-Durocher et al., 2010). Half of the ponds are warmed, with the degree of experimental warming in line with one of the scenarios put forward for warming of the Northern Hemisphere by the end of this century (IPCC, 2007). An electric heating element connected to a thermocouple maintained the water temperature in the heated mesocosms at 3-5°C above that in the ambient mesocosms. A total of 16 ponds were sampled were included with each pair including a warmed and an ambient pond (Figure 3.1). The mesocosms were established in December 2005, with heating beginning in September 2006, and set up with natural organic substrates, fauna and flora to replicate natural shallow lake systems (Jones et al., 2002; Liboriussen et al, 2005; Yvon-Durocher et al., 2015, 2010) (full species lists are available in Yvon-Durocher et al, 2010).

Sampling was carried out between spring 2013 and spring 2015 (January, April and August), approximately 8 years after warming began. Two main components of the nitrogen cycle were measured from the mesocosms; anaerobic denitrification and aerobic nitrification.

Prior to the main experimental analysis, preliminary experiments were completed to investigate the potential of the freshwater mesocosms to carry out anammox. Though we carried out measurements on several occasions across different seasons, no anammox was detected in any of the mesocosms. Details of the methodology and potential reasons for lack of detection will be discussed later in the chapter.

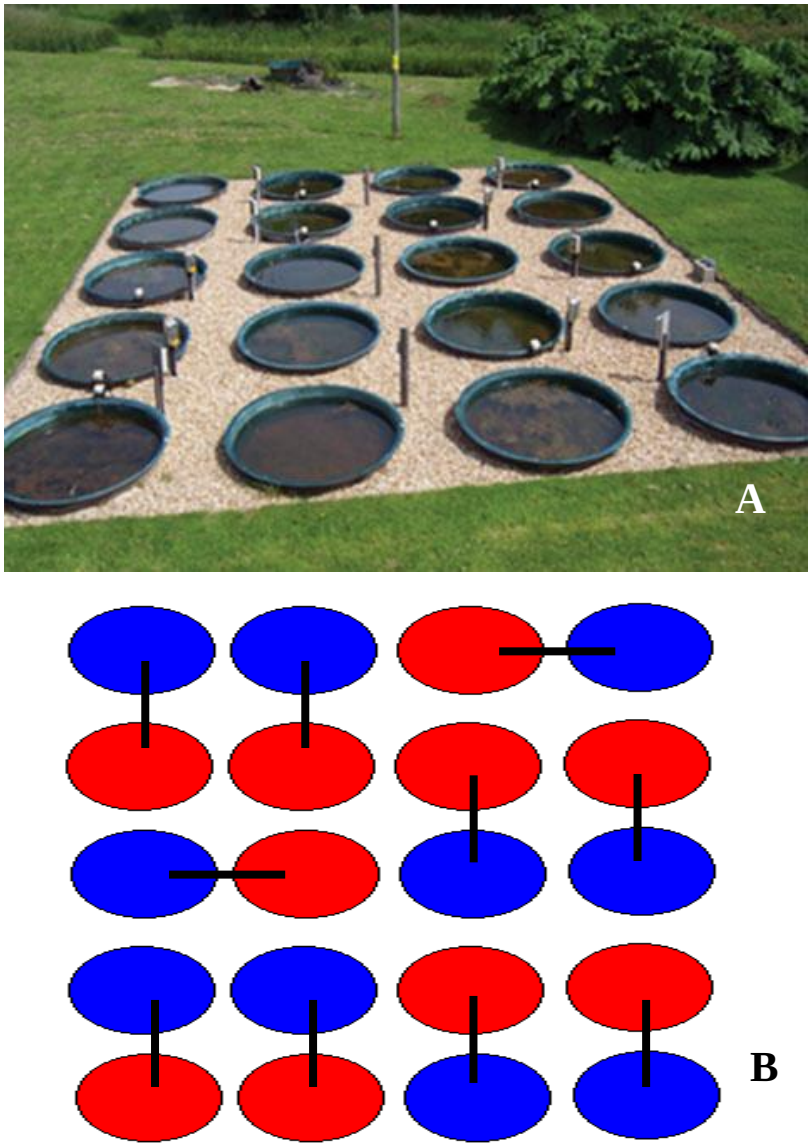

Figure 3.1 Photograph of experimental mesocosms (A) and experimental warming and ambient pairs design (B). Red circles indicate warmed mesocosms and blue circles indicate ambient mesocosms.

To investigate the effects of long term warming, two different experiments were carried out for both processes. The first experiment investigated the difference in rates of both denitrification and nitrification from the long term warmed and ambient mesocosms at *in-situ* conditions. The second experiment investigated the effects of short term exposure to a range of temperatures

with sediments from both heated and ambient mesocosms, again for both denitrification and nitrification, using a thermal gradient block (Figure 3.2).

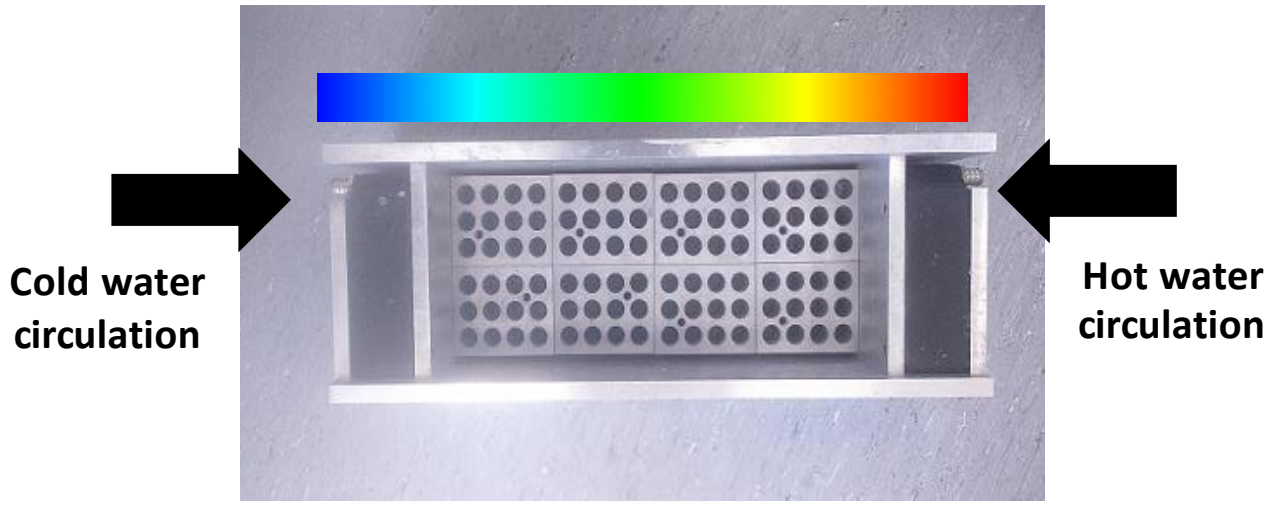

Figure 3.2 Solid aluminium thermal gradient bar used for short term temperature experiments. Heated water circulation at one end and cooled water circulation at the opposite end created a thermal gradient along the bar. Gradient block is shown open but was fitted with insulation and an aluminium lid during the incubations.

3.2.2 Water nutrient analysis

Water samples were collected at the time of sediment collection for microbial process measurements from each of the 16 mesocosms (8 heated, 8 ambient) to compare background nutrient concentrations in the two treatments. A minimum of 5 mL of mid-depth mesocosm water was filtered through a pre-rinsed sterile syringe filter (Whatman, Sigma Aldrich) and immediately frozen (-5^{o}C) for later processing in the laboratory. Water nutrient samples were analysed using a segmented- flow auto analyser (Skalar) and standard colourimetric techniques (SKLAR, San $^{++}$ System, Flow Access software 1.2.5) for NO_3^-, NO_2^-, NH_4^+ and PO_4-.

3.2.3 Sediment sampling for denitrification and Anammox

To measure the potential for denitrification and anammox, sampling was conducted in spring, summer and winter over 2013-2015. A total of 6 intact sediment cores were collected from each experimental mesocosm using 60 ml truncated syringes which were refrigerated 5^{o}C (< 1 h). In the laboratory, the top 2 cm of each cores was homogenised. Separate vials were used to measure potential anammox and denitrification activity.

3.2.4 Measuring potential rates of activity of denitrification and anammox and the thermal response of denitrification

For *in-situ* temperature response time series measurements, homogenised sediment (1 g) was placed into 3 mL gas-tight vials (Exetainer, Labco) which were then transferred into an anoxic hood (CV204, Belle Technology) filled with Oxygen free Nitrogen (OFN, 99.998%, British Oxygen Company) (Lansdown et al., 2012). Corresponding mesocosm water (1 mL) that had been vigorously bubbled for 20 minutes with OFN was added to each vial to make anoxic slurries which were then sealed with an OFN headspace (1 mL). The vials were placed back into the corresponding mesocosms to pre-incubate for 24 hours to ensure that any natural $^{14}NO_x$ (NO_3^- + NO_2^-) and O_2 was reduced (Risgaard-petersen et al., 2004; Trimmer et al., 2003).

For the thermal block experiment (denitrification only), all samples were prepared in the same method for the *in-situ* experiment. However, the slurries were in larger gastight vials (12 mL, Exetainer, Labco) with 2 g of homogenised sediments and 2 mL of corresponding mesocosm water, leaving an 8 mL headspace of OFN. The sediments were exposed to a range of temperatures created using an aluminium thermal gradient block containing aluminium racks in which the 12 mL vials fitted into. A final temperature range between 11^{o}C and 37.5^{o}C was obtained. The thermal gradient within the block was created using a heated water bath (Grant TC120) at one end and a chilling unit (Grant RC350G) connected to the opposite end. To obtain

the widest temperature range possible the thermal gradient had to be completed in three separate ranges, 11-16^{o}C (chiller: 2^{o}C, heated water bath: 20^{o}C), 16-36^{o}C (chiller: 2^{o}C, heated water bath: 80^{o}C), 31-37.5^{o}C (chiller: 25^{o}C, heated water bath: 60^{o}C).

After the pre-incubation period, the sediment slurries were spiked with deoxygenated $Na^{15}NO_3^-$ (98 ^{15}N atom %, Sigma Aldrich) by injecting through the butyl septa 160 µL of 1570 µM for the 3 mL *in-situ* vials or 160 µL of 3140 µM for the 12 mL thermal gradient vials to give a final concentration of 200 µM in the slurry porewater. Microbial activity was stopped by injection of formaldehyde solution (50 µL for the *in-situ* and 100 µL formaldehyde for the thermal gradient experiments, 38% w/v.) (Trimmer & Nicholls., 2009). Each vial was an independent sample for a particular time point (n = 8) within the time series *in-situ* experiments. For the thermal gradient block, each vial represented an independent sample at a particular temperature (n = 6). For production of N_2O and ^{15}N-labelled N_2 over background measurements, parallel slurries were prepared in the same method as above at the same time for both experiments but with the addition of formaldehyde at the beginning of the experimental incubations (after the pre-incubation) without the addition of $Na^{15}NO_3^-$. These background samples act as references for natural abundance ^{15}N and the rate of denitrification was calculated by the excess of ^{15}N-labelled N_2 ($^{29}N_2$ + $^{30}N_2$) for denitrification and anammox ($^{29}N_2$) (Thamdrup & Dalsgaard, 2000).

Similarly, N_2O production was measured excess above reference samples. Individual samples for *in-situ* rates were stopped at 0, 2, 4, 8, 16, 32, 64 hours and samples for short term thermal response rates were killed after 24 hours with background samples killed at 0 hours. The thermal block is a single time-point incubation (Trimmer et al., 2006). Details of experimental design can be seen in figure 3.3.

Experiment	Sampling month	Time points/ Temperatures	Number of Replicates per time point	Number of ponds
In situ denitrification	April August January	0, 2, 4, 8, 16, 32, 64 (hours)	3	8 warmed (3-5oC above ambient) & 8 ambient
In situ nitrification	April August January	0, 2, 4, 8, 16, 32, 64 (hours)	3	8 warmed (3-5oC above ambient) & 8 ambient
Thermal gradient denitrification	April August January	0, 24 (hours) 11.5-37.5°C (n = 23 temps)	3	6 warmed (3-5oC above ambient) & 6 ambient
Thermal gradient nitrification	August January	0, 16 (hours) 11.5-37.5°C (n = 23 temps)	3	6 warmed (3-5oC above ambient) & 6 ambient

Figure 3.3 Experimental design for denitrification and nitrification from experimental mesocosms including *in-situ* (orange boxes) and thermal gradient experiments (yellow boxes).

3.2.5 Gas analysis ($^{15}N_2$ and N_2O) and calculations for denitrification and anammox potential

Samples for both the *in-situ* and thermal gradient block experiments for denitrification potential were measured for N_2O concentration using a gas chromatograph fitted with a micro electron capture detector (GCμ/ECD, Agilent Technologies UK Ltd., South Queensferry, U.K.; Nicholls et al., 2007). Headspace N_2O concentrations were calculated from peak areas using a known standard concentration (Scientific and Technical Gases) and the total amount in the vial (headspace plus slurry) was corrected for temperature, pressure and solubility (Weiss & Price, 1980; Yamamoto et al., 1976).

Isotopic analysis of the production of ^{15}N-N_2 ($^{29}N_2$ + $^{30}N_2$) gas from the denitrification experiments, and $^{28}N_2$ and $^{29}N_2$ from anammox activity, were measured from the headspace by continuous-flow isotope ratio mass spectrometry (Thermo-Finnigan, Delta Matt Plus) previously described in Nicholls & Trimmer (2009).

To determine both denitrification and anammox activity, the several calculations are carried out. The mole fraction of the added substrate (98% ^{15}N atom) and measured excess N_2 production are used to calculate production via denitrification and anammox (Bo Thamdrup & Dalsgaard, 2002). With the addition of ^{15}N (in the form of $Na^{15}NO_3^-$), denitrification has the ability to produce $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ via random isotope pairing, calculated using the following equations:

$$D_{tot} = P_{30} \times F_N^{-2}$$

$$D_{30} = D_{tot} \times F_N^2$$

$$D_{28} = D_{tot} \times (1-F_N)^2$$

$$D_{29} = D_{tot} \times 2 \times 1 \times (1-F_N) \times F_N$$

D_x denotes the production of N_2 via denitrification. P_{30} represents the total N_2 produced in the vial measured by mass-spectrometry. F_N represents the ^{15}N mole fraction of the source compound ($Na^{15}NO_3^-$, or $Na^{15}NO_2^-$, 98% ^{15}N).

Anammox can only produce $^{28}N_2$ and $^{29}N_2$ assuming one molecule is obtained from $Na^{15}NO_x$ added to the samples and the other from $^{14}NH_4^+$ in the slurries (Dalsgaard et al., 2012). The following equations are required to determine the production via anammox:

$$A_{29} = P_{29} - D_{29}$$

$$A_{28} = A_{29} \times F_N^{-1} \times (1-F_N)$$

$$A_{tot} = A_{29} + A_{30}$$

$$RA = A_{tot} / (D_{tot} + A_{tot})$$

Where A_x denotes the production of N_2 via anammox. RA denotes that relative contribution of anammox to the total production of N_2 gas produced and can be multiplied by 100 to obtain anammox contribution as a percent (*ra* %).

Following the calculations above, rates of excess production of ^{15}N-N_2 were calculated as the slope of production P_{30} (linear regression) against time of the incubations.

Once all gas measurements were completed, the vials were opened and sediments were dried to a constant weight at 80^{o}C and gas production rates were normalised to dry mass (Lansdown et al., 2012; Shelley et al., 2014).

3.2.6 Sediment sampling for nitrification

Following the same method for sediments collected for denitrification, sediments were also collected to determine the temperature sensitivity or characteristic of potential nitrification activity.

3.2.7 Measuring potential rates of activity and thermal response of nitrification

For *in-situ* temperature response and the thermal gradient response of nitrification, 2 g of homogenised surface sediment was transferred into 12mL gas tight vials with aerated corresponding mesocosm water (2 mL) before being sealed with air headspace (~8 mL). To quantify nitrification, sediments were incubated with ^{15}N-ammonium (98 ^{15}N atom % Sigma Aldrich) and tracked its oxidation to $^{15}NO_x^-$ (Small et al., 2013). 122 µL of 1.83 mM $^{15}NH_4Cl$

was injected through the butyl rubber septa of each vial to give a final concentration of 90 µM in the water. Once vials were injected with ^{15}N tracer, samples were incubated in the same method as above for denitrification for both *in-situ* and thermal gradient experiments. As with the measurements for denitrification, parallel reference were prepared in the same method as above at the same time for both experiments but with the addition of formaldehyde at the beginning of the experimental incubations without the addition of $^{15}NH_4Cl$. These background samples act as references for natural abundance ^{15}N and the rate of nitrification (ammonium oxidation) was calculated by the excess of ^{15}N-labelled NO_x^- (Thamdrup & Dalsgaard, 2000). Similarly, N_2O production was measured excess above reference samples. Nitrification potentials under *in-situ* conditions were measured with a time series experiment and for the thermal response of nitrification the single time-point was used (16 hours). All vials were injected with 100 µL formaldehyde (38% w/v) to stop microbial activity. Details of experimental design for both experiments can be seen in figure 3.3.

3.2.8 Gas and nutrient (NO_x^-) analysis for nitrification

As previously mentioned, nitrification has the potential to also produce N_2O gas. Before carrying out any other analysis, N_2O was measured from the headspace of each vial (same method as above for denitrification, GCµ/ECD). Each vial was then opened and sediments washed with ultrapure water to ensure all NO_x^- was extracted from the sediment slurries. Ultrapure water (2 mL) was added to each vial and then placed on a rotation table for 2 hours at 100 revolutions min^{-1}, before being centrifuged (1700 x g, 5 minutes) (Lansdown et al., 2012). The 4 mL (2 mL ultrapure water + 2 mL from slurry) supernatant was then removed, retained and the process was repeated once more, leaving us with 6 ml extractions for ^{15}N analysis with a 3 fold dilution. Following extraction of the supernatant, the sediment slurries were dried to a constant weight at 80^{o}C and nitrification rates were corrected for dry mass as above (Lansdown et al., 2012; Shelley et al., 2014).

To measure the oxidation rates of $^{15}NH_4^+$ several steps were required. Firstly, NO_3^- and NO_2^- concentrations were measured from the extracted water samples using a segmented- flow auto analyser (Skalar) and standard colourmetric techniques using certified standards for calibration. Once concentrations were determined, the reduction of NO_3^- to NO_2^- using a modified spongy cadmium method (Mcilvin & Altabet, 2005). 0.2 mL of Imidazole was added to 5 mL of sample and incubated on an orbital shaker for 2 h (150 rpm). Samples were then transferred into 3mL gas-tight vials, sealed with no headspace, then a 0.5 mL headspace helium headspace was introduced (as above). Sulphamic acid was injected through the septa to reduce NO_2^- to N_2 (100 µL of 4 mM sulphamic acid in 4 M HCL). Samples were placed back onto the orbital shaker overnight (as above) to allow gases to equilibrate. Following conversion into ^{15}N-N_2, samples were measured by continuous-flow isotope ratio mass spectrometry (Thermo-Finnigan, Delta Matt Plus) for $^{29}N_2$ as above. Concentrations of $^{29}N_2$ were calculated using a calibration curve of different concentrations of N_2 in the range of the samples and different atom % of $^{15}N_2$ obtained using the same method converting NO_3^- to N_2 (Lansdown et al., 2016).

3.2.9 Deriving apparent Activation Energies

The rates of the measured processes were log transformed and the incubation temperatures were converted to $1/kT$, where k is the Boltzmann's Constant (8.62×10^{-5} eV K^{-1} (T)) and T is the absolute temperature in Kelvin. The natural log rates were plotted against standardised temperature ($1/kT$-$1/kT$c, where $1/kT$c is the average $1/kT$ for the thermal range included), on an Arrhenius plot where the negative slope of the regression line gives an estimate of the apparent activation energy in electron volts (eV) where 1 eV is equivalent to 96.49 kJ mol^{-1}. Plotting in this manner centres the inverse temperature around zero (Perkins et al., 2012). This calculated 'apparent' activation energy is used as an empirical index of temperature response of each process as this will always be lower than the theoretical sensitivity of biogeochemical

reactions to temperature. This is because other environmental factors come into play such as connectivity to other microbial processes.

3.2.10 Statistical analysis

Random mixed effect models were carried out to determine either effect of treatment (with season as random effect and treatment as fixed) or effect of season (with treatment as random effect and season as fixed) on potential rates in the *in situ* experiments. To compare differences between seasons with potential denitrification in the *in situ* experiments, a Kruskal Wallis ANOVA followed by a post Hoc Tukey test or Dunn's method, depending or normality of data, was undertaken. The effect of temperature was determined by the activation energy calculated in the method above (3.2.9).

3.3 Results

3.3.1 Water nutrient analysis

Water nutrient analysis was carried out at the same time of sediment sampling for microbial nitrogen transformations and statistical significance was calculated between the two treatments within season and also between seasons. The lowest nutrient concentrations were observed in the summer where, for example, the concentration of NO_3- was below the limit of detection (Appendix, Table 3.1). For NO_2^-, NO_3^- and NH_4^+, no significant difference was observed when the warmed treatments from the different seasons were compared ($p > 0.05$). However, PO_4^+ was significantly greater in the warmed mesocosms in spring compared to summer and winter ($f = 4.34$, $df = 2$, $p < 0.05$). Whereas much more seasonality was observed in the ambient treatment mesocosms. NO_2^- was significantly greater in the winter ($f = 22.87$, $df = 2$, $p < 0.0001$), NO_3^- and PO_4^+ were both significantly greater in spring ($f = 8.48$, $df = 1$, $p < 0.01$ and $f = 6.75$, $df = 2$, $p < 0.005$ respectively). The only calculated significant difference in nutrient concentrations between the two treatments within season was observed in spring when the

concentration of NO_3^- was significantly greater in the ambient treatment compared to the heated (f = 14.04, df = 1, $p < 0.002$). All other within season comparisons between treatments for NO_2^-, NO_3^-, NH_4^+ and PO_4^+ were non-significant ($p > 0.05$).

3.3.2 *In-situ* potential rates of anammox

Though experiments were carried out on several occasions across the different seasons, no anammox activity was detected in any of the mesocosms, heated or ambient.

