ANAMMOX IN A TEMPERATE ESTUARY

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ABSTRACT

The seasonal variation of anammox is yet to be comprehensively studied, unlike denitrification, the more traditional sink for fixed nitrogen.

A seasonal study of anammox, denitrification and benthic oxygen consumption using the revised isotope pairing technique is presented in Chapter 2. Experimental temperature and NO_3^- concentration were kept constant throughout so that the capacity of the sediment for anammox could be estimated. Similar seasonal variations in the rates of anammox, denitrification and oxygen consumption suggest that anammox is controlled by the availability of organic carbon. Furthermore the effect of tidal inundation by overlying water rich in NO_3^- was investigated by measuring rates of anammox, denitrification and oxygen consumption at three tidal elevations throughout the year. A significant relationship between anammox and denitrification was established at each tidal elevation, which increased in strength as length of inundation decreased.

To complement this seasonal study, additional experiments were undertaken, which are described in Chapter 3, to determine how anammox, denitrification and sediment metabolism responds to variations in experimental NO_3^- concentration and temperature. There were significant increases in rates of anammox, denitrification and sediment metabolism with temperature until 20^oC when rates of anammox began to reduce. Furthermore there was significant variation in the response of all three processes to temperature in samples collected at different dates, which suggested that reduced bioavailability of organic carbon in the winter months was limiting the response to temperature.

In addition to exploring how inorganic N is cycled in estuarine sediments, the ability of estuarine sediments to oxidize urea via nitrite was examined using ^{15}N and ^{13}C labelled substrates. Results, which are presented in Chapter 4, indicate that urea added to anaerobic sediment slurries was rapidly hydrolysed to ammonium before being oxidized via the anammox pathway.

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DECLARATIONS

I hereby certify that this thesis is a record of the work carried out at the School of Biological and Chemical Sciences, Queen Mary, University of London, and that it has not been submitted in any previous application for a higher degree.

FIGURES AND TABLES

CHAPTER 1: INTRODUCTION

1.1. The Availability of Nitrogen on Earth?

Nitrogen is widely abundant in the Earth's biosphere, existing mainly as an inert diatomic gas, making up 78% by volume of the Earth's atmosphere. It adopts oxidation states in aquatic environments ranging from -3 (NH_4^+) to +5 (NO_3^-) whose oxidation and reduction are catalyzed by both heterotrophic and chemolithoautotrophic bacteria (Zehr and Ward, 2002). Nitrogen is vital for all life, as it is essential in the synthesis of amino acids and proteins, however most living organisms can only utilize fixed forms of nitrogen, which only make up a very small proportion of nitrogen on earth (Bernhard, 2010). As the majority of prokaryotes as well as all eukaryotes require fixed nitrogen, it is often the limiting nutrient (Tyrrell, 1999, Vitousek and Howarth, 1991), and therefore its availability can regulate primary production (Vitousek et al., 2002, Ryther and Dunstan, 1971, Canfield et al., 2010), which means it is intimately linked to the carbon cycle (Thamdrup, 2012) and therefore has the potential to regulate atmospheric CO_2.

The discovery of the Haber-Bosch process in the early 20th century, whereby N_2 is reacted with H_2 to produce ammonia (Erisman et al., 2008), drove nitrogen fixation on an industrial scale, largely to be used to produce crop fertilizers, increasing yields and enabling the human population to grow. Since the 1950s, the large increase in fertilizer use has led to a near-doubling of fixed N, drastically increasing bio-available nitrogen. However the widespread agricultural application has led to an unintended increase in inputs of fixed nitrogen to both aquatic and terrestrial

environments (Galloway et al., 2008, Galloway et al., 1995, Howarth, 2008, Diaz and Rosenberg, 2008). Humans also contribute to fixed nitrogen through the burning of fossil fuels and cultivation of legumes. Estimates suggest that anthropogenic inputs of fixed nitrogen now equal that which is biologically fixed (Howarth, 2008). This includes discharge of sewage effluent, which will increase in line with Earth's rising population (Peierls et al., 1991). These anthropogenic inputs can ultimately end up in coastal waters resulting in large phytoplankton blooms (Beman et al., 2005, Galloway et al., 1995, Diaz and Rosenberg, 2008).