3.3.3 *In-situ* potential rates of denitrification

When rates of $^{15}N_2$ production from the different seasons were combined and season fitted as a random effect, the effect of treatment was significant; sediments from the heated treatment had greater production rates of $^{15}N_2$ than the ambient treatment (X^2 = 11.43, df = 2, $p < 0.001$) (Figure 3.4A). Though greater production was observed in the heated treatments over ambient treatments in all seasons, statistical significance was only seen in summer between treatments (t = 4.3, df = 14, $p < 0.001$). When rates of denitrification from both treatments were put together and treatment fitted as a random effect, there was a significant effect of season (X^2= 40.28, df = 2, $p < 0.0001$). $^{15}N_2$ production was significantly faster in summer than both spring (H = 17.1, df = 2, p = < 0.001) and winter (H = 17.1, df = 2, p = 0.03), and there was no difference between production in spring and winter (Kruskal-Wallis One Way Analysis of Variance, followed by Post Hoc Tukey). The greatest rates of $^{15}N_2$ production were measured in summer with a rate of 3.19 (± 0.79) and 0.78 (± 0.11) nmol g^{-1} h^{-1} in the heated and ambient treatments respectively. The lowest rates were observed in spring from the ambient treatment of 0.17 (±0.05) nmol g^{-1} h^{-1}. Winter had the lowest rate observed for the heated treatment of 0.47 (± 0.15) nmol $^{15}N_2$ g^{-1} h^{-1}.

Rates of N_2O production were slightly greater in the heated treatments in summer and winter, but rates were greater in spring from the ambient treatment (Figure 3.4B). When rates of N_2O

production from the different seasons were combined and season fitted as a random effect, the effect of treatment was non-significant. When rates of N_2O from the different treatments were grouped together and fitted as a random effect, there was an overall significant effect of season ($X^2 = 8.38$, $df = 2$, $p = 0.01$). Overall, production rates of N_2O were significantly greater in the summer in both treatments. Average ambient winter N_2O production is significantly lower than both summer ($t = 5.01$, $df = 14$, $p = <0.001$) and spring ($t = -4.11$, $df = 13$, $p = 0.001$). With the heated treatment, average rates were significantly greater in the summer than spring ($t = -1.9$, $df = 14$, $p = 0.04$) and winter ($t = 3.31$, $df = 14$, $p < 0.01$) (Kruskal-Wallis One Way Analysis of Variance, followed by Post Hoc Tukey)

The highest average production rates of N_2O were measured in summer for both the heated and ambient treatment with 0.61 (± 0.27) and 0.22 (± 0.07) nmol g^{-1} h^{-1} respectively. The lowest average rates of N_2O production were observed in winter for both heated and ambient treatments of 0.07 (± 0.02) and 0.04 (± 0.01) nmol g^{-1} h^{-1} respectively.

The ratio of N_2O to N_2 was calculated to determine the number of N_2O molecules produced for every molecule of N_2 produced. N_2O is a bi-product of denitrification and allows us to investigate the efficiency of the denitrifying bacteria carrying out this process at the exposure temperatures. A positive ratio indicates more N_2O molecules were produced per N_2 and the greater the number, the greater the amount of N_2O produced per molecule of N_2.Controlling for season, fitting it as a random effect and treatment as a fixed effect, the effect of treatment was significant on the ratio of N_2O/ N_2 production ($X^2 = 4.79$, $df = 1$, $p = 0.03$), with the only significant difference observed in spring between the heated and ambient mesocosms ($U = 4$, $df = 13$, $p = 0.004$), with the greatest ratio observed in the ambient mesocosms (Figure 3.4C). Controlling for treatment and fitting it as a random effect, the effect of season on the ratio of N_2O/ N_2 production was highly significant ($X^2 = 16.72$, $df = 1$, $p < 0.001$). The ratio was significantly higher in spring than both summer ($t = 4.38$, $df = 13$, $p < 0.001$) and winter ($t = -$

3.27, $df = 13$, $p = 0.006$) in the ambient treatment. No significant effect was observed within the heated treatment between seasons. The ratio of N_2O to N_2 production was consistently greater in sediments from the ambient treatments compared to the warmed treatments across all season. The highest ratio was observed in spring for both treatments, with an average ratio of 0.36 (± 0.12) and 1.21 (± 0.32) for the heated and ambient mesocosms respectively. The lowest average ratios were in summer for both treatments of 0.17 (± 0.05) and 0.29 (± 0.09) for heated and ambient treatments respectively. These results suggest the effect of season is a more important influence than long term warming.

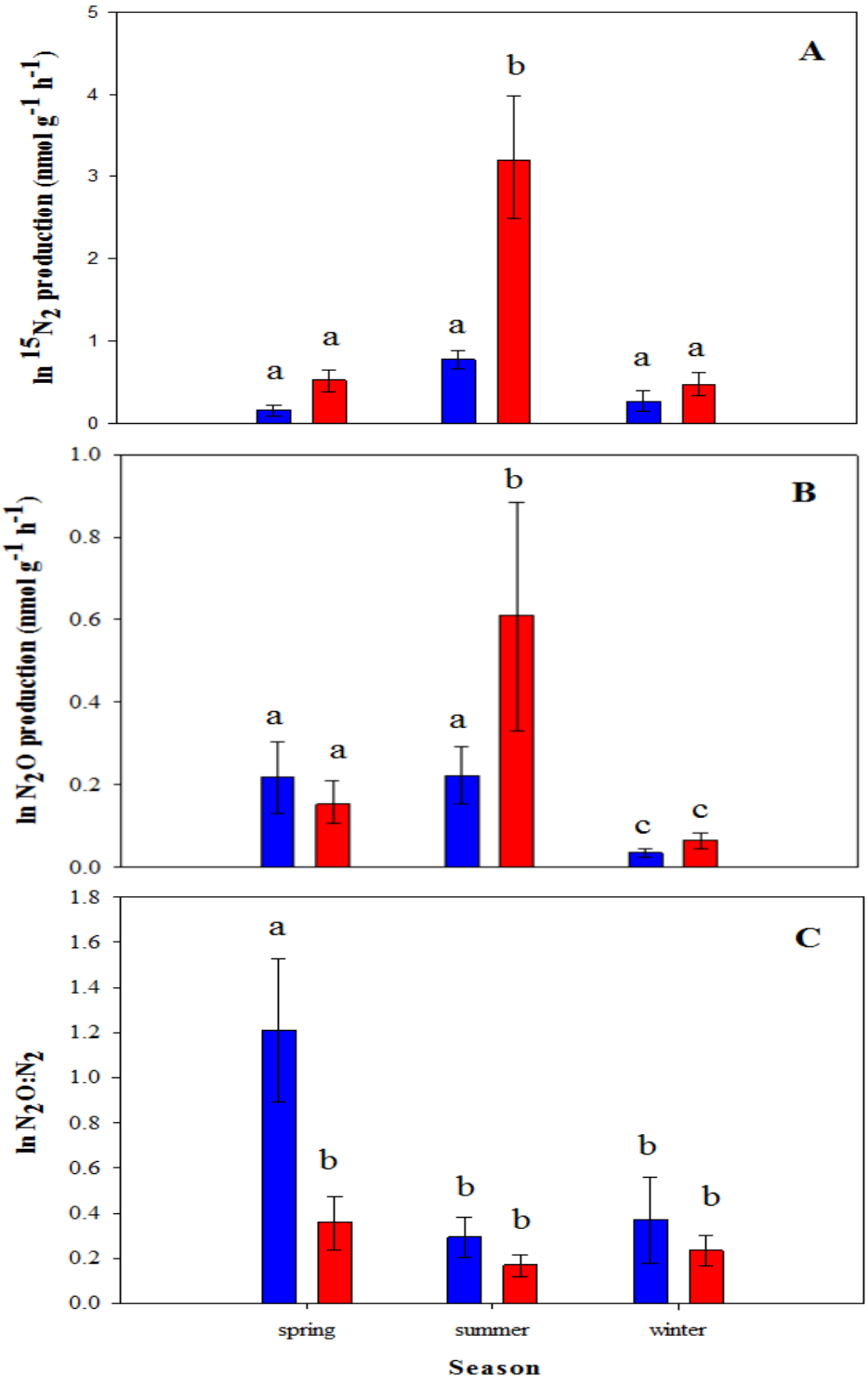

Figure 3.4 Production rates for A)$^{15}N_2$, B) N_2O and C) ratio of N_2O to N_2 for ambient (blue) and heated (red) treated mesocosms across seasons (means ± *se*, *n* = 8) through denitrification. Bars with different letters indicate results significantly different from one another ($p < 0.001$) within each figure.

3.3.4 *In-situ* seasonal potential rates of nitrification

Rates of net nitrification through oxidation of NH_4^+ were measured in spring, summer and winter in sediments from both heated and ambient mesocosms. Highest nitrification activity was measured in spring for both treatments, with very similar rates observed between the treatments (Figure 3.5). Rates of nitrification in sediments from the ambient treatment in summer and the heated treatment in winter were almost below detection levels. The only significant effect of treatment within season was shown in summer, with rates of nitrification greater from the heated treatment ($X^2 = 7.71$, $df = 1$, $p = < 0.01$). Within the heated treatment, spring nitrification rates were significantly greater than summer ($t = 3.86$, $df = 20$, $p < 0.01$) and summer nitrification rates were significantly greater than winter ($t = 2.73$, $df = 20$, $p < 0.03$) (ANOVA). A significant effect of season was also observed with ambient treatment sediments with spring having significantly greater rates of nitrification than summer ($t = 3.72$, $df = 17$, $p < 0.01$) and winter ($t = 2.54$, $df = 20$, $p < 0.01$) (ANOVA). These results suggest seasonal effects are much greater than those created by the treatment of warming.

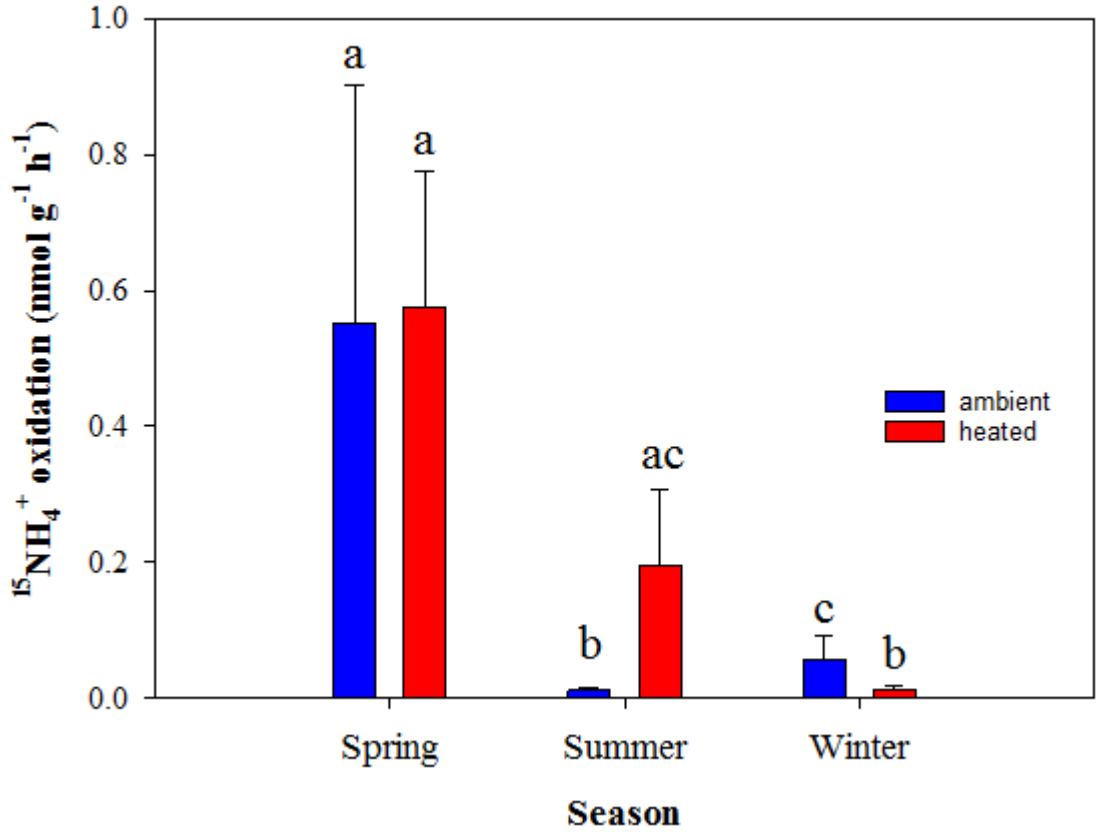

Figure 3.5 Average rates of nitrification (± *se*) through $^{15}NH_4^+$ oxidation for ambient and heated treated mesocosms across seasons (± *se*, *n* = 8) after long term moderate warming. Bars with different letters indicate results significantly different from one another ($p < 0.01$) within each figure.

3.3.5 Potential denitrification rates over a thermal gradient

Arrhenius plots can be used to derive an estimate of an apparent activation energy (Ea) or temperature sensitivity for a specific process over the linear range of production. The linear range of production of $^{15}N_2$ varied between the seasons, as such, both winter and spring have a linear temperature range between 11 to 16.5°C, whereas in the summer, the linear range lay between 11-15.5°C.

The short-term temperature sensitivity of denitrification ($^{15}N_2$ production) was measured in spring, summer and winter for both heated and ambient treatments (Figure 3.6) which demonstrated a clear positive effect of temperature on denitrification. Across all seasons, the short-term temperature sensitivity (Ea) was greatest in sediments from the heated treatments where slopes (activation energy) were steeper and the activity per gram of sediment were also greater. Further, the short-term temperature sensitivity for $^{15}N_2$ production was greatest (1.97 Ea) with sediments from the heated treatments in spring, followed by summer and then winter. The temperature sensitivity obtained from the ambient treatments were very similar for spring and winter but much greater in the summer (Appendix, Table 3.2). Production rates of $^{15}N_2$ from the ambient treatment were significantly lower in the winter than both summer ($Q = 4.43$, $df = 2$, $p < 0.001$) and spring ($Q = 4.65$, $df = 2$, $p < 0.001$; Kruskal-Wallis ANOVA, followed by Dunn's method post hoc analysis for non-normalised data). No significant difference in the intercept was determined between the seasons for the heated treatments. Therefore the effect of temperature was tested with season as a random effect and the results indicate that the effect of temperature was highly significant for the heated treatment ($X^2 = 39.82$, $df = 1$, $p < 0.0001$) on production rates of $^{15}N_2$.

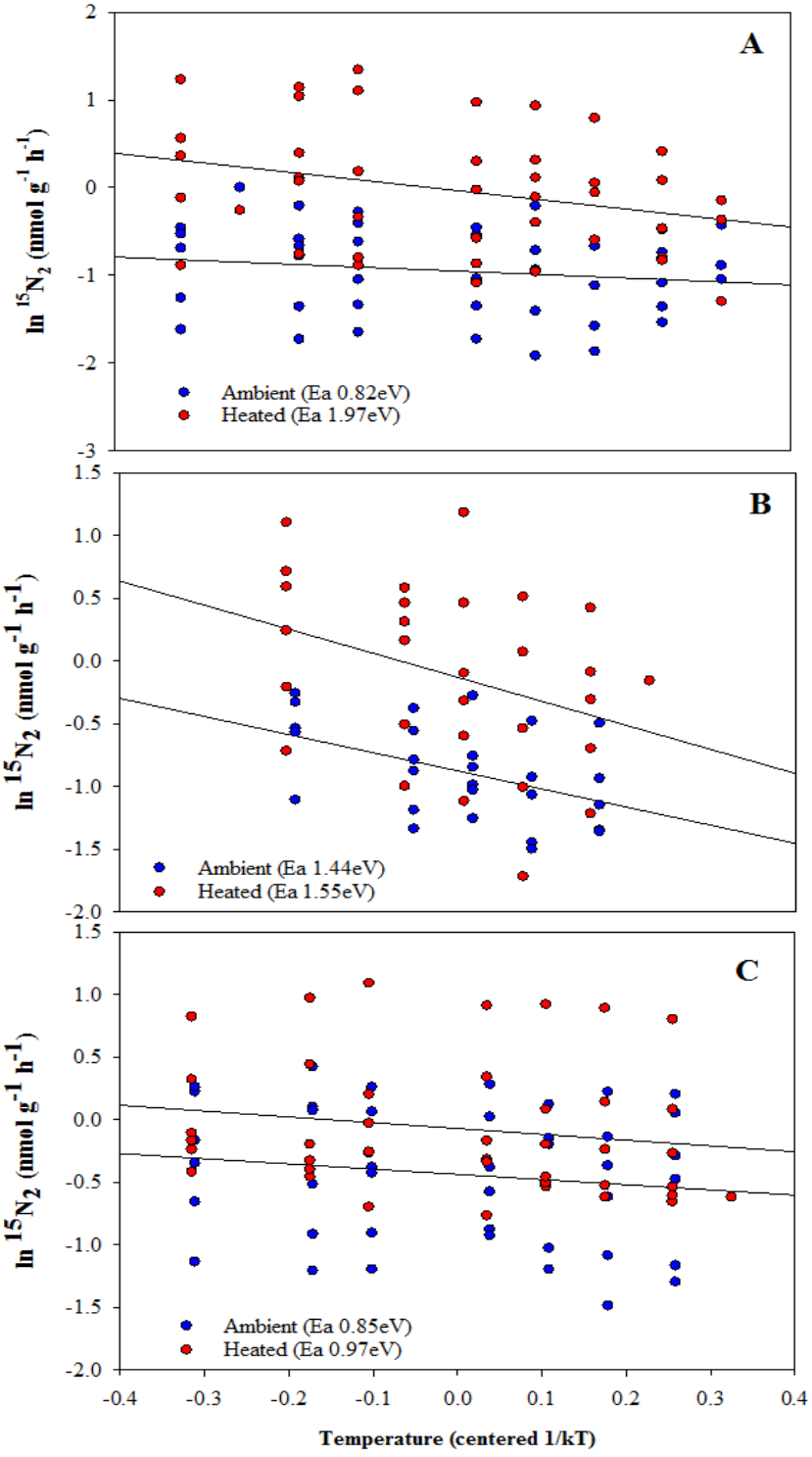

Figure 3.6. Arrhenius plots of natural log transformed $^{15}N_2$ production against incubation temperature through denitrification (centered 1/kT, where K = Boltzmann's constant and T is in Kelvin) for Spring (A), Summer (B) and Winter (C). Temperature increases from right to left.

Statistical significance of the short term temperature sensitivity for each treatment in each season is presented in Table 3.3 (Appendix). The most significant effect of temperature on the rate of $^{15}N_2$ production was observed in the heated treatments in spring and summer (Appendix, Table 3.3). However no significant difference was observed between the two treatments. This suggests a greater short term temperature sensitivity than long term warming effect created by the treatments. Even though the activation for the overall mean in the heated treatment sediments is much greater than that from the ambient treatment, the low r^2 values reduce any significant difference between the two.

The activation energies (Ea) for N_2O production showed little variation between the two treatments within spring and summer i.e. they are the same in spring and summer, but a large difference was observed between the heated and ambient treatments in winter (Figure 3.7). In both spring and summer, activation energies are greatest for sediments from the heated treatments, but the reverse is obtained in winter, with the ambient treatment showing a much greater sensitivity to temperature than sediment from the heated treatment (Appendix, Table 3.4). The greatest average production rates were observed in summer for both treatments, with the lowest average rates of N_2O production observed in winter for both treatments (Appendix, Table 3.4).

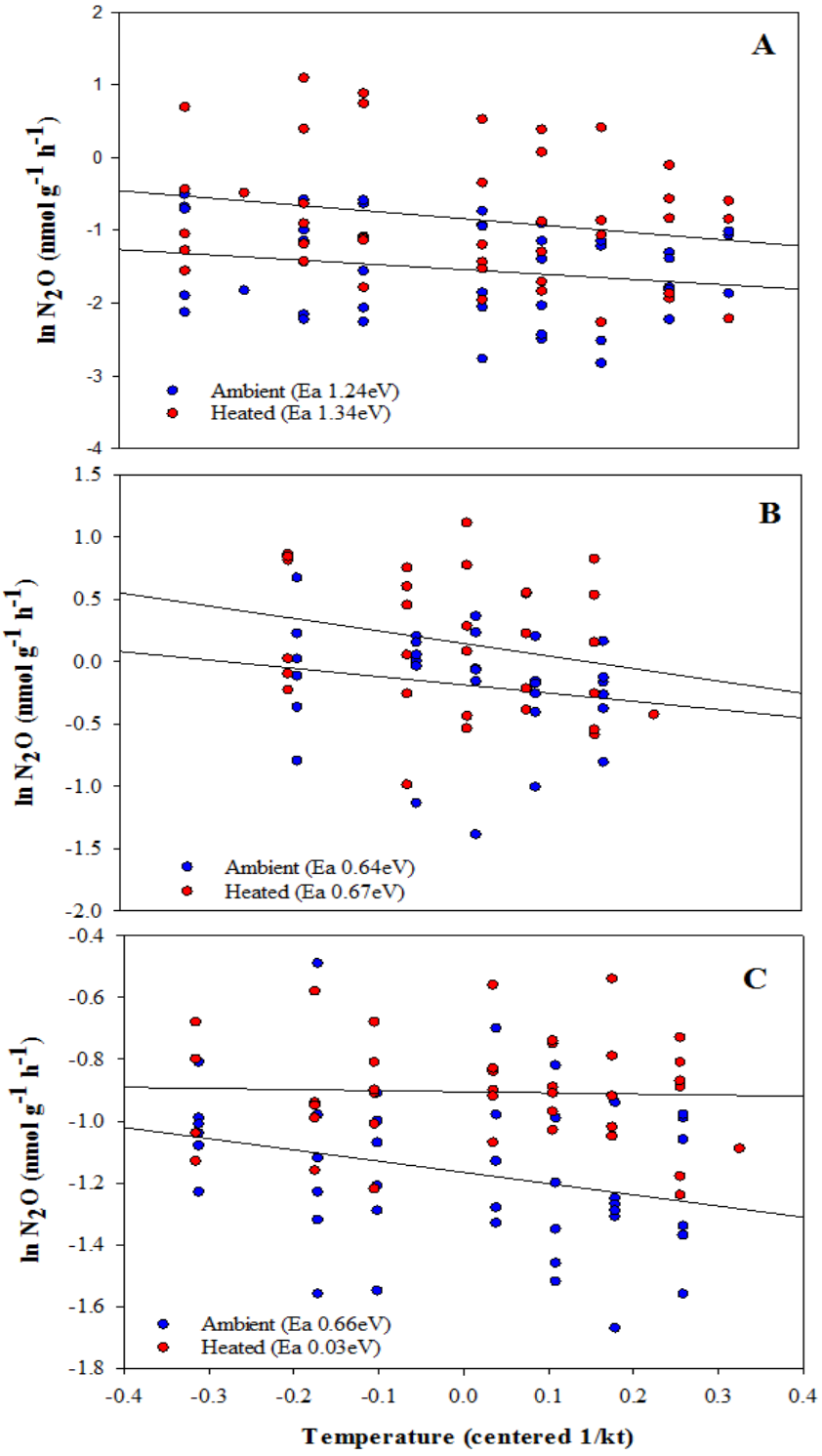

Figure 3.7 Arrhenius plots of natural log transformed N_2O production against incubation temperature through denitrification (corrected 1/kT, where K = Boltzmann's constant and T is

in Kelvin) for Spring (A), Summer (B) and Winter (C). Temperature increases from right to left.

As temperature increased, so did the production rates of N_2O. Statistical analysis of the effect of short term temperature exposure on N_2O production are summarised in Table 3.5 (Appendix). The greatest short term temperature sensitivity was observed from sediments collected in spring. The only non-significant effect of short term temperature was obtained in winter within the heated treatment. However, again, no significant effect of treatment was obtained within seasons indicating a stronger seasonal response for the production of N_2O from sediments that have had short term temperature exposure.

Rates of N_2O production were significantly greater from the ambient treatment in summer than both spring (Q = 8.42, df = 2, p < 0.001) and winter (Q = 7.46, df = 2, p < 0.001). N_2O production was also significantly greater in sediment from the heated treatment sediments in summer than both spring (Q = 5.75, df = 2, p < 0.001) and winter (Q = 7.73, df = 2, p < 0.001) determined by a Kruskal-Wallis ANOVA, followed by Dunn's method post hoc analysis for non-normalised data.

With the ratio of N_2O/ N_2 production there is a general trend of a constant ratio with sediments from the ambient treatments but an increase in the ratio towards the cooler temperatures from sediments that have had long term warming. This pattern is observed with sediments from all three seasons, except in the summer ambient treatment (Figure 3.8). Therefore, instead of activation energies, we get a negative response to temperature with a deactivation energy. Across all three seasons, the greatest effect of short term temperature exposure is observed with the heated treatments, with the largest deactivation energies (Appendix, Table 3.6). The largest ratios for both treatments were calculated from the summer season, indicating the greatest difference in the production rates of $^{15}N_2$ and N_2O. The lowest ratios occurred in winter for both treatments, suggesting the production rates of $^{15}N_2$ and N_2O were more similar than in

other seasons (Appendix, Table 3.6). The ratio of N_2O/ N_2 from the ambient treatment was significantly greater in summer than both spring ($Q = 6.25$, $df = 2$, $p < 0.001$) and winter ($Q = 10.49$, $df = 2$, $p < 0.001$). Spring was also significantly greater than winter from the ambient treatment ($Q = 4.75$, $df = 2$, $p < 0.001$). Similar results are seen from the heated treatment. Summer has significantly greater ratios than spring $Q = 5.78$, $df = 2$, $p < 0.001$) and winter ($Q = 10.01$, $df = 2$, $p < 0.001$) and spring has significantly greater ratios than winter ($Q = 4.93$, $df = 2$, $p < 0.001$). Results were determined by a Kruskal-Wallis ANOVA, followed by Dunn's method post hoc analysis for non-normalised data.

A highly significant response to short term temperature exposure, for N_2O/ N_2, was measured in spring from the heated treatment sediments ($p < 0.001$). A significant response to temperature is also seen in winter with the heated treatment ($p < 0.01$), though less significant than spring. In the warmer summer months, a significant response to temperature was seen in the ambient treatment ($p < 0.05$) (Appendix, Table 3.7). Although the ratios of N_2O/ N_2 was consistently greater from sediments in the ambient treatments than the heated sediments (Appendix, Table 3.7), this difference between treatments was non-significant.

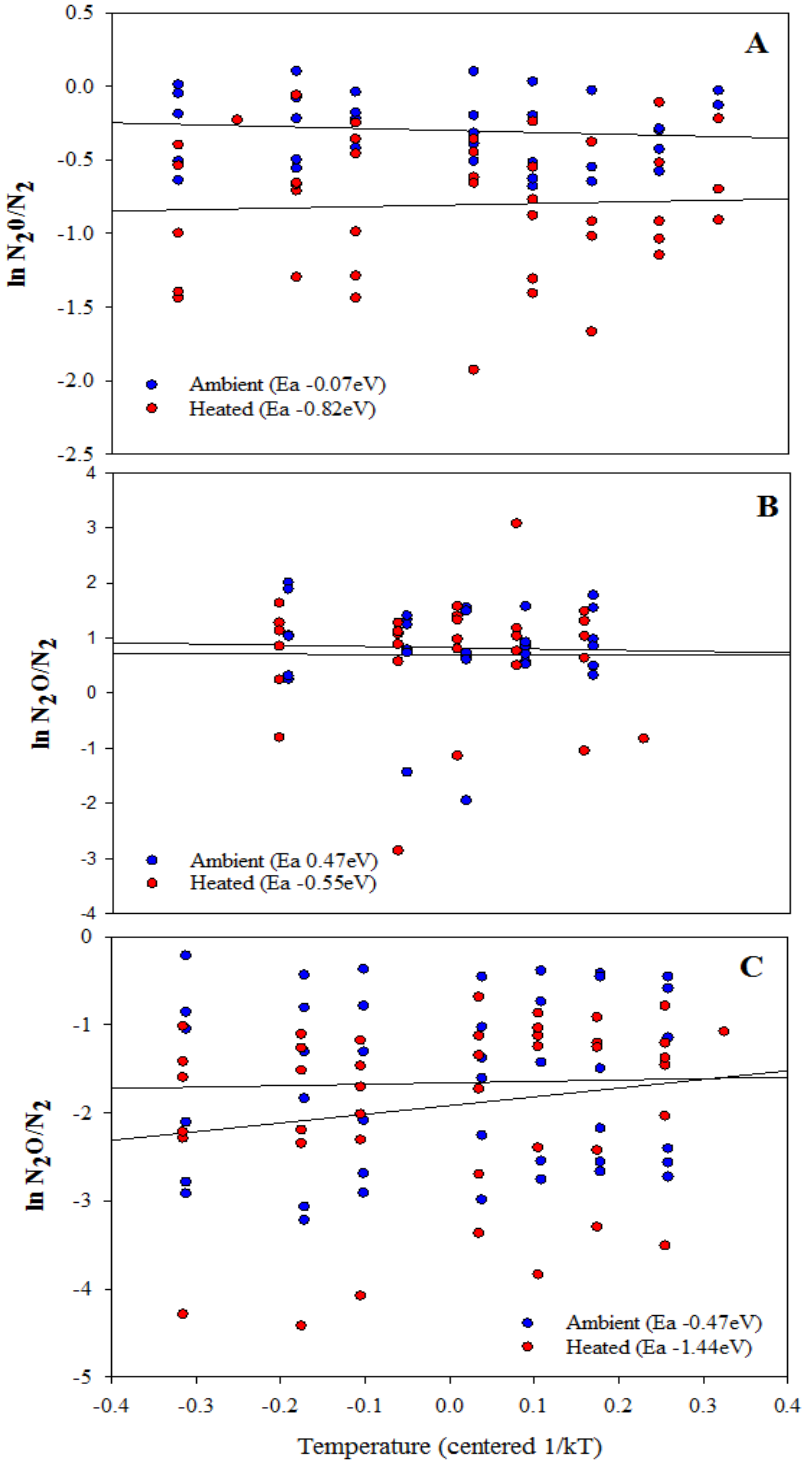

Figure 3.8 Arrhenius plots of the natural log transformed ratio of N_2O/ N_2 against incubation temperature through denitrification (corrected 1/kT, where K = Boltzmann's constant and T is in Kelvin) for Spring (A), Summer (B) and Winter (C). Temperature increases from right to left.

When the entire temperature range is considered for the thermal gradient (short term) temperature characteristics experiment for denitrification, production rates of $^{15}N_2$ (nmol g^{-1} h^{-1}) were significantly greater in sediments from the heated mesocosms when season is a random effect (X^2 = 187.73, df = 2, p < 0.001) (Figure 3.9A.). The average maximum rates of $^{15}N_2$ production were greater in the heated treatments in all season (Appendix, Table 3.8).

Seasonal effects were also observed. Within the ambient treatment, significantly higher production rates were measured from sediments analysed in winter and therefore greater temperature sensitivity was observed than in summer (p < 0.001) and spring (p < 0.001). Spring $^{15}N_2$ production rates were also significantly greater than those in summer (p < 0.001). The same significance was observed within the heated treatment. Winter had significantly higher rates of $^{15}N_2$ production than both summer (p < 0.001) and spring (p < 0.001) and spring had significantly higher rates than summer (p < 0.001) (Appendix, Table 3.9).

Similarly to production of $^{15}N_2$, production of N_2O (nmol g^{-1} h^{-1}) from short term temperature exposure was significantly greater in sediments from the heated treatment when season was treated as a random effect (X^2 = 8.84, df = 2, p < 0.003). Average maximum rates of N_2O production rates were greatest in the heated treatments in all seasons (Appendix, Table 3.10) (Figure 3.9B).

Seasonal effects were also observed for rates of N_2O production with short-term temperature exposure. For the heated treatment, rates were significantly greater in summer than spring (p < 0.001) and winter (p < 0.001). With rates in spring also significantly greater than those in winter (p < 0.05) (Appendix, Table 3.11). The same trend was again seen in sediments from the heated mesocosms. Rates of N_2O production were significantly greater in the summer than spring (p < 0.001) and winter (p < 0.001), with rates in spring significantly greater than those in winter (p < 0.001) (Appendix, Table 3.11).

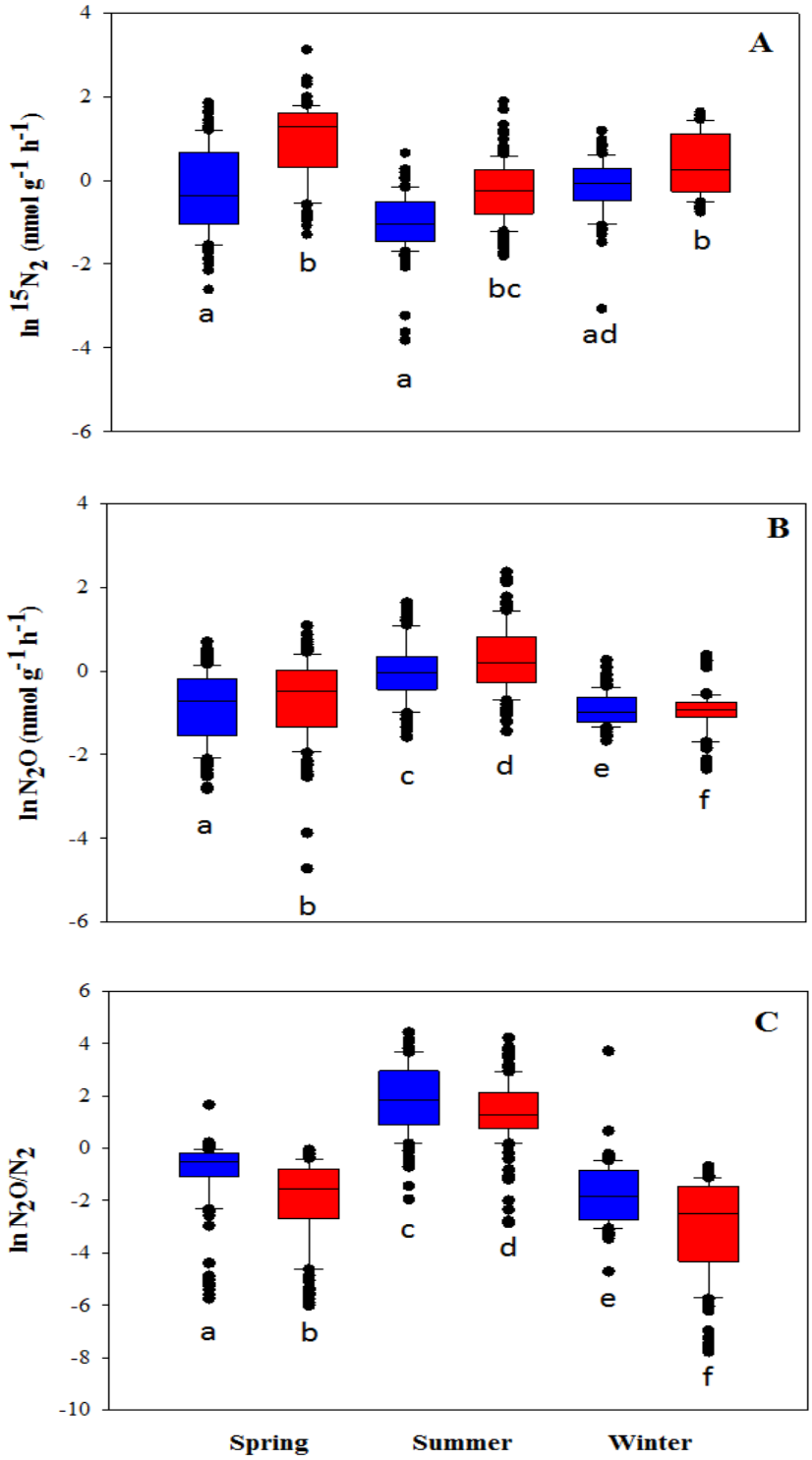

Figure 3.9 Box and whisker plots showing the production of A) $^{15}N_2$, B) N_2O and C) the ratio of N_2O/ N_2 through denitrification from mesocosm sediments. Sediments (warmed mesocosm – red, ambient mesocosm – blue) were exposed to a thermal gradient (11.5 – 36.5 ^{o}C) and each box and whisker plot shows the natural log production across this thermal gradient created by the thermal gradient block. Bars with different letters indicate results significantly different from one another within each figure.

When the effect of season is removed, and the ratio of N_2O/N_2 considered, significantly greater ratios were observed in sediments from the ambient mesocosms ($X^2 = 68.03$, $df = 2$, $p < 0.001$). The maximum average ratios are always greatest from the ambient treatment when broken down by season (Figure 3.9C, Appendix: Table 3.12).

Seasonal effects were also observed for the ratio of N_2O/N_2. Within the heated and ambient treatments, the ratios were significantly greater in summer than spring ($p < 0.001$) and winter ($p < 0.001$) and spring ratios also significantly greater than those in winter ($p < 0.001$) (Appendix, Table 3.13).

3.3.6 Short-term temperature sensitivity of nitrification

For rates of nitrification with short term temperature exposure, the thermal range was divided into 2 temperature ranges to ensure the linear range was used to determine activation energies. In the first temperature range of 11.5-24^{o}C, no significant effect of temperature was observed on the rate of nitrification in summer and winter (Figure 3.10). Apparent activation energies indicated a slight thermal sensitivity in both treatments in summer and winter, with low r^2 values confirming very little thermal response within this range (Appendix, Table 3.14). The heated treatment in winter shows a decrease in nitrification rates with increasing temperature, suggesting a deactivation. All other activation energies suggest an increase in activity with increasing temperature within this range. A significant difference was observed between seasons ($p < 0.0001$) with significantly higher rates in summer than winter for ambient ($p < 0.0001$) and heated ($p < 0.0001$) treatments (Appendix, Table 3.15). This again indicates a stronger seasonal response than long-term warming response from the treatment.

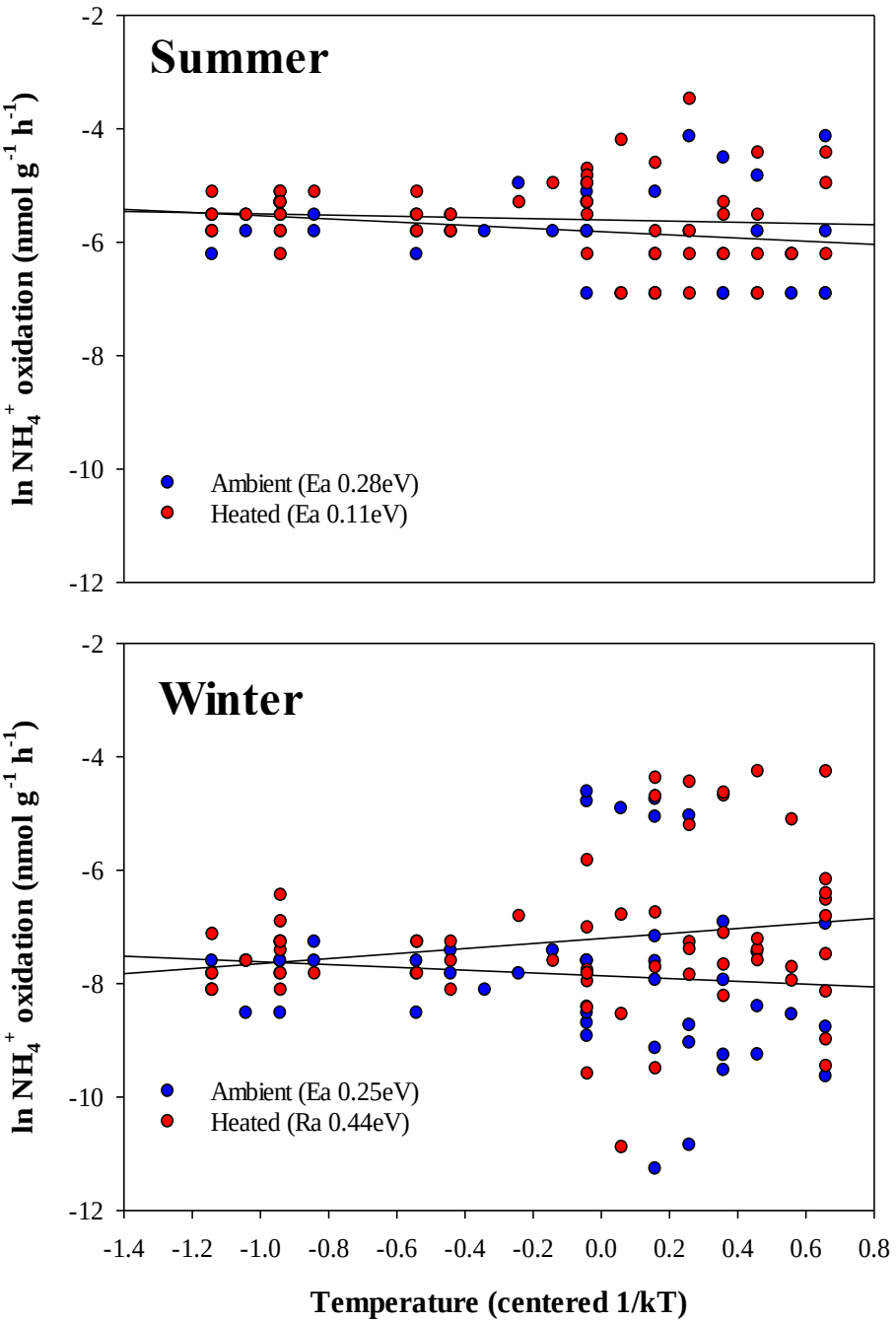

Figure 3.10 Arrhenius plots of natural log transformed nitrification rates (as NH_4^+ oxidation) against incubation temperature (corrected 1/kT, where K = Boltzmann's constant and T is in Kelvin) between 11.5-24°C in summer (A) and winter (B). Temperature increase from right to left.

In the higher thermal gradient range of 27-37.5^{o}C, an increase in net nitrification activity with increasing temperature in both treatments in summer and winter was observed (Figure 3.11). An increase in apparent activation energies confirms there is a greater response of nitrification at this higher temperature range (Appendix, Table 3.14), with the greatest activation energies observed in summer. No significant difference was observed in the rates of nitrification between the heated and ambient treatments within summer or winter. However, increasing temperature had a significant positive response to summer heated and ambient treatments ($p < 0.0001$) and for the winter ambient mesocosms ($p < 0.003$) (Appendix, Table 3.15). This suggests that at the higher temperature range, both treatments show greater thermal sensitivity in summer and confirming seasonal importance to this process.

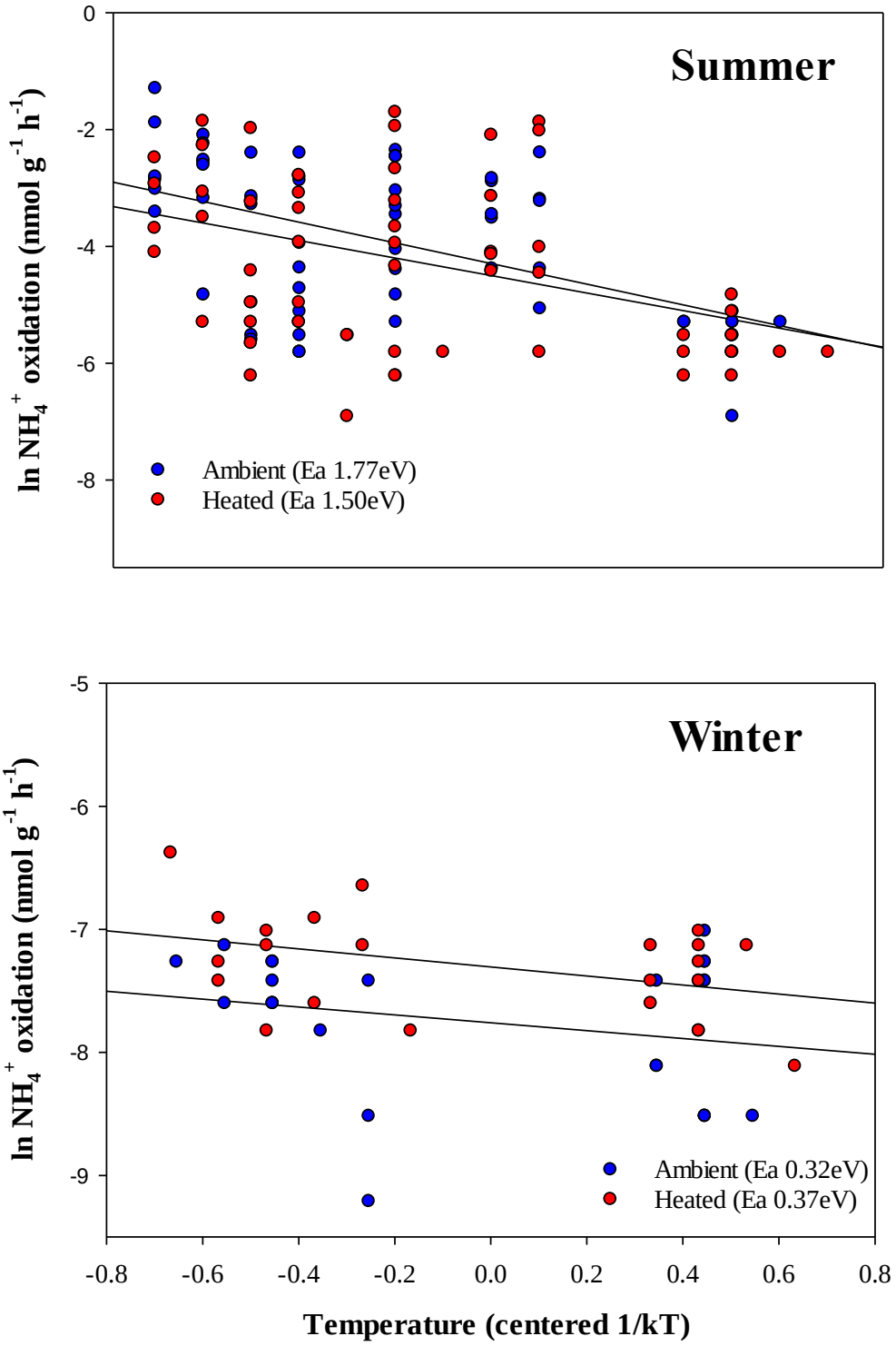

Figure 3.11 Arrhenius plots natural log transformed nitrification rates (as $^{15}NH_4^+$ oxidation) against incubation temperature (corrected 1/kT, where K = Boltzmann's constant and T is in Kelvin) between 27-37.5°C in summer (A) and winter (B). Temperature increases from right to left.