1.2. The ecological significance of nitrogen

The increased availability of fixed nitrogen in the biosphere due to anthropogenic activity has caused an imbalance between nitrogen fixation and nitrogen removal processes (Galloway et al., 1995, Canfield et al., 2010). The result of this imbalance in aquatic, estuarine and coastal environments is hypernutrification. Where increased levels of fixed nitrogen, and nutrient enrichment occurs in an aquatic ecosystem, the result can be a large increase in primary production, which is apparent as excessive algal and vegetative growth known as eutrophication, resulting in hypoxic and anoxic conditions in coastal waters (Diaz and Rosenberg, 2008). As the over-stimulated algal or vegetative matter die, they fall to bottom waters and fuel microbial respiration (Rabalais et al., 2002), which consumes dissolved oxygen, causing severely oxygen-depleted conditions. Furthermore, toxic algal blooms of some types of *dinoflagellates* have been shown to cause the death of fish and shellfish (Howarth, 2008). Nitrogen removal processes can limit the availability of

fixed nitrogen and can therefore have the potential to attenuate the problems directly linked to hypernutrification (Bernhard, 2010).

The nitrogen cycle, deemed by Thamdrup (2012) the most complex biogeochemical cycle on Earth, involves a myriad of biogeochemical processes. Some generate bioavailable nitrogen by fixing atmospheric N_2. The only organisms that can access the vast pool of N_2 in the atmosphere are nitrogen-fixing prokaryotes (Paerl and Zehr, 2000), which use the enzyme nitrogenase to catalyze N_2 to fixed forms of nitrogen. While others return the nitrogen back to the atmosphere through a series of metabolic pathways (Bernhard, 2010, Zehr and Ward, 2002). Many of the processes can only be carried out by specialised microbial communities with specific environmental oxygen requirements; therefore the availability of these processes can be stipulated by the local environmental conditions.

1.3. Nitrogen removal pathways

Until recently it was understood that the only biological process capable of removing fixed nitrogen from the environment is the two-step process of nitrification followed by denitrification , summarised in Figure 1.1 (Trimmer et. al, 2003). Each step is mediated by different microbial communities (Zehr and Ward, 2002). Nitrification couples the aerobic oxidation of ammonium to the aerobic oxidation of nitrite, producing nitrate, which can then be reduced anaerobically by the denitrifying community to N_2 gas through a series of intermediaries (Zumft,

1997, Zehr and Ward, 2002). The more recent discovery of the anaerobic oxidation of ammonium coupled to the reduction of nitrite (anammox) (Thamdrup and Dalsgaard, 2002, Dalsgaard and Thamdrup, 2002, Trimmer et al., 2003) redefined our understanding of how fixed nitrogen is removed from the environment; crucially this process negates the requirement of an anaerobic step therefore providing a more efficient pathway of N_2 removal (Figure 1.2).

In nitrification, ammonium derived from the decomposition of organic matter is first oxidized aerobically to nitrite (equation 1.1) before being fully oxidized to nitrate (equation 1.2). To date, no single organism has been discovered that can complete the whole reaction, but instead each stage is undertaken by different groups of bacteria and archaea. Nitrification is a chemolithoautotrophic process, requires oxygen to proceed, and is present throughout the water column of aquatic and marine environments. It also occurs in the upper oxic layers of benthic sediments as well as in many terrestrial environments such as soil and grasslands.

$$NH_4^+ + O_2 + H^+ + 2e^- \longrightarrow NH_2OH + H_2O \longrightarrow NO_2^- + 5H^+ + 4e^- \qquad (1.1)$$

$$2NO_2^- + O_2 \longrightarrow 2NO_3^- \qquad (1.2)$$

Equations 1.1. and 1.2. The process of nitrification involves two principle steps. Firstly the aerobic oxidation of NH_4^+ to NO_2^- is catalyzed by ammonium monoxygenase and hydroxylamine oxidoreductase (1.1). This is followed by the oxidation of NO_2^- to NO_3^- (1.2).