Rates of nitrification were measured over a thermal gradient ranging from 11.5 to 37°C in sediments taken from both the heated and ambient mesocosms in summer and winter (Figure 3.12). When considering the entire temperature range, the greatest average rates of nitrification rates were measured in summer for both the heated and ambient treatments (0.023 ± 0.0019 and 0.018 ± 0.0015 nmol N g^{-1} h^{-1}, respectively, n =143). With rates in summer significantly greater than those in winter (Appendix, Table 3.14). The lowest average rate of nitrification was observed in winter from the ambient treatment (0.0007 ± 0.0002 nmol N g^{-1} h^{-1}). The heated treatment in winter had a slightly higher average rate of 0.0015 (± 0.0003) nmol N g^{-1} h^{-1}. No significant effect was observed within season between treatments.

Very little thermal response was observed for rates of nitrification within treatments in either summer or winter (activation energies, Appendix: Table 3.17). The ambient in both summer and winter and heated in summer show a marginal increase in rates of nitrification with increasing temperature when looking at the entire range. The heated in winter however, shows an overall decrease in nitrification activity with increasing temperature. However, to calculate accurate apparent activation energies we need to consider the temperature range where production is linear. To do this, the temperature range was divided into two distinct sections of linear production. These ranges are 11.5-24°C and 27-37.5°C for both treatments in summer and winter.

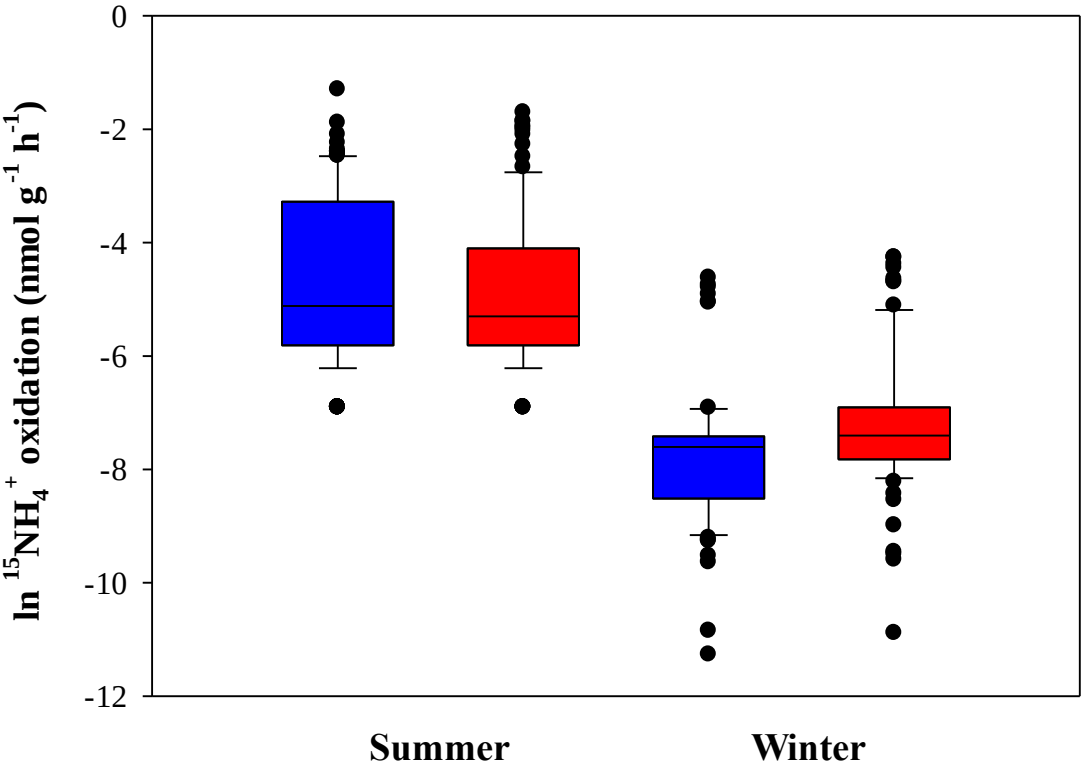

Figure 3.12 Nitrification rates through $^{15}NH_4$ oxidation for ambient (blue boxes) and heated (red boxes) treated mesocosms across seasons after short term exposure to a range of temperatures in a thermal gradient bar (± *se*, *n* = 8).

3.4 Discussion

Evidence of global warming altering the nitrogen cycle has been well documented through both direct effects (e.g. increased metabolic rate) or indirectly (e.g. availability of substrates and synergistic effects with respiration and photosynthesis (e.g. Canion et al., 2014; Veraart et al., 2011; Yvon-Durocher et al., 2010). The dominant controlling factors of denitrification and nitrification are temperature, NO_3^-, dissolved oxygen and carbon substrates (Holtan-Hartwig et al., 2002). The present study investigates direct temperature effects on denitrification and nitrification in sediments from warmed (long-term, 4°C) mesocosms. From the present study we found the thermal sensitivity of both denitrification and nitrification appeared to be more

affected by immediate temperature exposure, with significant effects of season. The effect of treatment (long-term warming vs ambient) appears to less important in determining rates of nitrogen conversion.

3.4.1 Denitrification

A significant effect of treatment was observed with rates of N_2 production, with greatest rates measured in the heated treatment from the *in-situ* experiment. It is expected that greater denitrification would be observed at warmer temperatures as most biochemical reactions increase with warmer temperatures due increased metabolic activity (Arrhenius, 1915., Boltzmann, 1872). Previous mesocosm experiments have shown denitrification to double with an increase of 3^{o}C, with only a 1^{o}C temperature rise required to increase denitrification by 24-28% in freshwater sediments (Veraart et al., 2011) which corresponds to the findings in the present study. Conversely, there was no significant effect of treatment on the rates of N_2O production within the *in-situ* experiment. This is in contrast to previous studies which have found increases in N_2O production in water-logged grassland sediments after 4 years of warming, due to a decrease in the ratio of the genes involved with N_2O reduction (*nosZ*) and NO_2^- reduction (*nirK*) reduction (i.e. *nosZ*/*nirK)* (Cantarel et al., 2012). However, evidence suggests lower temperatures (< 4^{o}C) may have greater net fluxes of N_2O due to reduced activity of the enzyme responsible for the reduction of N_2O (Holtan-Hartwig et al., 2002). This suggests that rates of N_2O production can stay relatively constant whereas the process of reducing N_2O to N_2 could increase with increasing temperature due to different optimal temperatures of the enzymes involved.

When the sediments from the two treatments were exposed to a range of temperatures to characterise their short-term temperature response, there was a significant positive effect of temperature, but no significant effect of treatment was observed within the linear range of N_2

or N_2O production. Activation energies for rates of N_2 and N_2O were in the range of 0.97-1.97 eV and 0.66-1.34 eV respectively. These are slightly higher than those recorded for denitrification in soils (0.28-0.81 eV) but are more in line with those measured from marine sediments (0.53-1.28 eV) (Canion et al., 2014; Holtan-Hartwig et al., 2002). The non-significant effect of treatment suggests short term temperature exposure is more important than the long term warming and potentially no adaptation to temperature of the microbial communities in these sediments has occurred over the ~10 years of warming. No evidence of a physiological response was observed, with similar thermal sensitivities obtained from sediments in both the heated and ambient mesocosms. However, the capacity to produce N_2 and N_2O from sediments has increased as the intercepts (production at mean temperature, nmol g^{-1} h^{-1}) are consistently greater from sediments within the heated treatment. When systems are limited in substrates required for metabolism, the microbial communities may not invest in adaptations to changes in temperature (Brin et al., 2016). The mesocosms in the present study are not directly influenced by outside sources of organic matter, relying on *in-situ* primary production and decomposition, so are therefore relatively nutrient limited and nitrate (the substrate) is very low. This may explain the limited effect of treatment in the linear range of the short term temperature exposure experiment. Several other studies have shown other microbial communities to have little to no adaptation to increased temperatures (Hartley et al., 2008; Rinnan et al., 2009; Vicca et al., 2009). Rates of N_2 and N_2O continued to increase after the initial linear phase with maximum rates measured between 35.5–37.5^{o}C for N_2 and 16.5-37.5oC for N_2O. When exposed to these higher temperatures, sediments from the heated treatment had significantly greater production of N_2 and N_2O than those from the ambient treatment. The response observed at the higher temperatures suggests a thermal adaptation of the denitrifying communities has occurred in response to long term warming and those in the heated treatment have higher metabolic activity than those from the ambient. Aquatic

organisms tend to not be exposed to rapid changes in temperature due to the high specific heat capacity of water (Wallenstein & Hall, 2012). However, temperate aquatic systems are exposed to a range of temperatures throughout the annual cycle and the organisms can therefore be adapted to a broad thermal range. We may only have observed a significant effect of treatment during the short term temperature exposure at the highest temperature as we have exposed them to temperatures they do not normally reach. The denitrifiers in the heated treatment may have a slight advantage over those in the ambient treatment. Long term thermal adaptations could include changes in e.g. community structure, gene expression, a change in protein structure affecting enzyme efficiency or abundance of either the organisms or the key genes involved in biochemical processes (Adams et al., 2010; Pörtner et al., 2006; Van der Gucht et al., 2007).

Calculating the ratio of N_2O/ N_2 allows us to investigate the efficiency of denitrification. A greater ratio indicates that more molecules of N_2O are produced per molecule of N_2. For each experiment that was carried out, the ratios were more greatly influenced by the production of N_2 rather than N_2O, as N_2 production had a stronger thermal response. The ratio of N_2O/ N_2 from the *in-situ* long term temperature experiment measurements were significantly affected by treatment, with the greatest ratios calculated in sediments from the ambient treatments. The ratios from the heated and ambient treatment sediments decreased with increasing temperature in the short-term exposure experiments, though this was non-significant. Whilst no significant effect of treatment was observed in the linear range, the intercepts were always greatest from the sediments in the ambient treatment. We only observe a significant effect of temperature at the highest temperatures with short-term temperature exposure. The response of a decrease in the ratio of N_2O/ N_2 with increasing temperatures has previously been measured but literature for aquatic systems is lacking with most studies having been carried out using soils (Avalakki et al., 1995; Bailey & Beauchamp, 1973; Keeney, Fillery, & Marx, 1979).

Significant seasonal variations were observed in the production of N_2 and N_2O. Within the long-term temperature experiment, greatest rates of N_2 production were observed in sediments analysed in the summer and greatest production of N_2O was observed in spring. Conversely, across the thermal gradient, production rates of N_2 were significantly greater in winter with N_2O rates significantly greater in the summer sediments. The ratio of N_2O/ N_2 were significantly greater in spring for both the *in-situ* experiment and the initial linear range of the short-term thermal sensitivity experiment. However, when the entire thermal gradient is considered, the greatest ratios are observed in summer. Previous studies have shown that seasonal variations of denitrification in freshwater systems has been most strongly correlated to NO_3^- availability with enhancement from increased temperature (Hasegawa & Okino, 2004; Pattinson et al., 1998). Seasonal sampling of sediments along the river continuum of Swale-Ouse, UK, had greatest rates of denitrification in spring which was significantly correlated with higher concentrations of NO_3^- and temperature (Pattinson et al., 1998). Eutrophic, stratified lakes have shown similar trends, with denitrification greatest in the winter and spring, when NO_3^- concentrations are higher and low to negligible in the summer due to increased primary production. Both these studies suggest NO_3^- to be the dominant factor controlling denitrification, whether in a eutrophic system or with large influxes of NO_3^- from field run off. Due to our method of slurry incubations, our samples were not limited by NO_3^- availability, so other factors will need to come into play. Greater denitrification in summer could be related to increased organic matter deposition after the spring bloom providing more organic carbon to the sediments. Warmer temperatures also have a positive effect on the availability of organic carbon for heterotrophic metabolism by increased rates of decomposition (Brin et al., 2015; Andy Canion et al., 2014; Isaksen & Jørgensen, 1996). However, the high rates in spring of N_2O from the *in-situ* experiments and greatest production of N_2O from sediments collected in the winter is a slight anomaly.

Results from these two experiments, as well as previously published findings, suggest as temperatures increase we will see an increase in production of both N_2 and N_2O, however, from the current study we can also expect to see a greater occurrence of complete denitrification with deceasing ratios of N_2O/ N_2.

3.4.2 Nitrification

No significant effect of treatment for nitrification was observed for either *in-situ*, long term thermal response or the short term temperature exposure experiment using a thermal gradient bar. Season appears to be more important than long term exposure with significantly greater rates in sediments analysed in spring. A minor thermal response was only observed in the higher thermal range of 27-37.5^oC, with rates significantly greater in sediments in the summer. The rates of nitrification presented in this study are much lower than those presented in the literature. For example, rates of nitrification in Lake Superior, a large oligotrophic lake, ranged between 18-34 nmol N L^{-1} d^{-1} (Small et al., 2013). These values are regarded as low in comparison to other lake systems that have rates ranging from 60-480 nmol N L^{-1} d^{-1} in the summer and 230-335 nmol N L^{-1} d^{-1} in the winter (Carini & Joye, 2008). The Lake Superior study suggested low rates were positively correlated with low abundances of ammonium oxidising archaea and nitrite oxidising bacteria due to competition with other heterotrophic and autotrophic organisms that also utilise NH_4^+ as a substrate (Small et al., 2013). In river sediments, nitrification was completely inhibited by additions of organic carbon due to more competitive heterotrophic bacteria utilising available NH_4^+ (Strauss & Lamberti, 2000) however, this limiting factor was removed by using slurry experiments and providing ample NH_4^+. Another possibility for the low rates of nitrification observed is the link with denitrification. Coupled nitrification-denitrification is often observed at oxic-anoxic interfaces within sediments, as nitrification produces NO_3^- which denitrification requires as a substrate (An & Joye, 2001; Seitzinger et al., 2006). Though our slurries were oxic, there may still have

been micro pockets of anoxia where denitrification could have utilised NO_3^- that had been produced through nitrification, therefore underestimating the rates of nitrification. Dissimilatory reduction of nitrate to ammonium (DNRA) is another process within the nitrogen cycle which may have caused an under estimation with our measured rates of nitrification. DNRA bacteria are predominately heterotrophic that reduce NO_3^- back into NH_4^+ (Hardison et al, 2015). We did not measure DNRA in this study due to the small possibility of detectable rates. DNRA is favoured in environments with high labile organic carbon and low NO_3^- concentrations, where it can outcompete denitrification (Tiedje, 1988; Hardison et al, 2015). Additionally, direct measurements of DNRA from freshwater systems are scarce in the literature, therefore very little is known of the controlling factors in these environments (Burgin and Hamilton, 2007; Giblin et al, 2013). The experimental mesocosms could have potentially favoured DNRA due to low concentration of NO_3^-. However, these are closed systems with very little input from external sources, which also suggests organic carbon content would be low. In these conditions of potentially a low ration of organically available carbon to NO_3^-, denitrification is favoured (Bonin, 1996; Nijburg et al, 1997; Silver et al, 2001).

We did however, see seasonal variation for nitrification. Previous studies have shown seasonal fluctuations of nitrification are positively correlated with NH_4^+ release from sediments due to increasing temperatures and subsequently increase rates of decomposition (Sheibley et al., 2003). The greatest rates were observed in spring, which marry up with spring blooms and increased rates of photosynthesis during spring have been positively correlated with increased rates of nitrification due to increased oxygen concentration of overlying waters (An & Joye, 2001). The low nitrification rates observed in summer could have been due to lowered oxygen concentrations caused by increased respiration. Nitrification is an aerobic process, requiring the presence of oxygen to oxidise NH_4^+ (Kemp et al, 1990; Rysgaard et al, 1994). Seasonal variations in nitrification rates were measured in Chesapeake Bay sediments, with highest rates

observed in November (70.4 µmol m^{-2} h^{-1}), reducing by up to 50% in April to negligible in the summer months, which positively correlated with oxygen concentration of the overlying water in those sampling months (Kemp et al, 1990). In addition to potentially low oxygen concentration in the summer reducing nitrification activity, sunlight can also hinder this process. Ammonium oxidising archaea (AOA) and bacteria (AOB) have been observed to be inhibited by sunlight (French et al., 2012; Merbt et al., 2012) and with longer and generally sunnier days in the summer, there is more potential for this inhibition to occur. The experimental mesocosms in this study were shallow and therefore did not provide depth for which these nitrifying organisms to remain out of the sunlight. Though many studies have detected nitrification in marine euphotic zones (e.g. Beman et al, 2012; Clark et al, 2008; Raimbault & Garcia, 2008), a more recent study, covering a 7500 km transect from the equatorial Pacific Ocean to the Arctic Ocean, maximum rates of nitrification were observed at 1-0.1% of light depth (Shiozaki et al, 2016). The organisms responsible for nitrification, AOA and AOB are widespread and have been recorded in a variety of systems from marine and coastal areas (Beman et al, 2008; Shiozaki et al, 2016), estuarine sediments (Beman and Francis, 2006; Mosier & Francis, 2008), hot springs (Hatzenpichler et al, 2008) and freshwater sediments (Francis et al, 2005; Heermann et al, 2008) to mention a few. Environmental requirements of the two nitrifying microorganisms differ. AOB have a low affinity for NH_4^+ and are therefore more dominant in regions with high NH_4^+ concentrations, whereas AOA have a higher affinity and can thrive in low NH_4^+ environments (Martens-Habbena et al, 2009). The experimental systems used in this study had low NH_4^+ concentrations throughout the year and could suggest AOA may be the dominant nitrifyers in the system due to their ability to thrive in low NH_4^+ environments. The high concentration of NH_4^+ used in the sediment slurries (90 µM in the vial) were much greater than the concentrations naturally found in the systems (0.4-5.2 µM NH_4^+). We used a high concentration in the experiment to remove NH_4^+ limitation, but

this may have inhibited AOA activity. Several species of AOA isolated from the environment have shown maximum growth rates at low concentrations of NH_4^+ and growth inhibition at higher concentration. An AOA species (*C. Nitrososphaera gargensis*) isolated from hot springs with concentrations of 5.9 µM NH_4^+, thrived at 0.14-0.8 mM concentrations but showed inhibition at 3.2 mM NH_4^+ (Hatzenpichler et al, 2008). Similarly, *N. maritimus*, an isolated marine species, had a maximum growth rate of 0.78 day^{-1} in a medium with a NH_4^+ concentration of 0.5 mM (Könneke et al, 2005). However, gene expression of the amoA gene responsible for Archaeal nitrification, has been reported in concentration as high as 10 mM NH_4^+ (Treusch et al, 2005). This suggests a flexibility in the capability of AOA activity at different concentrations, but potentially a preference for low NH_4^+ concentrations. Molecular analysis would need to be carried out to determine the dominant nitrifying communities present in the experimental mesocosms.

Even though emissions of N_2O through nitrification has been well documented across a variety of both terrestrial and aquatic systems (Baulch et al., 2012; Dore & Karl, 1996; Parton et al., 1996; Small et al., 2013; Sutka et al., 2006) we did not have any detectable rates of N_2O in either the long-term or short-term temperature response experiments. It has commonly been found that < 1% of nitrified N is actually converted into N_2O in laboratory studies on soils (e.g. Klemedtsson et al., 1988; Maag & Vinther, 1996). If this is the case, with the low rates of nitrification that we observed, any N_2O produced would be below the limit of detection.

3.5 Conclusions

The current study has considered the potential rates of nitrogen transformations after a period of prolonged moderate warming to enable us to investigate the potential long term effect of climate change and global warming. The direct comparison between heated and non-heated experimental mesocosms allowed us to remove other confounding effect such as allochthonous

carbon and nutrient inputs and focus on the effect of temperature. The long term response of denitrification appears to have increased the capacity of these systems to produce N_2 and N_2O, however, there has been no significant physiological adaptation with similar thermal sensitivities observed between the two treatments. The response of potential rates of nitrification appeared to be negligible, either to long term warming or short-term temperature response, reasons for which are unclear. The low rates of nitrification are of interest as it would be expected they would provide the substrates required for denitrification within the mesocosms. The rates measured in the present study would not be sufficient to support denitrification and so further experiments are required. Though more difficult, *in-situ* rates would be beneficial to investigate the coupling between these two processes.

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Chapter 4: Effect of temperature on benthic and pelagic nitrogen transformations in the Eastern Tropical North Pacific Oxygen Minimum Zone (ETNP OMZ)

4.1 Introduction

Oxygen minimum zones (OMZ) are stratified bodies of water in the ocean with oxygen near or below detection limits (typically < 1.5-2 µM (Beman et al., 2012; Karstensen et al., 2008). The formation of OMZ's occurs when respiration, associated with the breakdown of organic matter, requires more oxygen than can be resupplied in areas of poor water circulation (Karstensen et al., 2008; Lam & Kuypers, 2011). These areas are prominent contributors to the global nutrient cycles, specifically the nitrogen cycle (Beman et al., 2012; Dalsgaard et al., 2012) as they are responsible for removing large amount of fixed nitrogen from the biosphere. Even though they only constitute ~ 0.1% of the total ocean volume (Codispoti, 2007), they are estimated to be responsible for 20-50% of all oceanic nitrogen loss (Codispoti et al., 2001; Gruber & Sarmiento, 1997). Two processes are known to be involved in the removal of fixed nitrogen from the marine environment: denitrification and anaerobic ammonium oxidation (anammox).

Briefly, denitrification is the microbially mediated reduction of nitrate (NO_3^-) to di-nitrogen gas (N_2) through a series of steps performed by different bacteria (Falkowski, 1997). Canonical denitrification is complete reduction of either NO_3^- or NO_2^- through to N_2 gas (Codispoti, 2007) by a single organism (respiratory denitrification), though some bacterial have the ability to reduce NO_3^- and NO_2^- but do not produce N_2 gas as a final product (non-respiratory denitrification) (Tiedje, 1998). The full denitrification pathway is as follows (Kalkowski & Conrad, 1991; Zumft, 1997):

$$NO_3^- \rightarrow NO_2^- \rightarrow NO + N_2O \rightarrow N_2$$

Anaerobic ammonium oxidation (anammox) also produces N_2 via the anaerobic oxidation of ammonium (NH_4^+) using nitrite (NO_2^-) (Dalsgaard et al., 2012) with the following transformation:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$$

Although anammox produces N_2, it does not produce the greenhouse gas (GHG) nitrous oxide (N_2O), which is an intermediate in the denitrification pathway. N_2O is a highly potent GHG, with radiative forcing ~300 times that of CO_2 and leads to the reduction of ozone in the stratosphere (Ravishankara et al., 2009; Wright et al., 2012). Oceanic N_2O production is classified as natural production as it is away from anthropogenic influences of nutrient loading, with production rates in the range of 1.8-5.8 Tg y^{-1}, while the anthropogenic influenced N_2O production from coastal systems, estuaries and freshwaters is in the range of 0.5-2.9 Tg y^{-1} (IPCC, 2007). Globally, approximately a third of all natural N_2O emissions are from the oceans, and the majority of that is released into the atmosphere from OMZ's (Bange, 2006; Naqvi et al., 2010). As oceanic water temperatures increase under global warming, the amount of dissolved oxygen is decreasing, and rates of respiration are increasing, causing expansion of OMZ's (Keeling et al., 2010; Stramma et al., 2008; Vázquez-Domínguez et al., 2007). Not only could this lead to enhanced removal of fixed nitrogen from the oceans, but also increased N_2O emissions, a potential positive feedback mechanism.

Denitrification is an important process to understand as it removes biologically available nitrogen from systems, which may lead to reduction in primary productivity and, ultimately, to reduction in carbon sequestration (Seitzinger, 1988). As mentioned above, OMZ's are significant sites of nitrogen removal, and with evidence of their expansion (Stramma et al., 2008), this will lead to even greater rates of nitrogen loss. The temperature response of nitrogen removal has previously been investigated in marine and estuarine sediments. Denitrification generally responded more strongly to temperature than anammox, with greater contribution to

production of N_2 through denitrification (Canion et al., 2014; Rysgaard et al., 2004). Both studies suggest slow growing anammox bacteria to be psychrophilic (thriving at colder temperatures) and denitrifying bacteria to be psychrotrophic (able to survive and potentially thrive in cold temperatures). Given the increasing size of OMZ's and the potential for increased rates of denitrification with warming, we could witness an even greater loss of reactive nitrogen from the oceans. This loss will remove the reactive nitrogen for primary production and lead to reduced draw down of CO_2 from the atmosphere, leading to further warming.

Whilst many studies have reported N_2 production and the relative contributions of denitrification and anammox to the total N_2 production, very few have reported N_2O production. The ratio of N_2O/ N_2 is of interest because of the GHG properties of N_2O. If, as predicted, rates of denitrification increase with warmer temperatures, how will the efficiency of the process be effected? As mentioned above, N_2O is an intermediate that can be released during denitrification, which means it is not fully reduced to N_2. It is the reduction steps that release energy for the denitrifying organism and losing this intermediate makes the process less energy efficient. A decrease in the efficiency of denitrification will lead to more of the intermediate N_2O being released and a positive feedback for global warming. The temperature response of N_2O production was investigated in soils where they found increased N_2O production with warming temperatures between 5-20^oC (Holtan-Hartwig et al., 2002; Schaufler et al., 2010). To my knowledge, no one has explored the thermal sensitivity of N_2O production rates in marine environments, let alone in an OMZ.

Biological nitrogen fixation (BNF) is the biological conversion of abundant N_2 gas into biologically available inorganic nitrogen compounds, namely NO_2^-, NO_3^- and NH_4^+ (Zumft, 1997). Most systems are limited in nitrogen and BNF is the main natural source of these compounds into the biosphere (Galloway et al., 1995). Rates of BNF from the North Pacific ranged between 70 to 2800 µmol N m^{-2} d^{-1} (Montoya et al., 2004). Published rates of marine

nitrogen fixation are rare, especially those which focus on thermal sensitivity. A general rule of biological processes is that as temperatures increases so does enzyme activity (Brown et al., 2004). Therefore, it could be predicted that the rate of nitrogen fixation will increase with warming. It is unclear whether the rate of BNF will balance the losses of nitrogen through processes such as denitrification and anammox in a changing climate. It could be suggested that rates of BNF will be insufficient to keep up nitrogen losses, relying on energy obtained from photosynthesis, which, as we have seen, responds less to warming than respiration. Denitrification and anammox are forms of respiration (Tiedje, 1998) and therefore could respond more to warming.

We investigated the thermal sensitivity of denitrification and anammox as forms of N loss in both sediments and the water column and BNF in the upper oxic waters of the Eastern Tropical North Pacific Oxygen Minimum Zone (ETNP OMZ), the largest in the world (Paulmier & Ruiz-Pino, 2009), from 70 to 120 km off the coast of Guatemala. Production rates of N_2 through denitrification and anammox and N_2O production through denitrification were measured over a range of temperatures close to and above ambient using the isotope pairing technique to investigate nitrogen losses. To determine nitrogen uptake, the rates of nitrogen fixation was also measured in concentrated plankton surface waters over a range of temperatures close to and above ambient using the stable isotope $^{15}N_2$. Measuring these processes will allow us to investigate the nitrogen in and nitrogen losses from this system.

4.1.1 Aims and Hypothesise

We hypothesised with increasing temperature, we would see an increase in metabolic activity of anammox, denitrification and BNF. The aim of this work was to analyse the thermal sensitivity of OMZ sediment slurries and water column samples to produce N_2 and N_2O through denitrification and anammox, with different substrates in controlled conditions, and how these N losses may be balanced out with water column BNF.

4.2 Methods

4.2.1 Sampling sites

Several sampling sites were selected between 70-150 km off the coast of Guatemala where both sediment and water collection was carried out between 28/12/13 – 10/2/2014 (Figure 4.1). A standard conductivity–temperature–depth (CTD) rosette, comprising 24 Niskin (20 L) bottles and a Sea-Bird 24 electronics system (fluorimeter, altimeter, PAR and oxygen sensors, etc.) was used to collect water and a multi-corer (Mega Corer, OSIL, U.K.) was used to recover intact cores of sediment and overlying water. Site selection for denitrification and Anammox was predominately based on oxygen concentration (to ensure we were within the OMZ) and water density (to ensure organic matter was available for microbial activity). Figure 4.2 shows water column oxygen concentration and density with depth and horizontal lines indicate sampling depths. Sampling sites for surface water nitrogen fixation were aided by NO_3^- and PO_4^- concentrations (Figure 4.3).

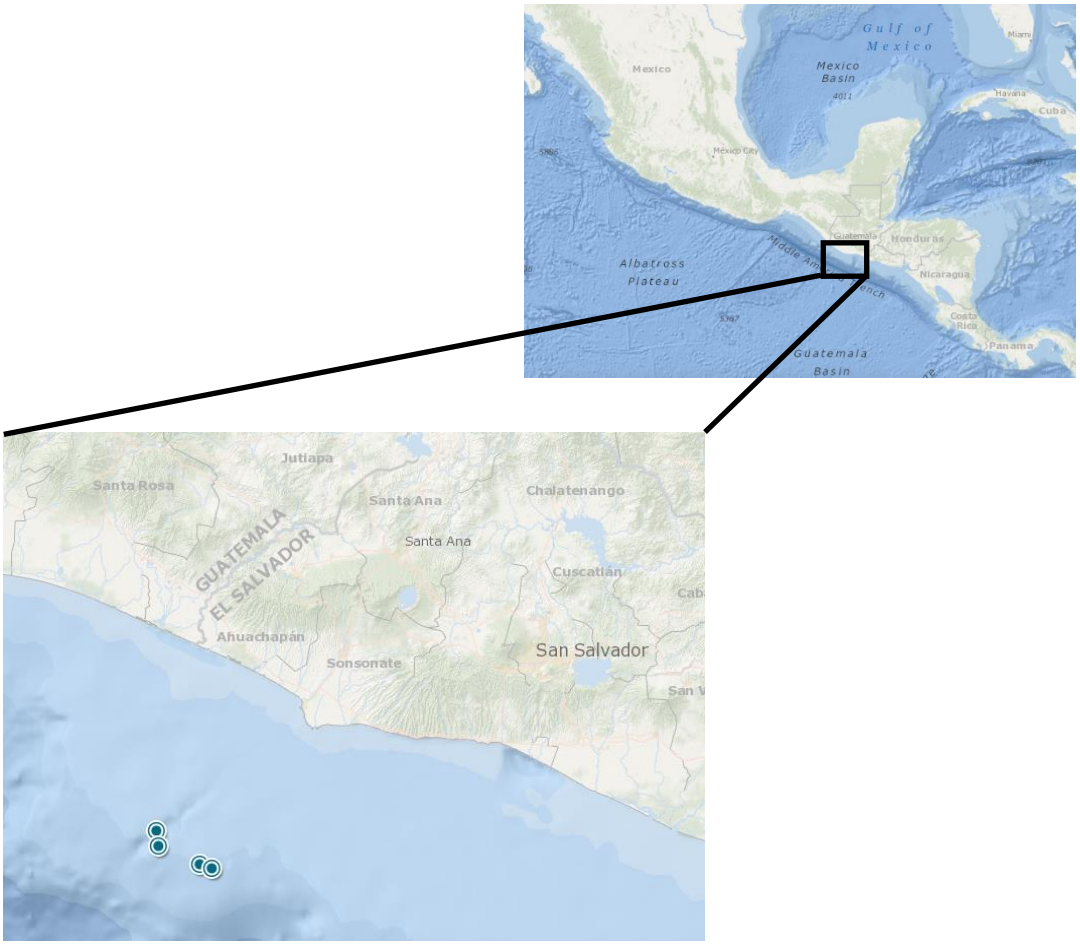

Figure 4.1. Location map of selected sites for sampling approximately 70-150 km off the coast of Guatemala. Blue dots indicate specific sampling sites.

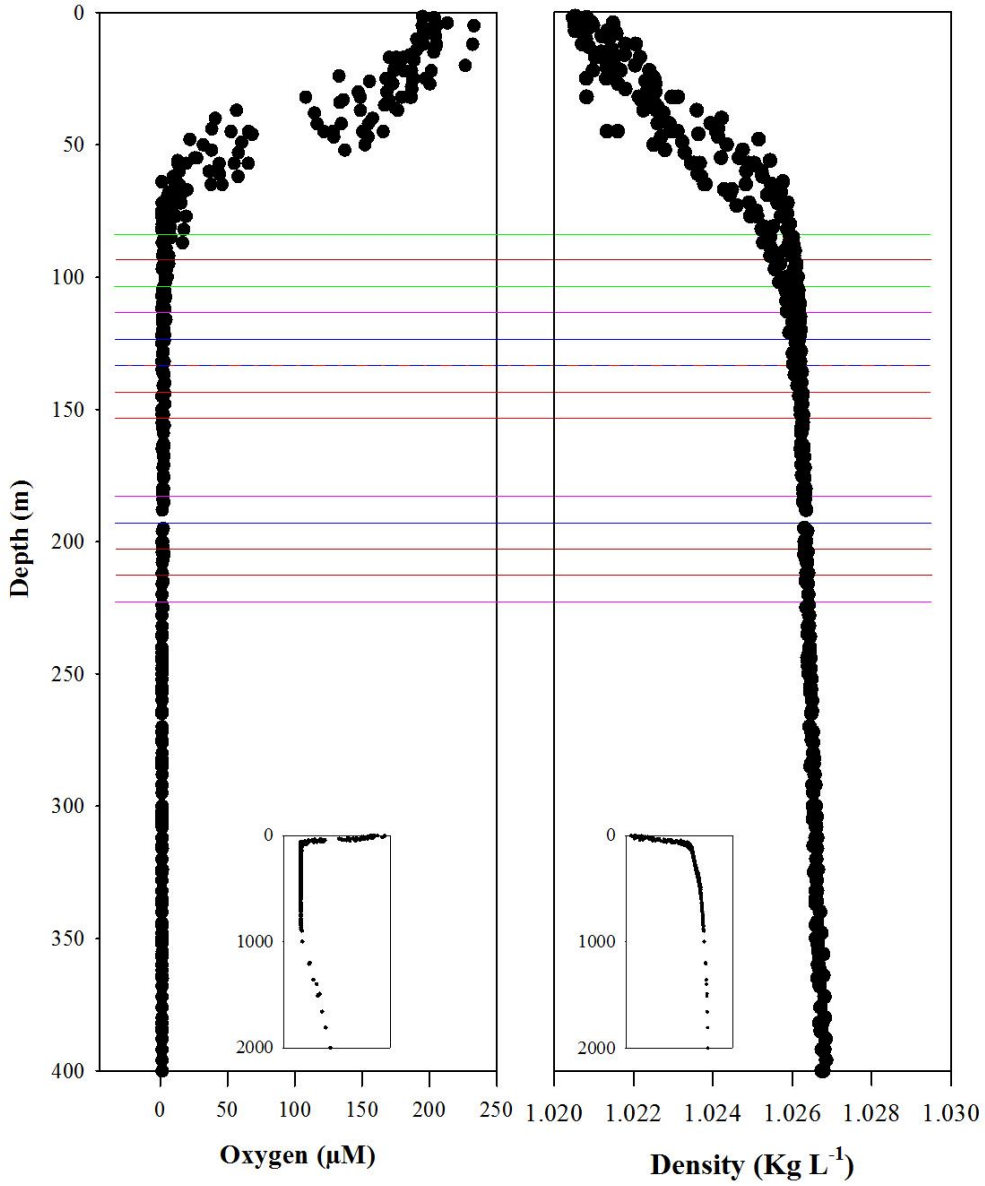

Figure 4.2. Water column profiles constructed based on CTD sensors measurements, with the top 400 m shown in the main panel and all data points (0-2000 m) shown in in-set plots. The coloured lines indicate sampling depths for water column N_2 production (denitrification and anammox); the coloured lines indicate the different sampling sites, with at least two depths collected from each site.

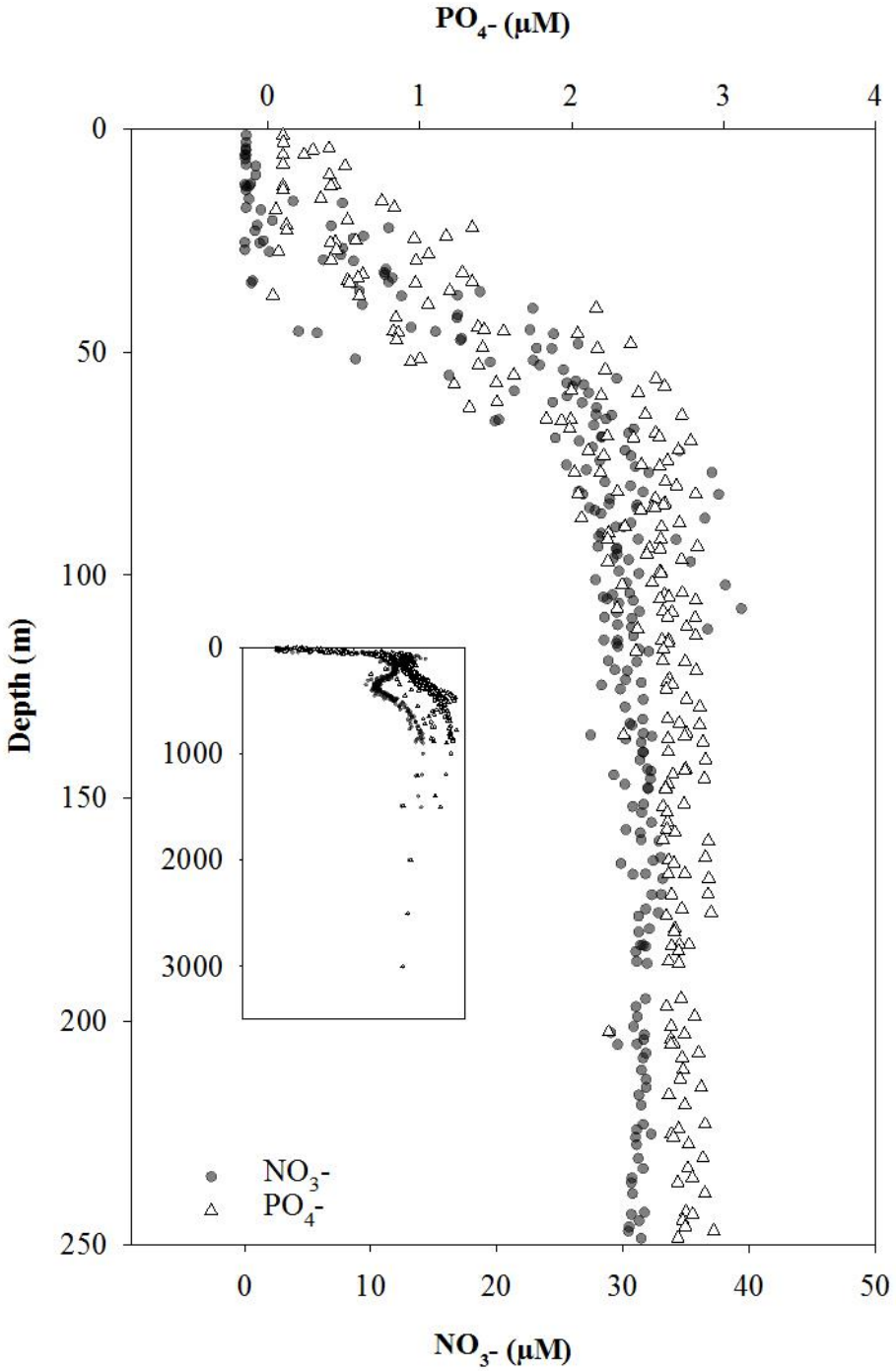

Figure 4.3. Water column profiles for NO_3^- and PO_4^-, with the top 250 m shown in the main panel and all data points (0-3000 m) shown in in-set plots. These profiles were used to determine nitrogen fixation sampling depths. (Sample processing carried out by cruise technician, Dr Ian Sanders, on site).

4.2.2 Sample collection and preparation for denitrification and anammox measurements in sediments

Sediments for potential denitrification and anammox rates were collected from the ocean floor using a Mega-Corer, with up to six cores at a time (Figure 4.4 left). The Mega-Corer allowed us to retrieve intact sediment cores overlaid with undisturbed bottom-water. At each site, surface sediments (0-2cm) were collected from all six sediment cores, transferred into zip-lock bags and quickly moved into an anoxic hood (CV204, Belle Technology) filled with oxygen free nitrogen (99.998%, OFN, British Gas Company). Additional water from ~10m above the ocean floor was collected using a CTD (conductivity –temperature-depth) rosette system with 20L Niskin bottles at the same sites of sediment collection. This water was later used to prepare sediment slurries for the temperature characteristic experiments (Figure 4.4 right).

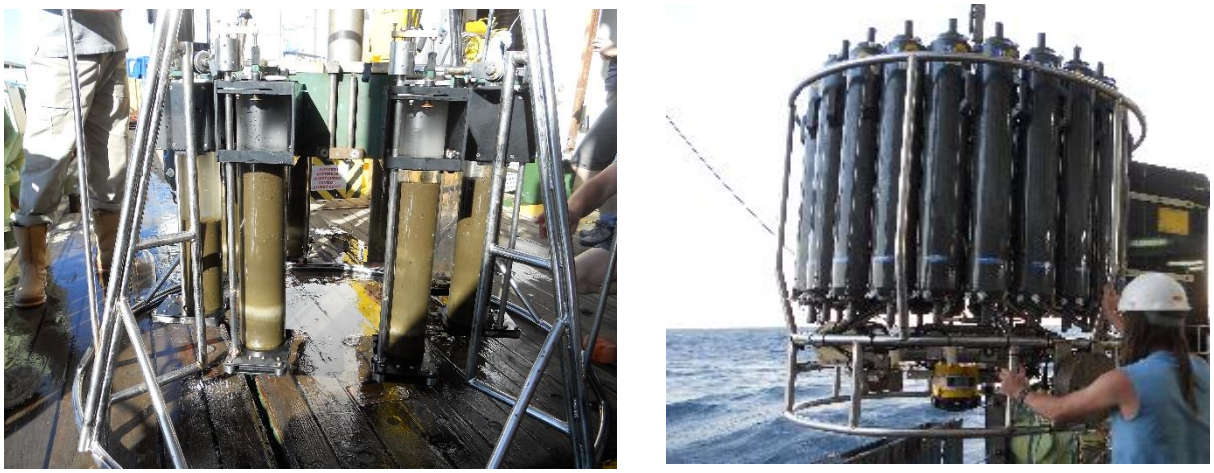

Figure 4.4. Sediment multi-corer to collect large intact sediments (left) and rosette niskin bottles used to collect water from the ocean floor (right).

Sediments were homogenised and slurries were prepared by the addition of degassed (OFN for 20 minutes) bottom water from the same site. The amount of water to sediment varied on the water content of the sediments but was typically between 2-4 mL of water mixed with 6-8 mL of sediment. The slurries were then transferred into 12 mL gas-tight vials (Exitainer, Labco). Once sealed, the gas tight vials were placed at a range of temperatures over night to ensure that any natural $^{14}NO_3^-$ or other oxidants present would have been reduced along with any traces of oxygen (Risgaard-Petersen et al., 2004; Trimmer et al., 2003). To begin the experiment, each vial was injected through the rubber butyl septa with deoxygenated $Na^{15}NO_3^-$ or $Na^{15}NO_2^-$ (98 ^{15}N atom %, Sigma Aldrich) to a final concentration of 100µM in the water. The two experiments were conducted to determine if the species of $^{15}NO_x$ (NO_3^- or NO_2^-) affected the potential activity of denitrification and anammox. The experiment was repeated at 5 sites, with 3 replicates at each time point. Slurries were incubated at 5, 12, 15, 20 and 24^oC for 0, 0.5, 1, 2, 4 and 8 h. Once the experiment was complete, microbial activity was stopped with the addition of 200 µL formaldehyde (38% w/v) (Trimmer & Nicholls., 2009). Rates of N_2 and N_2O production were measured from sediments exposed to a range of temperatures close to and above the ambient temperature the sediments would normally be exposed to.