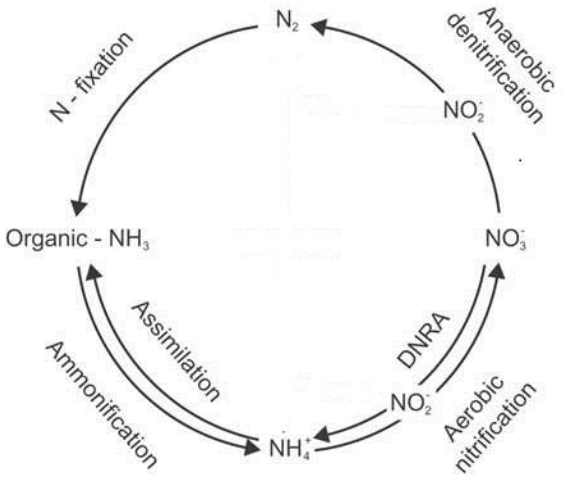


Figure 1.1: Schematic diagram of the traditional nitrogen cycle pre-dating the discovery of anammox (Trimmer et al., 2003).

Denitrification is a largely heterotrophic process performed primarily by facultative aerobes, who, in the absence of oxygen, sequentially metabolise NO_3^- through a series of intermediates (NO_2^-, N_2O) and finally to N_2 gas (Zumft, 1997). Equation 1.3 shows the overall reaction.

$$C_6H_{12}O_6 + 4NO_3^- + 4H^+ + O_2 \rightarrow 6CO_2 + 8H_2O + 2N_2 \quad (1.3)$$

Equation 1.3. The complete denitrification of NO_3^-, whereby NO_3^- is reduced through a series of intermediates ($NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$) to N_2.

For the respiration of NO_3^- to proceed rather than O_2, the conditions need to be anoxic; this limits the locations of denitrification to areas of low oxygen in aquatic ecosystems such as below the surface oxic layers of aquatic sediments (Rysgaard et al., 1993, Dong et al., 2000, Cabrita and Brotas, 2000) or in the anoxic water columns of the oxygen minimum zones (OMZs) of the ocean (Ward et al., 2009).

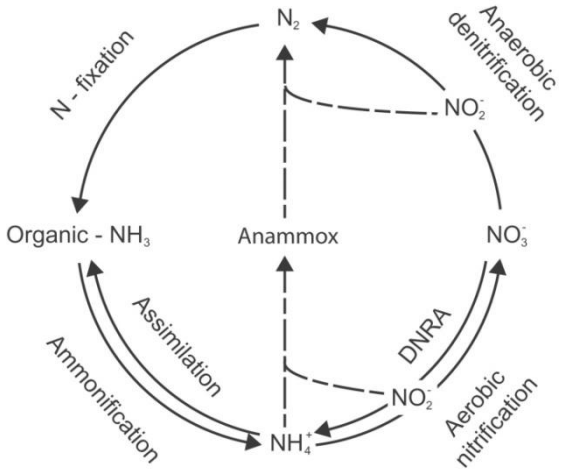


Figure 1.2: Schematic diagram of the nitrogen cycle which illustrates how the presence of anammox negates the requirement of an aerobic process, unlike the nitrification-denitrification pathway of N removal (Trimmer et al., 2003).

Within aquatic sediments, the nitrification and denitrification pathways are coupled across the oxic/anoxic interface (Henriksen, 1988). Nitrate, produced in the oxic layers of sediments by nitrification, can diffuse into the anoxic layers of sediments and subsequently be reduced anaerobically to N_2. Furthermore when nitrate concentration in the overlying water is high, it can diffuse into the anoxic layers of sediments and be reduced by the nitrate-reducing community (Meyer et al., 2008).

1.4. Anammox

An unexplained loss of NH_4^+ in an anoxic water column of a Norwegian Fjord was reported by Richards (1965a, 1965b). In 1977, Broda predicted the existence of a microorganism capable of carrying out anaerobic ammonium oxidation via NO_3^- or NO_2^- based on thermodynamic calculations. Decades later, the anaerobic oxidation of ammonium coupled to the reduction of nitrite or nitrate (anammox) (equation 1.4) was detected, in a fluidized bed reactor (Mulder et al., 1995), and the substrate characterised as NO_2^- rather than NO_3^- by van de Graaf et al. (1995). It was later identified in the environment for the first time in marine sediments (Thamdrup and Dalsgaard, 2002).

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (1.4)$$

Equation 1.4. The anaerobic coupling of NH_4^+ oxidation to the reduction of NO_2^-.

Importantly, anammox does not require an aerobic step in order to generate N_2 (Figure 1.2.). However, as anammox is an anaerobic process, this limits the locations where it can exist. Strous et al. (1999), by using enrichment cultures, demonstrated that the anammox pathway is inhibited by as little as 1μM O_2, although in a marine environment, the inhibitory oxygen concentration can be considerably higher at up to 10μM O_2 (Jensen et al., 2008). Areas where oxygen is sufficiently low generally occur in one of two distinct aquatic ecosystems. Firstly in aquatic sediments, which become anoxic within a few mm of the surface (Revsbech et al., 2006, Binnerup,

1992), and secondly in areas of very low oxygen concentration in oceans called oxygen minimum zones (OMZs) (Dalsgaard et al., 2003). Both have similar ecophysiologies, whereby the oxygen required for the heterotrophic metabolisation of organic matter is greater than can be supplied through diffusion from either the surface of marine or estuarine sediment or the oxic water columns surrounding the OMZs. To date, the anammox reaction and affiliated bacteria have been detected in estuarine (Nicholls and Trimmer, 2009, Trimmer et al., 2003, Risgaard-Petersen et al., 2004a), marine (Rysgaard et al., 2004, Engström et al., 2005, Dalsgaard and Thamdrup, 2002, Thamdrup and Dalsgaard, 2002) and riverine sediments (Zhao et al., 2013, Zhang et al., 2007) and also in the anoxic water columns of lakes (Hamersley et al., 2009, Schubert et al., 2006) and OMZs (Dalsgaard et al., 2003, Ward et al., 2009) throughout the world.

Anammox bacteria are chemolithotrophic autotrophic microbes deriving energy from the oxidation of NH_4^+ coupled to the reduction of NO_2^-. Although cultures of anammox bacteria have been difficult to isolate, enriched cultures have been obtained from waste-water reactors (Kartal et al., 2010). A survey of the species of bacteria that carry out anammox (Thamdrup, 2012) describes how they appear to be genetically linked, with marine anammox being dominated by the *Candidatus* Scalindua species (van de Vossenberg et al., 2012). Five genera, which have been enriched (Jetten et. al., 2009) are listed in van de Vossenberg et al. (2012), who describe how they form a monophyletic order which branches deeply into the phylum Plancomycetes.

Much of our understanding of anammox has been obtained through the study of estuarine (Trimmer et al., 2003, Rysgaard et al., 1993, Risgaard-Petersen et al., 2004, Rich et al., 2008, Dong et al., 2009, Nicholls and Trimmer, 2009) and marine sediments (Rysgaard et al., 2004, Engström et al., 2005, Dalsgaard and Thamdrup, 2002).

The relative contribution of anammox to total N_2 production has been widely used as a measure of the potential for anammox in investigations across different ecosystems, and this is denoted as *ra*%. Substantial variation in *ra*% between shallow estuarine and deep marine sediments has been found. An increase in the relative importance of anammox to denitrification (*ra*%) with ocean depth was proposed by Dalsgaard et al. (2005) and supported by Engström et al. (2005). More recently, by examining available data from previous studies on aquatic sediments, Trimmer and Engström (2011) demonstrated that there is a strong trend in ra% with depth until an *ra*% of approximately 50%, when it plateaus; although there was a one-off reading of 80% (at 700m). However the increase in *ra*% with sediment depth was generally not because of an increase in the rates of anammox, but instead resulted from a reduction in denitrification activity, attributed to a decrease with depth in the availability of organic carbon, essential in heterotrophic denitrification but not directly required for autotrophic anammox.