4.2.3 Sample collection and preparation for denitrification and anammox measurements in the water column

Rates and temperature characteristics of denitrification and anammox in the water column of the OMZ were also measured. Specific depths within the water column were determined based on oxygen concentrations and water density (Figure 4.2) following previous studies (De Brabandere et al., 2014). Briefly, we focused on the top of the OMZ where oxygen was almost at a minimum concentration and the density reached a peak, with a second depth selected within the core of the OMZ. Water was dispersed directly from the Niskin bottles into 1 L serum

bottles, which were overflowed 3 times to minimise contact with the atmosphere and oxygen contamination. This was then vigorously bubbled with helium for 20 minutes (BOC) to minimise oxygen concentration and transferred into 12 mL gas tight vials (Exetainer, Labco), overflowed 3 times and sealed with no headspace. The filled glass vials were then pre-incubated at the desired temperature for 20 minutes to ensure the water was at this temperature before beginning the experiment. To begin the experiment each vial was injected through the septa with 50 µL of degassed, concentrated stock of $Na^{15}NO_2^-$ (2.4 mM, 98 ^{15}N atom %, Sigma Aldrich and helium (BOC)), to a final concentration in the vial of 10 µM. To stop microbial activity, samples were injected with 50 µL ZnCl (50% w/v, Sigma Aldrich). Sampling sites, depths, incubation temperatures and time points can be seen in Table 4.1.

Table 4.1. Description of sampling sites and experimental design for water column anammox and denitrification activity.

Site	Sea bed depth (m)	Latitude (ºN)	Longitude (ºW)	Sampling depth (m)	Incubation Temperature (ºC)	Time points (h)
1	121	1331.02	9121.62	85, 105	6, 12, 15, 20, 24	0, 3, 6, 12, 24, 48
2	179	1326.31	9122.5	135, 140, 155,		
3	382	1324.68	9122.69	125, 135, 195		
4	1504	1318.68	9123.73	120, 185, 225		
5	503	1316.3	9108.06	90, 205, 220		

4.2.4 Gas sample analysis for denitrification and anammox activity

Gas samples from the headspace (100 µL) were measured for N_2O concentration using a gas chromatograph fitted with a micro-electron capture detector (GC/µECD, Agilent Technologies UK Ltd., South Queensferry, U.K.; (Nicholls et al., 2007). N_2O concentrations were calculated from peak areas using a known standard concentration (Scientific and Technical Gases) and

the total amount in the vial (headspace and slurry) was corrected for temperature, pressure and solubility (Weiss & Price, 1980; Yamamoto et al., 1976).Water samples were headspaced just before gas analysis by introducing 2 mL He (analytical-grade helium) using a two-way valve and a gastight syringe (Hamilton). The total concentration of the gas measured was corrected in the same way as in the sediment samples.

Production of ^{15}N-labelled N_2O was measured using a gas-chromatograph isotope ratio mass spectrometer (GC/IRMS) coupled to a modified pre-concentration unit (Precon). 100 µL was sub-sampled from the headspace of the original samples using a gas tight syringe (Hamilton) into air filled gas tight vials (12 mL) and processed following the method previously descried in detail in Trimmer et al, (2006). Briefly, cryo-focusing removes most of the CO_2 and allows separation of N_2O from the CO_2. The GC/IRMS then analyses the specific mass to charge ratios of $^{44}N_2O$, $^{45}N_2O$ and $^{46}N_2O$ (Mander & Zaman, 2015; Trimmer et al., 2006). The concentration of N_2O measured by the GC was multiplied by the contribution of ^{15}N-N_2O measured by the IRMS to obtain the concentration of labelled ^{15}N-N_2O.

Isotopic analysis of the production of N_2 ($^{28}N_2$, $^{29}N_2$ and $^{30}N_2$) gas from the vials was measured from the headspace by continuous-flow isotope ratio mass spectrometry (Thermo-Finnigan, Delta Matt Plus) as previously described in Trimmer and Nicholls (2009). For the sediment samples (or slurries), once all gas measurements were completed, the vials were opened and sediments were dried to a constant weight at 80^{o}C. Gas production rates were corrected for dry mass (Lansdown et al., 2012; Shelley et al., 2014).

4.2.5 Calculations for denitrification and anammox

The mole fraction of the added substrate (98% ^{15}N atom) and measured excess N_2 production are used to distinguish between production via denitrification and anammox (Bo Thamdrup &

Dalsgaard, 2002). With the addition of ^{15}N, denitrification has the ability to produce $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ via random isotope pairing, calculated using the following equations:

$$D_{tot} = P_{30} \times F_N^{-2}$$

$$D_{30} = D_{tot} \times F_N^2$$

$$D_{28} = D_{tot} \times (1-F_N)^2$$

$$D_{29} = D_{tot} \times 2 \times 1 \times (1-F_N) \times F_N$$

D_x denotes the production of N_2 via denitrification. P_{30} represents the total N_2 produced in the vial measured by mass-spectrometry. F_N represents the ^{15}N mole fraction of the source compound ($Na^{15}NO_3^-$, or $Na^{15}NO_2^-$, 98% ^{15}N).

Anammox can only produce $^{28}N_2$ and $^{29}N_2$ assuming one molecule is obtained from $Na^{15}NO_x$ added to the samples and the other from $^{14}NH_4^+$ in the slurries (Dalsgaard et al., 2012). The following equations are required to determine the production via anammox:

$$A_{29} = P_{29} - D_{29}$$

$$A_{28} = A_{29} \times F_N^{-1} \times (1-F_N)$$

$$A_{tot} = A_{29} + A_{30}$$

$$RA = A_{tot} / (D_{tot} + A_{tot})$$

Where A_x denotes the production of N_2 via anammox. RA denotes that relative contribution of anammox to the total production of N_2 gas produced and can be multiplied by 100 to obtain anammox contribution as a percent (*ra* %).

Following the calculations above, rates of excess production of ^{15}N-N_2 were calculated as the slope of production P_{30} (linear regression) against time of the incubations. These equations

were used to calculate both sediment and water column production of N_2 gas. The only difference was the source compound: for sediment, both $^{15}NO_3^-$ and $^{15}NO_2^-$ were used, whereas only $^{15}NO_2^-$ was used for the water column incubations.

4.2.6 Sample collection and preparation for nitrogen fixation

Given that the availability of fixed N in any ecosystem is ultimately governed by the net balance between N fixation and N_2 gas production, the temperature dependency of nitrogen fixation in the water column was also investigated. Characterisation of the water column through nutrient analysis aided site selection, though site selection was more importantly based on being close to the coast to where more organic matter would be available for microbial processes. Water samples for nutrient analysis were collected using CTD rosette and 20 L Niskin bottles as above and were analysed using a segmented- flow auto analyser (Skalar) and standard colourimetric techniques (SKLAR, San $^{++}$ System, Flow Access software 1.2.5) for NO_3^-, NO_2^-, NH_4^+ and PO_4-. Surface water samples (10m) were collected at dusk using the CTD rosette and 20 L Niskin bottles as above. Water was collected at this depth, top of the euphotic zone, where concentrations of nitrate and phosphate were at a minimum, as shown by the water column characterisation (Figure 4.3).

Water from the Niskin bottles was directly transferred into 30 mL serum bottles, which were overflowed 3 times and left with 1 mL headspace. At the same time, a plankton net was hauled through the top 20 m to collect a concentrated sample of plankton (~2000 times concentrated). 1 mL of the concentrated plankton was added to each 30 mL serum bottle before sealing with a butyl stopper. As the low nitrate and phosphate upper waters of the OMZ have low abundances of planktonic nitrogen fixing organisms, adding the concentrated sea water allowed us to increase the number of nitrogen fixing organisms in the sample to better determine the temperature dependency of the process. Once sealed, the serum bottles were placed at the

incubation temperatures for 30 minutes to ensure they reached temperature before the experiment began. The 30 minute temperature adaptation period was based on preliminary test to ensure adequate time for 30 mL water to reach the experimental temperature. To begin the experiment, 0.5 mL of 20 nmol $^{15}N_2$ gas was injected through the septa into each serum bottle. The $^{15}N_2$ was produced from pure cultures of denitrifying bacteria. The bacteria were grown in a growth medium with $Na^{15}NO_3^-$ (98 ^{15}N atom %, Sigma Aldrich) as substrate which then produced $^{15}N_2$ gas through denitrification (Details of pure culture bacterial growth can be seen in chapter 2). Samples were incubated in the dark, at 5 temperatures (15, 20, 24, 30, 35^{o}C), which straddled ambient surface water temperature (24^{o}C) and were sacrificed over a time series (maximum 18 hours). There were 2 replicates at each temperature and the process was carried out at 4 different sites. Samples were harvested by filtration on to (GF-F, Whatman) using a filtration tower and vacuum pump. Filter papers were placed into individual cryo-vials, immediately frozen in liquid nitrogen to ensure microbial activity was quickly stopped and transferred to -80^{o}C till processing back in the on-land laboratory.

4.2.7 Sample analysis for Nitrogen fixation

On return to the UK, filter papers were removed from the freezer (-80^{o}C) and dried for 24 hours at 80^{o}C. Eight discs were cut out from each filter, placed into ultra-clean tin capsules (Elemental Microanalysis, UK) and run through an elemental analyser (Flash EA 1112, Thermo-finnigan), coupled to a continuous flow isotope ratio mass spectrometer (CF/IRMS; Finnigan MAT $Delta_{Plus}$, Thermo-Finnigan). The amount of $^{15}N_2$ incorporated into the biomass of the nitrogen fixing organisms on the filter paper was calculated using standard reference material (Caesin, Elemental Microanalysis Ltd, Devon, UK). To calculate excess nitrogen fixation, background (natural abundance) values were subtracted from samples incubated with

^{15}N. We obtained natural abundance values from filtering control water samples with no addition of $^{15}N_2$ that were treated in the same way as the $^{15}N_2$ addition samples.

4.2.8 Deriving apparent Activation and Deactivation Energies

In order to calculate the temperature dependency of each process, natural Log transformed rates of each process was plotted against standardised incubation temperatures ($1/kT$, where k is the Boltzmann's Constant ($8.62x10^{-5}$ eV K^{-1} (T)) and T is the absolute temperature in Kelvin) on an Arrhenius plot. The temperature was centred (1/kT-1/kTc, where 1/kTc is the average 1/kT for the thermal range included), and the negative slope of the regression line gives an estimate of the apparent activation energy in electron volts (eV) where 1eV is equivalent to 96.49 kJ mol^{-1}. Plotting in this manner centres the inverse temperature around zero (Perkins et al., 2012). This calculated 'apparent' activation energy is used as an empirical index of temperature response of each process as this will always be lower than the theoretical sensitivity of biogeochemical reactions to temperature. This is because other environmental factors come into play such as connectivity to other microbial processes.

4.2.9 Statistical analysis

To determine whether there were any significant differences between N_2 and N_2O production rates with either NO_3^- or NO_2^- additions to sediments, paired t-tests (two tailed) were performed and the P-values are reported. To test for a significant effect of temperature on $^{15}N_2$ production and $^{15}N_2$ fixation, we used linear regression analysis. For all processes, site was fitted as a random effect in a mixed effect model to determine an overall population estimate for the temperature characteristics.

4.3 Results

4.3.1 Oxygen and density water profiles

At all 5 sites, high-resolution (~10 m) gas and nutrient vertical profiles were constructed from the water column. Figure 4.2 shows the profiles of oxygen and density with depth for each of the 5 sites, which were used to characterise the water column and determine exact sampling areas for further analysis.

4.3.2 Temperature characteristic of potential rates of denitrification and anammox in sediments

With the addition of $^{15}NO_3^-$, sediment slurries showed almost no response in production of N_2 via denitrification with increasing in temperature (Figure 4.5A). This response is marginal with an apparent activation energy of only 0.014 eV (Table 4.2) and was non-significant ($p > 0.05$, Table 4.3). However, when the average rate of N_2 production via denitrification is calculated with $^{15}NO_3^-$, the maximum rate was measured at 6^{o}C, with a rate of 4.25 (± 0.42) nmol g^{-1} h^{-1}, and the minimum rate observed at 12^{o}C (3.45 ± 0.46 nmol g^{-1} h^{-1}).

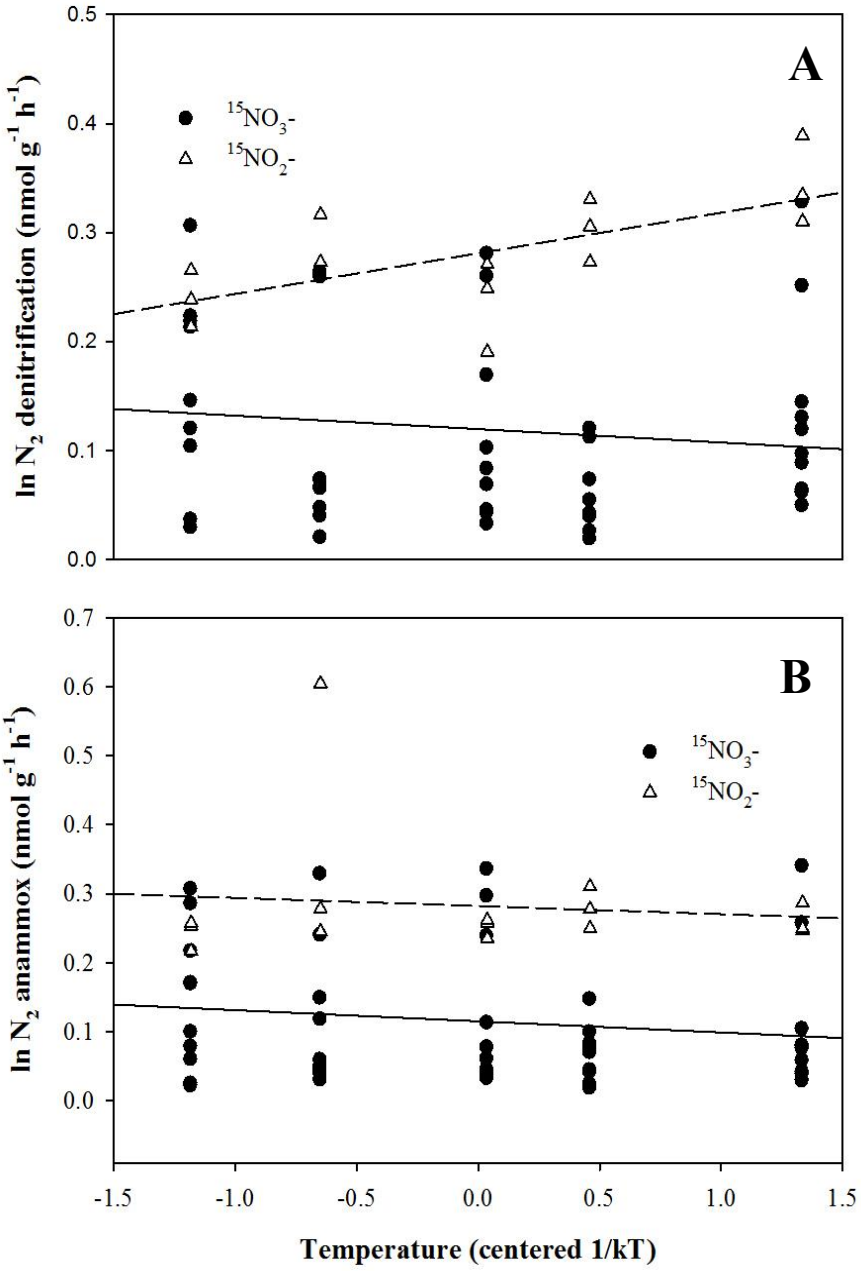

Figure 4.5. Arrhenius plots for the thermal sensitivity of natural log N_2 production rates from sediments via denitrification (A) and anammox (B) from both $^{15}NO_3^-$ and $^{15}NO_2^-$ addition experiments at all sites against incubation temperature (corrected 1/kT, where K = Boltzmann's constant and T is in Kelvin). Temperature increases from right to left.

Incubations with the addition of $^{15}NO_2^-$ showed a stronger, though negative, thermal response, with a significant decrease in N_2 production through denitrification with increasing temperature ($p < 0.05$, Table 4.3). Though, again, the actual thermal response was only marginal, with an activation energy of -0.04 eV (Table 4.2). The maximum rate of N_2 production was observed at 12°C with 7.36 (± 1.82) nmol g^{-1} h^{-1}. Overall production of N_2 through denitrification was significantly greater in the sediment slurry incubations with $^{15}NO_2^-$ additions ($p < 0.001$, Table 4.4). Based on the average across the entire temperature range, production rates of N_2 through denitrification were 40% greater from incubations with $^{15}NO_2^-$ than $^{15}NO_3^-$.

The production of N_2 through anammox increased marginally for both $^{15}NO_3^-$ and $^{15}NO_2^-$ with increasing temperature (Figure 4.5B); however, the apparent activation energy was low for both treatments. With the $^{15}NO_3^-$, the activation energy was 0.02 eV and with $^{15}NO_2^-$ the activation energy was even lower at 0.008 eV (Table 4.2). Overall, there was no significant effect of temperature with either $^{15}NO_3^-$ or $^{15}NO_2^-$ for the production of N_2 through anammox ($p > 0.05$, Table 4.3). These results suggest anammox had a very weak thermal response in these type of sediments. With $^{15}NO_3^-$, the maximum average rate of N_2 production via anammox was measured at 15°C and was 4.19 (± 0.42) nmol g^{-1} d^{-1}. Incubations with $^{15}NO_2^-$ had much greater average rates of N_2 production through anammox of 8.99 (± 2.43) nmol g^{-1} d^{-1} at 12°C. Average production rates of N_2 through anammox were 70% greater with $^{15}NO_2^-$ than with $^{15}NO_3^-$ incubations. Significantly greater production of N_2 through anammox was determined for incubations with $^{15}NO_2^-$ additions than sediments incubated with $^{15}NO_3^-$ ($p < 0.001$, Table 4.4). These results suggest a preference of nitrite over nitrate as a nitrogen source.

Anammox had significantly greater overall contribution to N_2 production with NO_2^- than NO_3^- ($p < 0.001$, Table 4.4). In sediments with $^{15}NO_3^-$, the contribution of anammox to N_2 production reached a maximum at 15°C, with 55.72% (± 2.07), and sediments incubated with $^{15}NO_2^-$ had a maximum average contribution of anammox to N_2 production at 24°C, with 70.49% (± 1.15)

(Figure 4.6). The average contribution to N_2 production from nitrite across the thermal gradient was 65.72% (± 0.93) and for nitrate, 55.39% (± 0.76). No significant temperature effect was observed with either treatment ($p > 0.05$, Table 4.4).

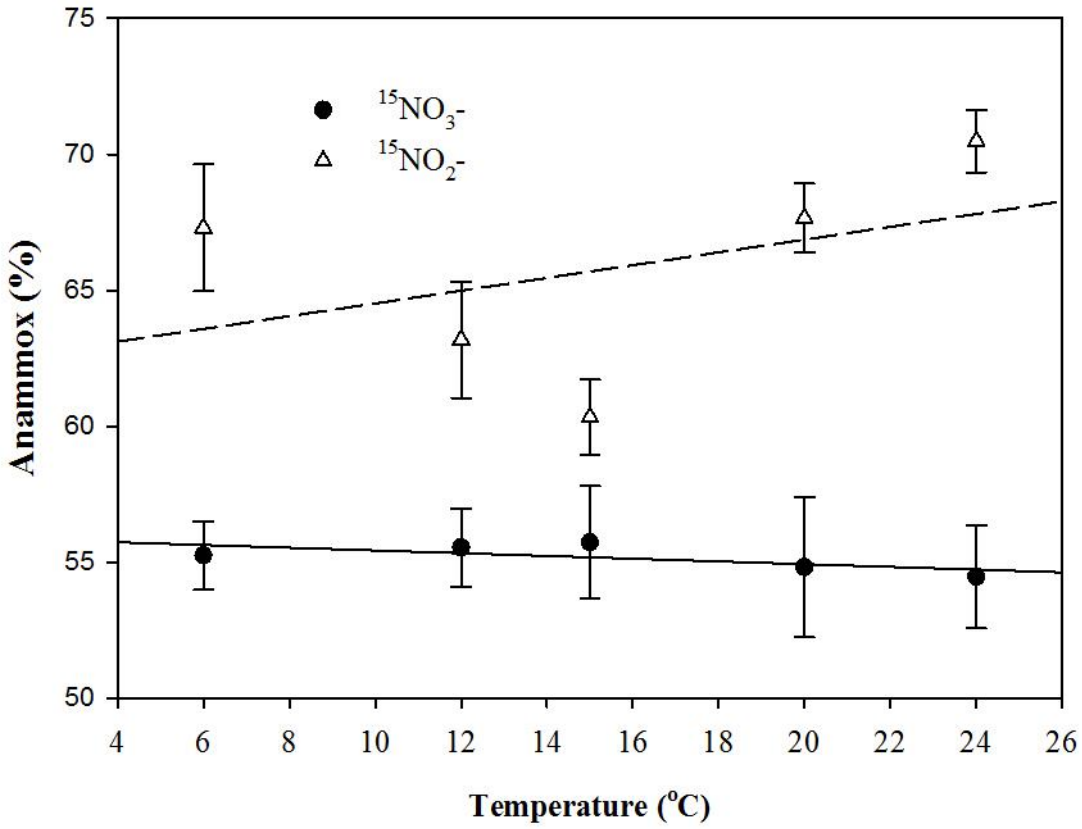

Figure 4.6. Average percentage contribution of anammox (± *se*, *n* = 18) to total N_2 production from both $^{15}NO_3^-$ and $^{15}NO_2^-$ addition experiments against incubation temperature (^{o}C) from sediments.