1.5. Anammox and carbon

Although anammox does not directly require a supply of organic carbon, as mentioned previously, it does require a supply of NO_2^- and NH_4^+, the production of

which is mediated by largely heterotrophic processes in estuarine sediments (Zehr and Ward, 2002). The concentration of NO_2^- in aquatic ecosystems is generally two orders of magnitude lower than NO_3^- (Trimmer et al., 2005) in both the overlying water and sediments; consequently, the anammox community must ultimately rely on other biogeochemical processes to provide a supply of NO_2^- (Dalsgaard and Thamdrup, 2002). Of particular importance to anammox in this process are the heterotrophic organisms, which carry out NO_3^- reduction and which have been reported to produce the majority of NO_2^- in anoxic sediments (Meyer et al., 2005). With generally high concentrations of NO_3^- and NH_4^+ in estuarine sediments, it is probable that availability of carbon will regulate the supply of NO_2^- for anammox (Trimmer et al., 2003, Engström et al., 2005, Trimmer and Engström, 2011).

This theory is supported by the work of Trimmer et al. (2003), who established a positive linear relationship between the carbon content of the sediment and the potential for anammox, when studying sediments from the Thames Estuary, UK, with a similar correlation across a large range of estuaries (Nicholls and Trimmer, 2009). This may indicate that although anammox does not directly require a supply of organic carbon, it indirectly needs its availability, so that the heterotrophic production of NO_2^- can proceed. More specifically, Rooks et al. (2012) demonstrated a relationship between CO_2 production and anammox over a depth profile in estuarine sediments. As the production of CO_2 is directly related to sediment metabolism, and therefore the availability of organic carbon (Glud, 2008), this would suggest that anammox is utilizing NO_2^- from heterotrophic processes.

Rysgaard et al. (2004) suggested that, as denitrification generates NO_2^- as an intermediate, which could be supplied to the anammox community, there should be a positive correlation between rates of anammox and denitrification. The techniques for quantifying anammox also provide measurements of denitrification. Thus in 2011, Trimmer and Engström re-examined a wide range of previous data from investigations into anammox, and established a positive linear relationship between rates of anammox and denitrification. More recently Zhao et al. (2013), when studying the seasonal variation in riverine sediments, also observed a highly significant relationship between anammox and denitrification; further evidence that denitrification supplies at least some of the NO_2^- for anammox. Furthermore rates of NO_2^- production have been shown to balance consumption in the Thames Estuary (Trimmer et al., 2003), which would suggest a much tighter coupling between NO_2^- production and anammox than may be expected in marine sediments, where consumption is considerably slower than production leading to an accumulation of NO_2^- (Dalsgaard and Thamdrup, 2002, Rysgaard et al., 2004). This may explain the increase in *ra*% with water depth mentioned previously, as marine sediments may not be so heavily reliant on NO_2^- produced through denitrification.

1.6. Anammox in Estuarine sediments

The current study focussed on the contribution of anammox to N_2 production in estuarine sediments. Estuaries are areas of intense biogeochemical processing as they receive terrestrial inputs of fixed nitrogen transported from rivers before reaching coastal waters. Many studies have demonstrated an estuary's ability to

attenuate fixed nitrogen from the overlying water before reaching coastal waters (Dong et al., 2009, Nedwell et al., 1999, Trimmer et al., 2003, Ogilvie et al., 1997, Jørgensen, 1989, Cabrita and Brotas, 2000). A recent study examining the potential for anammox in estuarine sediments throughout the S.E. of England reported its presence at every sampling location (Nicholls and Trimmer, 2009). In their study, Nicholls and Trimmer found the relative contribution of anammox to N_2 production (*ra*%) ranged from <1% to 11%. Rooks et al. (2012) however, observed an even higher *ra*% of >30% at the Medway Bridge Marina, S.E. England. Clearly anammox has significant potential to aid in the removal of fixed nitrogen.