The temperature sensitivity of N_2O production from the sediment slurries was also calculated for $^{15}NO_3^-$ and $^{15}NO_2^-$ incubations (Figure 4.7). A significant decrease of N_2O production rates with exposure to increasing temperatures was observed with additions of $^{15}NO_3^-$ ($p < 0.002$, Table 4.4). The mean maximum production rate for N_2O was 44.92 (± 15.64) nmol g^{-1} h^{-1}, at 6^{o}C. Conversely, sediments incubated with $^{15}NO_2^-$ showed a non-significant response to

temperature for N_2O production rates ($p > 0.05$, Table 4.3), with a maximum of 80.35 (± 20.09) nmol g^{-1} h^{-1} observed at 20°C. A significant difference was observed in the rates of N_2O production between the two treatments ($p < 0.001$, Table 4.4), with greater production of N_2O occurring with $^{15}NO_2^-$ addition. Activation and deactivation energies suggest a similar response for N_2O production rates as N_2 production (Table 4.2). With increasing temperature, $^{15}NO_2^-$ additions showed an increase in the rate of production of N_2O, whereas additions of $^{15}NO_3^-$ showed a decrease in activity.

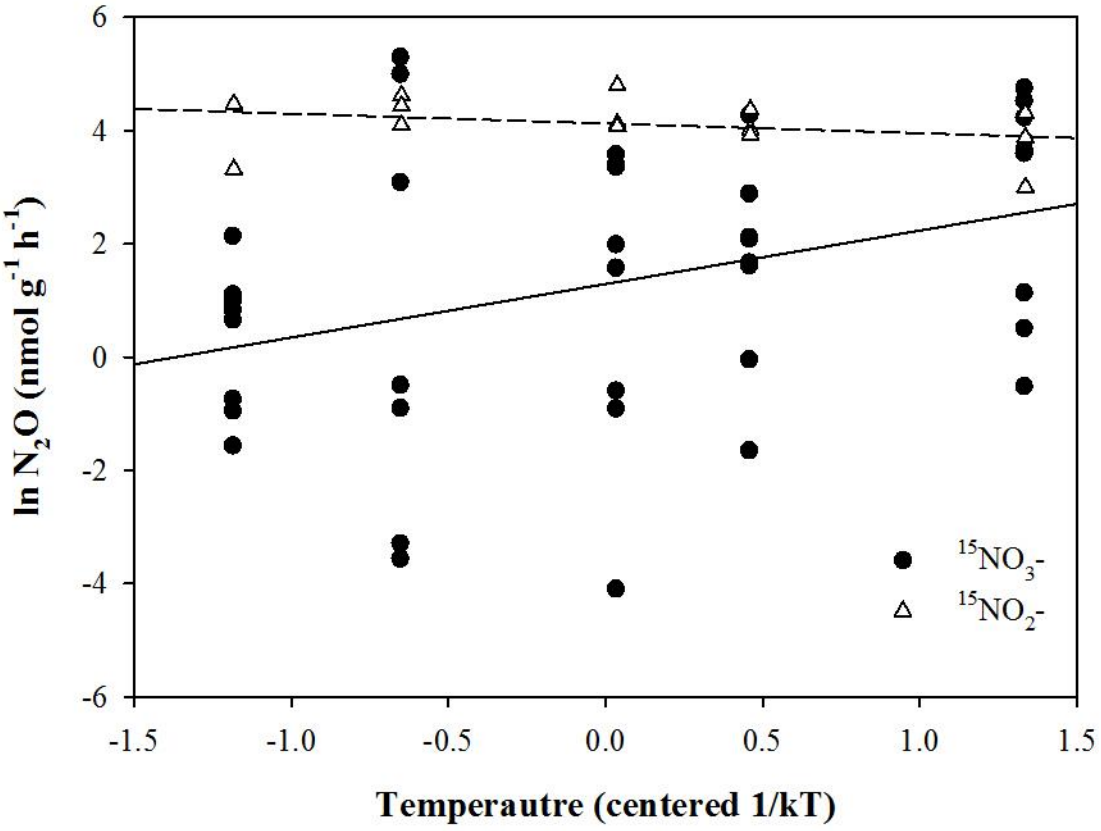

Figure 4.7 Arrhenius plot of log transformed N_2O production rates for $^{15}NO_3^-$ and $^{15}NO_2^-$ addition experiments against incubation temperature (corrected 1/kT, where K = Boltzmann's constant and T is in Kelvin) from sediments. Temperature increases from right to left.

Figure 4.8 shows the effect of temperature on the ratio of log N_2O to log N_2 production for both $^{15}NO_2^-$ and $^{15}NO_3^-$ additions. There was a significant decrease in the ratio with increasing temperature with $^{15}NO_3^-$ ($p < 0.003$), whilst, with $^{15}NO_2^-$, there was a significant decrease ($p < 0.03$, Table 4.3), with significantly greater ratios from the $^{15}NO_2^-$ addition experiment when comparing the intercepts at the mean temperature ($p < 0.001$, Table 4.4). Calculated activation energies of the ratios are 0.45 eV and -0.54 eV for $^{15}NO_2^-$ and $^{15}NO_3^-$ incubations, respectively (Table 4.2). The maximum average ratio for the $^{15}NO_3^-$ incubations was 5.7 (± 2.06) at 6°C, and for $^{15}NO_2^-$ incubations, the maximum average ratio was 10.6 (± 2.58) at 24°C.

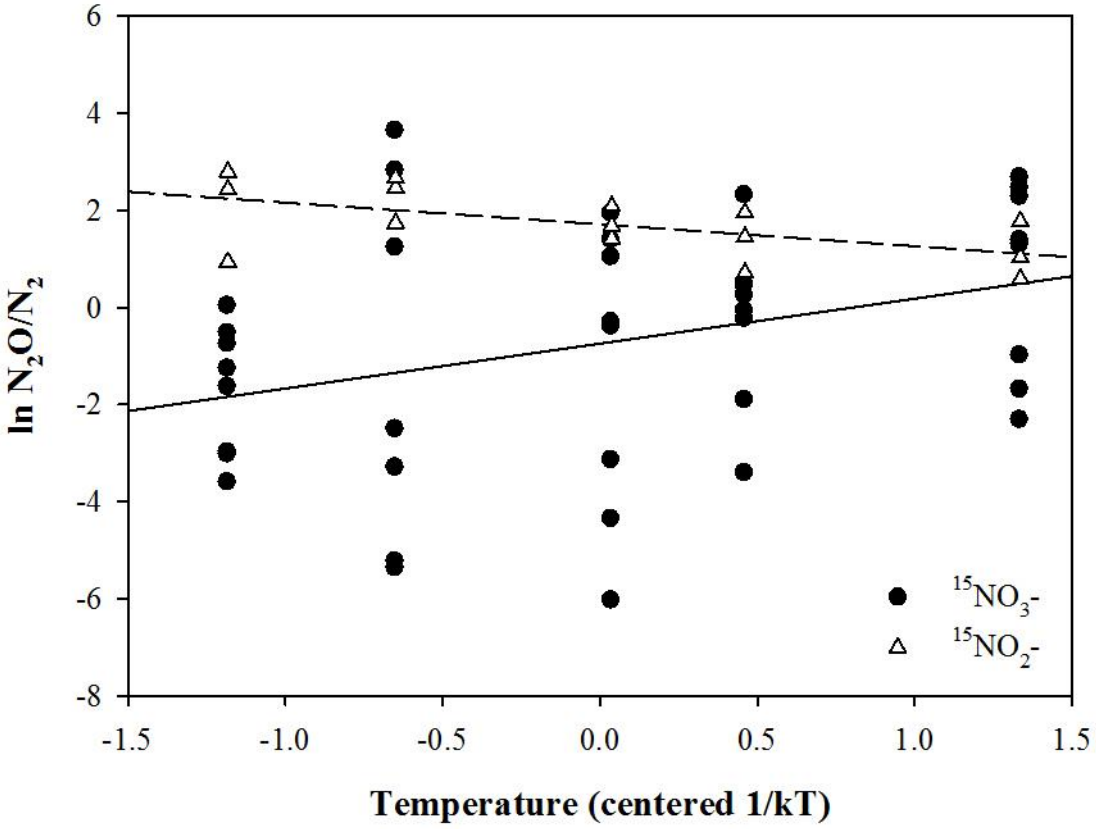

Figure 4.8 Arrhenius plot of the ratio of natural log N_2O to N_2 production rates for $^{15}NO_3^-$ and $^{15}NO_2^-$ addition experiments against incubation temperature (corrected 1/kT, where K = Boltzmann's constant and T is in Kelvin) from sediments. Temperature increases from right to left.

Table 4.2 Summary of apparent activation energy (Ea) in electron volts (eV) for sediment incubations (nmol g^{-1} h^{-1} $(1/kT)^{-1}$) and r^2 of the regression. A minus sign (-) indicates a negative response to temperature.

Treatment	Variable	Process	Ea (eV)	r^2
$^{15}NO_3^-$	ln N_2	Denitrification	0.014	0.02
$^{15}NO_3^-$	ln N_2	Anammox	0.02	0.02
$^{15}NO_3^-$	ln N_2O	Total	-0.63	0.05
$^{15}NO_3^-$	ln N_2O/ N_2	Total	-0.54	0.05
$^{15}NO_2^-$	ln N_2	Denitrification	-0.04	0.43
$^{15}NO_2^-$	ln N_2	Anammox	-0.008	0.01
$^{15}NO_2^-$	ln N_2O	Total	0.17	0.11
$^{15}NO_2^-$	ln N_2O/ N_2	Total	0.45	0.34

Table 4.3 Response of the rate of activity to temperature for N_2 production through anammox and denitrification, total N_2O production, and the ratio of N_2O to N_2 from $^{15}NO_3^-$ and $^{15}NO_2^-$ sediment slurry incubations.

Test	Treatment	Explanatory variable	Dependent variable	Random effect	*df*	X^2	*p*
Linear mixed effects model	$^{15}NO_3^-$	Temperature	ln $^{15}N_2$ production denitrification	site depth	1	1.07	> 0.05
Linear mixed effects model	$^{15}NO_3^-$	Temperature	ln $^{15}N_2$ production anammox	site depth	1	1.46	> 0.05
Linear mixed effects model	$^{15}NO_3^-$	Temperature	ln N_2O production	site depth	1	9.87	< 0.002
Linear mixed effects model	$^{15}NO_3^-$	Temperature	ln N_2O/ N_2	site depth	1	9.18	< 0.003
Linear mixed effects model	$^{15}NO_2^-$	Temperature	$^{15}N_2$ production denitrification	site depth	11	5.02	< 0.05
Linear mixed effects model	$^{15}NO_2^-$	Temperature	$^{15}N_2$ production anammox	site depth	11	0.36	> 0.05
Linear mixed effects model	$^{15}NO_2^-$	Temperature	N_2O production	site depth	11	1.54	> 0.05
Linear mixed effects model	$^{15}NO_2^-$	Temperature	ln N_2O/ N_2	site depth	11	6.62	<0.03

Table 4.4 Summary of statistical significance between $^{15}NO_3^-$ and $^{15}NO_2^-$ addition on rates of response variables (nmol g^{-1} h^{-1}) for sediment potential of denitrification and anammox.

Test	Response variable	*df*	*T*	*p*
Mann Whitney; Rank Sum	ln N_2 denitrification	62	673	< 0.001
Mann Whitney; Rank Sum	ln N_2 anammox	62	681	< 0.001
Mann Whitney; Rank Sum	Total ln N_2O	62	621	< 0.001
Mann Whitney; Rank Sum	Total ln N_2O/ ln N_2	62	596	< 0.001

4.3.3 Temperature characteristic of potential rates of denitrification and anammox in the water column

Water column incubations with the addition of $^{15}NO_2^-$ showed very little response to temperature for N_2 production through either denitrification or anammox (Figure 4.9), with corresponding activation energies of 0.003 eV and 0.0004 eV, respectively (Table 4.5). The non-transformed maximum average rate of N_2 production via denitrification was 0.93 (± 0.47) nmol L^{-1} d^{-1} at 24°C. The maximum average rate of N_2 production via anammox was 0.69 (± 0.23) nmol L^{-1} d^{-1} at 24°C. No significant effect of temperature was observed for N_2 production rates via either denitrification or anammox ($p > 0.05$, Table 4.6), with no significant difference observed between the contribution of N_2 production via denitrification and anammox ($p > 0.05$).

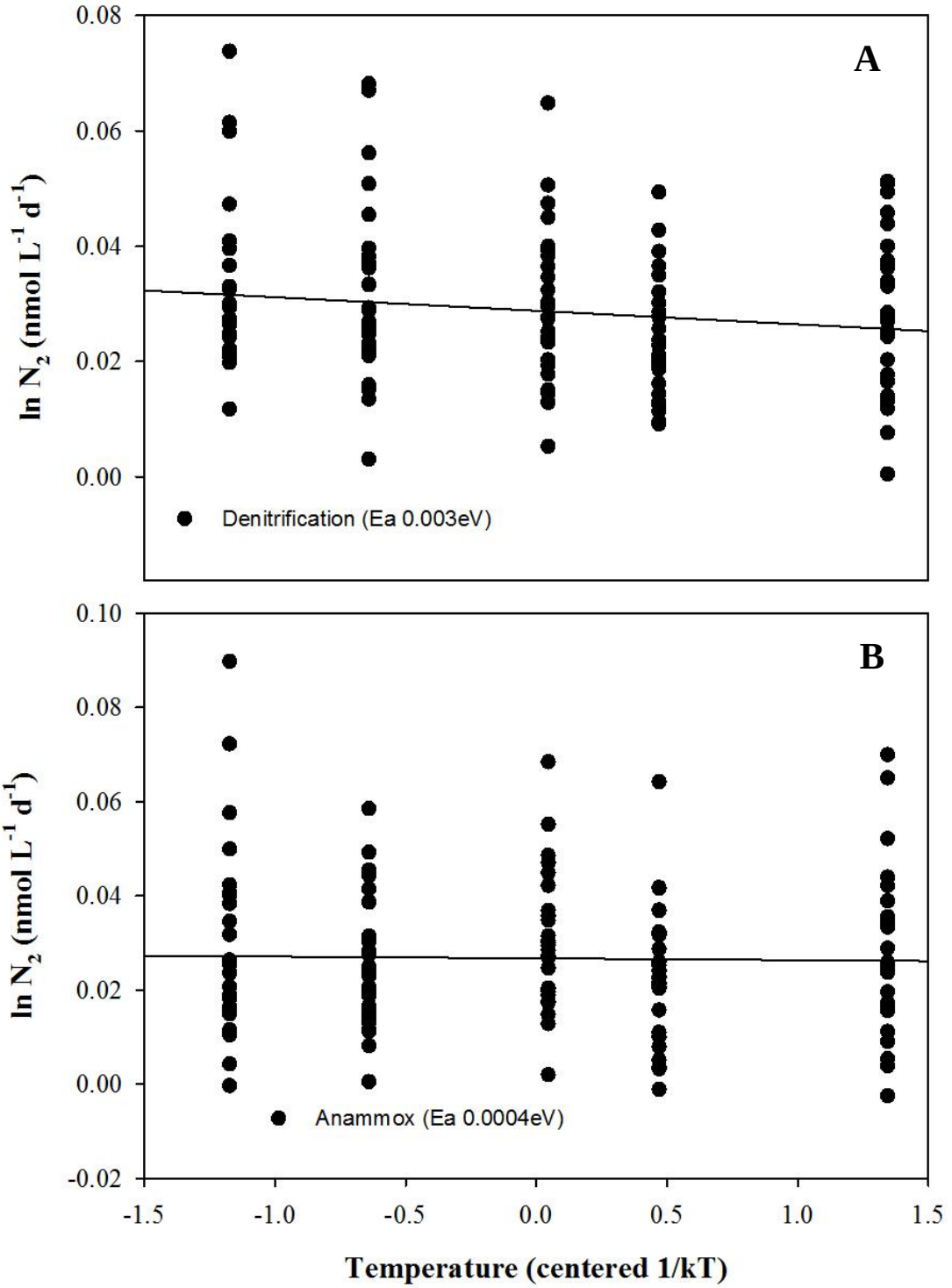

Figure 4.9 Arrhenius plots of natural log N_2 production rates through denitrification (A) and anammox (B) from water column incubations against incubation temperature (corrected 1/kT, where K = Boltzmann's constant and T is in Kelvin). Temperature increases from right to left.

The average contribution in the water column of anammox to total N_2 production shows very little, non-significant ($p > 0.05$) response to increasing temperature (Figure 4.10). The average contribution of anammox over the incubation temperatures was 51.09% (± 1.67).

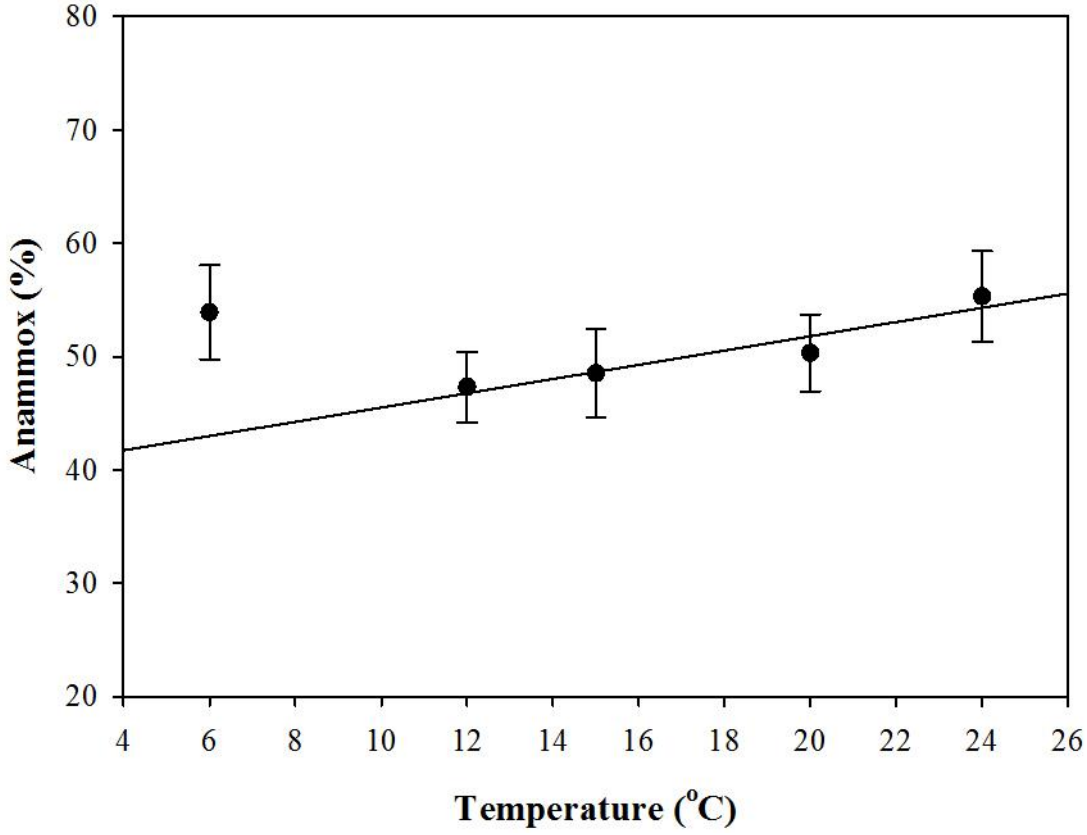

Figure 4.10. Average contribution of anammox (± *se*, *n* = 27) to total N_2 production from water column experiments over incubation temperatures.

Table 4.5 Summary of apparent activation energy (Ea) in electron volts (eV) for water column incubations (nmol L^{-1} d^{-1} $(1/kT)^{-1}$) and r^2 of the regression line for water column capacity for denitrification and anammox.

Variable	Ea (eV)	r^2
ln N_2 denitrification	0.003	0.03
ln N_2 anammox	0.002	0.005
Anammox % contribution	0.7	0.001

Table 4.6 Statistical analysis for the response in rates of N_2 production to temperature via denitrification and anammox (nmol L^{-1} d^{-1}) and the contribution of anammox to N_2 production rates in water column incubations. Linear mixed effect models, with temperature as the explanatory variable and random effect as water column depth (factor).

Dependent variable	*df*	X^2	*p*
ln N_2 Denitrification	1	3.08	> 0.05
ln N_2 Anammox	1	0.08	> 0.05
Anammox % contribution	1	0.98	> 0.05

4.3.4 Water column nitrogen fixation

Rates of nitrogen fixation, measured as the amount of $^{15}N_2$ incorporated into organic biomass showed an overall decrease with increasing temperature between a range of 15-35°C, though this was non-significant ($p > 0.05$, Figure 4.11A). No significant effect of site was observed, therefore rates from each site were averaged together by temperature ($p > 0.05$, Figure 4.11B),

and we observed an increase in $^{15}N_2$ fixation rates from 0.61 (± 0.16) ng L^{-1} h^{-1} at 15°C to 1.1 (± 0.2) ng L^{-1} h^{-1} at 20°C. This is followed by a decrease back down to 0.42 (± 0.11) ng L^{-1} h^{-1} at 24°C where it appears to plateau. Nitrogen fixation is significantly greater at 20°C than all other temperatures, with no significant differences observed between the other measured temperatures (Table 4.7). Due to the data not being suitable for a linear regression, no activation energy could be calculated, simply a peak of activity at 20°C. This indicates a potential optimal temperature of nitrogen fixation at 20°C, whereas on either side of this temperature rates of nitrogen fixation are significantly lower.

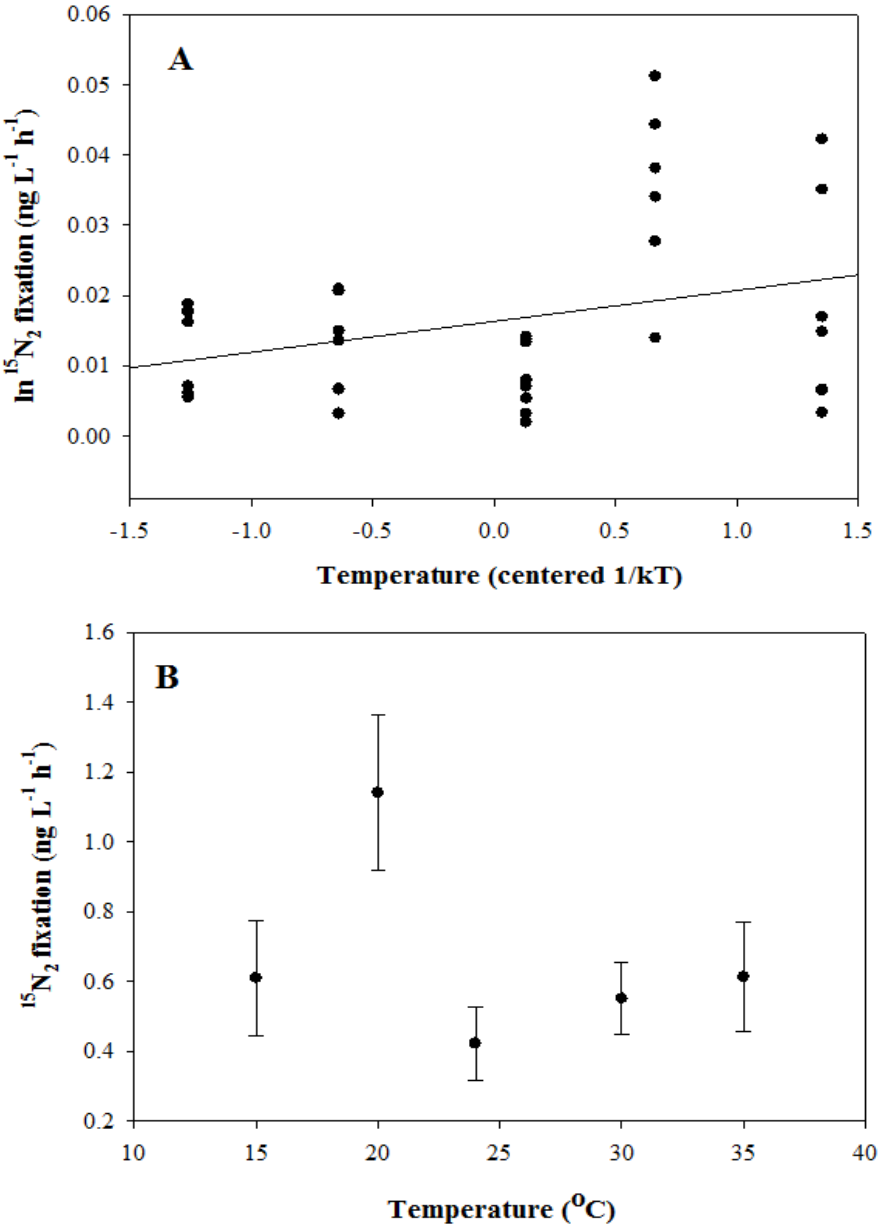

Figure 4.11 Arrhenius plot for rates of nitrogen fixation (as natural log $^{15}N_2$ incorporation) against incubation temperatures (corrected 1/kT, where K = Boltzmann's constant and T is in Kelvin) (A) and average rates of nitrogen fixation (± *se*, *n* = 8) (B) as $^{15}N_2$ incorporated into organic biomass (ng L^{-1} h^{-1}) of nitrogen fixing organisms against incubation temperature (^{o}C).

Table 4.7 Statistically significant results of nitrogen fixation rates between temperatures (One-way ANOVA followed by Holm-Sidak method pairwise comparison).

Temperature (°C)	*t*	*df*	*p*
20 vs15	3.09	34	< 0.05
20 vs 24	4.98	34	<0.001
20 vs 30	4.10	34	< 0.005
20 vs 35	4.04	34	< 0.005

4.4 Discussion

Rates of anammox and denitrification were measured in sediments and water column within the ETNP OMZ. Sediment rates of anammox and denitrification did not show strong thermal responses, with greatest rates at temperatures closest to the ambient temperature, suggesting narrow thermal optima for both processes. However, we did observe a difference in rates with different N substrates, with greater rates of denitrification with additions of NO_3^- and anammox rates were greatest with additions of NO_2^-. Water column rates of N_2 fixation had no significant thermal response, as did rates of nitrification.

4.4.1 Temperature characteristic of potential rates of denitrification

Our average rates of sediment N_2 production through denitrification (3.75 ± 0.26 nmol g^{-1} h^{-1} $^{15}NO_3^-$ additions and $^{15}NO_2^-$ additions 5.28 ± 0.66 nmol g^{-1} h^{-1}) are comparable to those found in other permanently cold sediments (Canion et al., 2014a; Dalsgaard & Thamdrup, 2002; Rysgaard et al., 2004). However, sediment N_2 production rates had marginal thermal responses from denitrification confirmed by their low activation energies. This limited thermal response was observed with both $^{15}NO_3^-$ and $^{15}NO_2^-$ additions. We measured optimum temperatures for maximum N_2 production through sediment denitrification at 6°C and 15°C with $^{15}NO_3^-$ and $^{15}NO_2^-$ additions respectively. This is lower than optimal temperatures in previous publications,

where the optimal temperature for denitrification ranges from 20-27°C in marine sediments, and is activity still occurring at 37°C (Brin et al, 2014; Canion et al., 2014a; Rysgaard et al., 2004). In slurry incubations from near shore Arctic sediments, denitrification had an optimal temperature of 25-27°C (Canion et al., 2014a), however, they determined organic substrate additions (acetate and lactate) to be a more dominant control for rates of denitrification than temperature alone. In marine Arctic sediments, with an average annual temperature of -1.7 to 4°C, a strong thermal response was calculated for denitrification rather than anammox, with an activation energy of 0.63 eV (Rysgaard et al., 2004). Temperate estuarine and shelf sediments have also shown denitrification to be positively correlated to temperature (Brin et al., 2014). Brin et al (2014) confirmed that the major drivers of denitrification activity in the estuarine and shelf sediments off the coast of New England were temperature and O_2 consumption. These sediments were exposed to natural temperature fluctuations between 3-24°C over a year and therefore the denitrifiers were also exposed to this range. In comparison, the sediments used in this study are from a permanently cold site, therefore are adapted to cold and not fluctuating temperatures. Another key factor for the limited thermal response in this study could be the availability of organic matter, a limiting factor for microbial growth, which has been observed in previous studies (Brin et al., 2014; Mark Trimmer & Nicholls, 2009). *In-situ* rates of denitrification were measured at the same sites as Brin et al (2014) with no thermal response, with reactivity limited by substrate availability (Heiss et al., 2012). Whilst our slurry incubations had concentrations of NO_3^- and NO_2^- above limiting values, other substrates, such as organic carbon, may have limited their potential thermal response. The only way to fully explore the thermal capabilities of such processes is to remove all limiting factors (Brin et al., 2014).

A decrease in production rates of N_2O through denitrification was observed with increasing temperature, with a maximum rate of 44.92 nmol g^{-1} h^{-1} at the lowest temperature (6°C), which

is closest to the average ambient temperature of 4°C. This thermal response was only observed with $^{15}NO_3^-$ incubations. Rates of N_2O production with $^{15}NO_2^-$ addition showed no thermal response but were significantly greater with a maximum rate double than that produced in $^{15}NO_3^-$ incubations (80.35 ± 20.09 nmol g^{-1} h^{-1}). Decreases of net N_2O production with increasing temperature have been observed in other systems, mainly soils (Holtan-Hartwig et al., 2002), potentially due to the enzymes involved with N_2O production and reduction. Studies of soils have suggested the enzymes involved in N_2O reduction had depleted activity at lower temperatures, having a greater optimal temperature than the enzymes responsible for N_2O production, resulting in a net flux of N_2O (Holtan-Hartwig et al., 2002). N_2O production rates were negligible above background levels in the water column incubations. The literature suggests that N_2O levels within the OMZ are supersaturated so the production from within our samples may have been swamped by background levels (Cohen & Gordon, 1978; Pierotti & Rasmussen, 1980). Furthermore, our rates of N_2 production were very low, so N_2O may have just been below the limit of detection.

The ratio of N_2O/ N_2 allows us to consider the efficiency of denitrification, as increased N_2O release means less N_2O has been reduced within the microbial cells and therefore potential energy is lost. The substrate additions had differing effects on the ratio; additions of $^{15}NO_3^-$ showed a significant decrease in the ratio with increasing temperature. This means as temperature increased, the number of molecules of N_2O produced, per molecule of N_2, decreased. The opposite was observed with $^{15}NO_2^-$ additions, where the ratio significantly increased with increasing temperature, so more molecules of N_2O were produced for every molecule of N_2 with warming. This is an interesting finding when compared to the N_2 production rates which suggest these organisms have a preference for NO_2^- even though this is potentially less energy efficient. The response of a decrease in the ratio of N_2O/ N_2 with

increasing temperatures has previously been measured but literature for aquatic systems is lacking with most studies having been carried out using soils (Avalakki et al., 1995; Bailey & Beauchamp, 1973; Keeney, Fillery, & Marx, 1979).

4.4.2 Temperature characteristic of potential rates of anammox

Anammox had optimum temperatures of 15°C and 12°C with additions of $^{15}NO_3^-$ and $^{15}NO_2^-$, respectively within sediment incubations. Though we found no significant thermal response of anammox activity, the optimum temperatures, where maximum rates of N_2 production were measured, are similar to those found in other sediment studies with optimum temperatures between 12-17°C (Canion et al., 2014a; Dalsgaard & Thamdrup, 2002; Rysgaard et al., 2004). Relatively few studies have examined the temperature response of anammox in sediments, but those studies have found anammox to have a thermal response in marine systems (e.g. Canion et al., 2014a; Canion et al., 2014b; Dalsgaard & Thamdrup, 2002; Rysgaard et al., 2004). However, there are exceptions in the literature: in estuarine and shelf sediments off the coast of New England, anammox had no thermal response (Brin et al., 2014), with organic matter and NO_3^- porewater concentrations being the main drivers of observed anammox activity. Trimmer & Nicholls (2009) found rates of anammox to be positively correlated to sediment organic carbon content as well as NO_3^- in the overlying water in estuaries along the east coast of the UK. Brin et al (2014) found that rates of anammox were positively correlated to rates of denitrification, limited by the rate of NO_2^- production via reduction of NO_3^- by denitrifying organisms. Our results of N_2 production through anammox were 70% greater with additions of NO_2^-, than when NO_3^- was the substrate, reducing its dependence on denitrification. This is further confirmed by the contribution of anammox to total N_2 production in our study. Anammox was slightly more dominant as a source of N_2 for both treatments and this contribution was constant across the temperature exposure with 55.39% and 65.75% with additions of $^{15}NO_3^-$ and $^{15}NO_2^-$ respectively. Published values for the contribution of anammox

to N_2 production can be as high as 80% in cold marine sediments incubated at *in-situ* temperatures (6°C) (Dalsgaard & Thamdrup, 2002). In contrast, temperate estuarine and shelf sediments have shown a dominance of denitrification with the contribution of anammox between 0 - 4% and 8 - 42% respectively, indicating that anammox was reliant on denitrification (Brin et al., 2014). The importance of NO_3^- concentration was also found in Chesapeake Bay estuarine sediments, with the greatest rates positively correlated with maximum NO_3^- concentrations (Rich et al., 2008).

Rates of N_2 production from the water column through anammox also showed no significant thermal response. Though an average maximum was observed 24°C with 0.69 nmol L^{-1} d^{-1}, they were much lower than rates measured from the sediments, suggesting sediments are more important sources of N_2 production. Our rates of anammox are closer to the lower end of previously published rates found in oxygen deficit waters (Dalsgaard et al., 2012; Thamdrup et al., 2006; Ward et al., 2009). Previously published rates of water column N_2 production through anammox found no correlation with concentrations of either NO_3^- or NO_2^- (Dalsgaard et al., 2012). The literature is limited in thermal responses of anammox in marine oxygen-depleted waters and so direct comparisons are difficult.

Anammox communities have been described as psychrophilic (Canion et al., 2014a; Canion et al., 2014b), which means that they have higher enzymatic activity at lower temperatures and therefore thrive at lower temperatures (Feller & Gerday, 2003). They are slow growing (Jetten et al., 1998) and therefore likely to respond more slowly to environmental changes (Dalsgaard et al., 2012). No dominance was observed in the relative contribution of either anammox or denitrification in the water column, with almost 50/50 contribution of the two processes to total N_2 production. Previously published data for the Eastern Tropical South Pacific OMZ, where rates of water column denitrification, though patchy, dominated and contributed to 72% of the N_2 production (Dalsgaard et al., 2012).

4.4.3 Water column nitrogen fixation

Measuring rates of nitrogen fixation are difficult in the open ocean as the spread of organisms are patchy and generally not in high concentrations (Zehr, 2011). Our method of concentrating the plankton sample before incubations was to encourage measurable rates. We collected the water at dusk to allow the nitrogen fixing organisms to generate maximum energy during the day to enable nitrogen fixation (diazotrophy) to occur during the night (Zehr, 2011). We also targeted areas with low concentrations of phosphate which could be an indication of nitrogen fixation (Bonnet et al., 2008), with the nitrogen fixing organisms requiring phosphate for protein and DNA synthesis (Hynes et al., 2009; Moutin et al., 2005) and low nitrate concentrations encouraging nitrogen fixation as new nitrogen is required in the system. However, we still measured rates much lower than those previously recorded and we did not measure iron which is important for the formation of nitrogenase enzyme required for N_2 fixation (Karl et al., 2002). In the South Pacific, rates of ~1-2 nmol L^{-1} d^{-1} have been recorded (Raimbault & Garcia, 2007) and rates between 0.9-2.9 nmol L^{-1} d^{-1} have been observed in the North Pacific upper mixed layers (4-14 m) (Dore et al., 2002). However, no measurable rates of N_2 fixation were found in one study carried out in the South Pacific central gyre, even with additions of both iron and phosphate, suggesting neither nutrient is limiting fixation here, rather they found low abundances of the gene (*nif H*) required to produce the enzymes to carry out this process (Bonnet et al., 2008).

The rates of nitrogen fixation in the present study were non-linear with temperature and peaked at 20^{o}C, close to the average ambient surface temperatures of 24^{o}C, suggesting the process has a narrow thermal niche. Increasing temperatures would be expected to increase rates of metabolic processes due to increased enzyme activity. Very few studies have considered the effect of temperature on rates of aquatic nitrogen fixation. One previous study found an increase in temperature (6^{o}C) did not significantly increase rates of nitrogen fixation in

Trichodesmium but was much more influenced by concentration of CO_2 for photosynthesis (Levitan et al., 2010). *Trichodesmium* are potentially one of the most important nitrogen fixing organisms in the open ocean (Capone et al., 2005; LaRoche & Breitbarth, 2005), and therefore been widely studied (Bonnet et al., 2008; Karl et al., 2002; Zehr, 2011). Laboratory experiments on the effect of temperature on *Trichodesmium* have shown optimal temperature between 24-30^{o}C, in line with optimal temperature found in this study, with maximum rates of 0.13 mmol N mol POC^{-1} h^{-1} d^{-1} at 27^{o}C when all other factors were controlled for (e.g. light, nutrients etc). However, this study was carried out with acclimation at experimental temperatures. Whereas short-term incubations have shown a linear increases of nitrogen fixation up to 36^{o}C (Staal et al., 2003) which is not in-line with our findings. With many different variables affecting the rates of nitrogen fixation, it is difficult for us to fully explain the low rates observed in the current study. Further investigation into the genes required for N fixation would allow to determine if the potential for measurable rates of N fixation exists at these sites. We can then further investigate the external limiting factors, such as PO_4^-, NO_3^- and iron on the rates at increasing temperatures.

4.5 Conclusion

The current study enabled us to investigate the thermal response of denitrification and anammox in both the water column and sediment within the ETNP OMZ. The rates of nitrogen loss and fixation in this study suggest the OMZ's to be a significant source of nitrogen in the form of N_2. The marginal thermal responses observed in this study may have been constrained by substrate limitation. Availability of organic carbon is a key factor in determining rates of both denitrification and anammox. Open ocean sediments are limited in organic substrates, relying on primary productivity in the surface waters to bring down nutrients and carbon. The low rates of nitrogen fixation observed in the currently study, may suggest very little new

nitrogen is entering the systems and therefore rates of primary productivity will be low, creating a knock-on effect further in the system. To try to reduce the limitation of organic substrates in the water column, we selected sites closer to shore where water density was greatest. However, in both sediments and water column, the greatest rates of denitrification and anammox were measured at temperatures closest to those experienced *in-situ*, which may also suggest the microbes involved are adapted to these temperatures with a narrow thermal niche and are not necessarily substrate limited. Substrate addition experiments with additional sources of carbon and exposure to increasing temperature will aid in determining if the responses are limited by substrate or microbe physiology.

4.6 References

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Chapter 5: Conclusions and suggestions for future work

The aim of the research presented in this thesis, was to investigate the thermal responses of different processes within the nitrogen cycle in both pure cultures and different environmental samples. The data presented in this study highlights the similarity of responses to temperature i.e. generally rates of nitrogen transformations increase with increasing temperature, but the degree of these responses are variable between systems and processes. In chapters two and three, we found strong thermal responses of denitrification to temperature, with both rates of N_2 and N_2O production increasing to an optimal temperature. However, we found very little thermal response of denitrification or anammox in marine sediments or water column of an oxygen minimum zone (chapter four).

There are two key differences between the laboratory pure culture experiments, the closed system experimental mesocosms and the marine oxygen minimum zones; substrate availability and ambient temperatures. Within chapter two, pure cultures of denitrifying bacteria were grown at room temperature in a media providing all required substrates in non-limiting concentrations. Here we observed an increase in metabolic activity with increasing rate of production of N_2 and N_2O up to an optimum temperature, after which, activity decreased. Ensuring all substrates for denitrification were available meant we could thoroughly investigate the thermal response in isolation, which is very difficult with environmental samples. In addition to increasing rates with increasing temperature, we also observed an increase in efficiency of denitrification with a decrease in the ration of N_2O/ N_2. This has wider implications of a more efficient excess nitrogen removal pathway from eutrophic systems as global temperatures increase with a relative reduced about of GHG emissions. Results from chapter two suggest even moderate-term exposure to different temperatures can alter denitrifiers thermal sensitivity in pure culture laboratory. Furthermore, this response varied between strains, highlighting the importance of molecular analysis on environmental samples.

In chapter three, the experimental mesocosms, sediments were exposed to a range of temperatures throughout the year as experienced are in a temperate climate. These systems are 'closed' in that they rely on organic substrates from primary production and cycling within the system and receive very little allochthonous contributions. We found an interesting differing responses of short-term temperature exposure and moderate to long-term warming. Short-term temperature exposure experiments allow us to investigate the capacity of the microbes in their present physiological state to adjust to temperature changes. Whereas long-term warming allows us to investigate potential physiological or community structure changes. With long-term warming of the experimental mesocosm sediments, we observed a significant effect of temperature, with greater rates of denitrification in the warmed mesocosms at *in-situ* temperatures. However, when sediments from both treatments were exposed to a range of temperatures, we only observed a significant difference between treatments at the highest temperature, which they are not exposed to when *in-situ*. These results may suggest an adaptation has occurred within the heated treatment denitrifying community. Molecular analysis on these sediments would allow us to determine if there has been a community shift in the warmed ponds to bacterial strains that are more efficient at higher temperatures or whether we see similar bacterial strains that have adapted to increase fitness when warmed. We also found significant effects of season on the rates of denitrification within the experimental mesocosms in both long-term warming and short term temperature exposure experiments. We measured the greatest rates of N2 and N_2O production in the warmer summer months with no effect of treatment. This seasonal variability highlights the importance of substrate availability, which are strong limiting factors for nitrogen cycling processes. The organisms present will only have the ability to increase their metabolic process if the substrates are available. However, the observed seasonal variability may also be influenced by changes in community structure. This again highlights the advantage of molecular analysis to identify if this is the

case, or whether other factors, such as substrate availability has a stronger influence. Molecular analysis is advantageous as it not only allows the identification of the species present through DNA analysis and sequencing but RNA analysis allows the identification of genes that are active at that point in time.

Chapter four measured anammox and denitrification in permanently cold sediments (~ 4^{o}C) and nutrient limited waters within the core of the OMZ that have relatively stable temperatures due to stratification. As seen in previously published studies, organic substrate availability can limit temperature responses (e.g. Brin et al, 2014; Heiss et al., 2012; Trimmer & Nicholls, 2009), which may have been the case within chapter four. However, potential rates in response to temperature may have also been reduced in the marine system due to a narrow thermal niche of the microbes responsible. As previously mentioned, these systems do not experience a wide range of temperatures, and it could be suggested that the microbes are specifically adapted to maximise fitness at their constant temperature. To investigate whether the responses observed in these three systems are controlled by substrate availability or natural ambient temperatures and their adaptations to this, we would need to carry out substrate addition experiments with temperature. This would reduce the effect of substrate limitation and allow us to investigate the full capacity of the microbe's potential at greater temperatures.

The nitrogen cycle is an important macronutrient cycle, controlling the distribution of life on Earth as it is a key nutrient required for primary production. It is an extremely complex cycle, with numerous steps, all mediated by different enzymes which have individual optimal temperatures. The thermal range of these enzymes and the rate at which they can adjust to warming will ultimately dictate how the different processes within the nitrogen cycle will change with the predicted changes in climate. This study has highlighted gaps in the literature as to the long-term and short-term warming effects on the various processes within the nitrogen but also the direct effects on the microbes carrying out these processes in aquatic systems,

especially in open oceans. Comparison of the thermal responses of organisms present in the nitrogen cycles and their ability to alter their metabolic processes in relation to temperature, also highlights the importance of substrate availability. We obersved stronger thermal responses in the pure cultures with no substrate limitation than we observed with the environmental samples. We did ensure enough N was available in the experiments, but did not have any additions of a source of carbon or any additional micro nutrients which may have been limiting, dampening the potential thermal responses.

Key future work which would aid in our understanding of the potential of the nitrogen cycle dynamics with increasing temperature, would be to include both substrate addition experiments and molecular analysis. For a greater understanding, these would be carried out in conjunction with one another. For environmental samples, seasonal sampling for the different processes would be carried out, including addition of increasing substrate concentrations (N and C source) at a range of temperatures. These samples would then be stored in a way to ensure community structure could be investigated. DNR and RNA analysis could be carried out, which would allow us to determine what organisms were present at the time of sampling, and the genes active at that particular time. To further this understanding, more processes within the nitrogen cycle would also be measured. Different components of the nitrogen are tightly connected to one another and we may have underestimated certain processes within our investigations. For example, nitrification within the experimental mesocosms may have been greater than measured, as due to practical restrains, we do not measure DNRA which can convert the product of nitrification back into NH_4^+.

5.1 Parallel research projects

During our six week cruise on the RSS James Cook investigating aspects of the nitrogen cycling in the North-Eastern Tropical Pacific oxygen minimum zone just off the Guatemalan

coastline, water samples were collected for molecular analysis within the water column by a colleague. This data includes gene copy numbers for nitrogen cycling genes and it is our hope to combine the molecular data with the process data and combine the findings in the near future.

Currently a meta-analysis on the experimental mesocosm is underway, including seasonal molecular analysis on both carbon and nitrogen cycling genes. This will compliment previous process measurements carried out by myself within this thesis and also previously published finding on carbon cycling within these systems (Yvon-Durocher et al., 2010; Yvon-Durocher et al., 2011).

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