1.7. Strata of sediment

The strata of estuarine sediments becomes anoxic after a matter of mm (Rooks et al., 2012, Revsbech et al., 2006). The availability of O_2 determines the oxidation state of the fixed nitrogen available within the sediment by controlling which metabolic inorganic nitrogen pathways can proceed (Figure 1.3). In the upper oxic layers of sediments, the nitrifying community aerobically oxidize ammonium, first to NO_2^-, followed by NO_3^- (Ward, 2000). This supply of NO_3^- can diffuse downwards into the anoxic layers of sediment where it can be reduced by the nitrate-reducing community (Meyer et al., 2005, Steif and de Beer, 2002). This includes the denitrifiers who preferentially respire O_2 when available, before switching to NO_3^- when conditions become anaerobic (Zumft, 1997). Both the reduction of NO_3^- and the aerobic oxidation of ammonium produce NO_2^- as an intermediate, so an increase in NO_2^- is usually observed just below the oxic/anoxic interface (Meyer et al., 2005,

Stief and de Beer, 2002). However fine scale micro-profiles indicate that the majority of NO_2^- in anoxic sediments results directly from the reduction of NO_3^- (Meyer et al., 2005). This pool of NO_2^- in the anoxic sediments can either be respired by the denitrifying community, or can be reduced coupled to the oxidation of ammonium by anammox, both ultimately producing N_2 gas, which is then lost from the ecosystem.

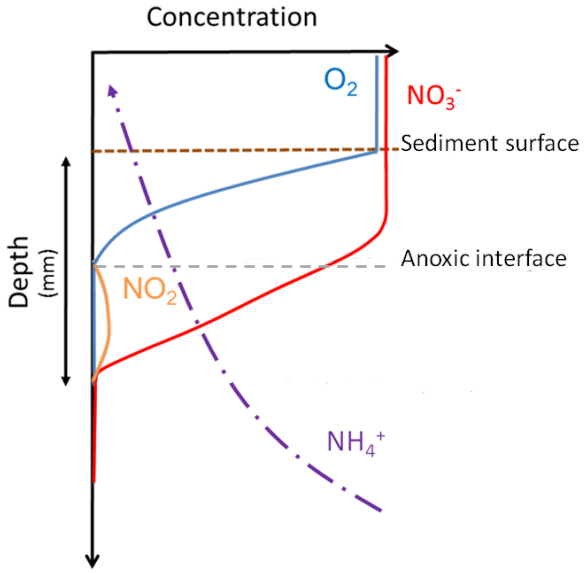


Figure 1.3: A diagrammatic representation of the idealised distribution of O_2, NO_3^-, NO_2^-, NH_4^+ throughout the sediment strata of an estuarine sediment. O_2 concentration declines rapidly as it is metabolically respired or consumed via biotic or abiotic reduction. NO_3^- concentration penetrates deeper into the anoxic layers of sediment where it is reduced. NO_2^- is produced as an intermediate of NO_3^- reduction creating an increase in NO_2^- concentration below the oxic . anoxic interface. Ammonium concentration decreases towards the surface of the sediment as it is utilized by anammox in the anoxic sediment and aerobic ammonia oxidizers in the oxic sediments.

As mentioned previously, a considerable increase in NO_2^- concentration occurs below the oxic layers of sediment. This is the narrow range of depths the anammox community have been shown to specifically inhabit (Rooks et al., 2012, Meyer et al., 2005), as they require a steady supply of NO_2^- (Risgaard-Petersen et al., 2004).

Furthermore Meyer et al. (2005) observed that the potential for anammox is directly related to production of NO_2^- in aquatic sediments, which, in turn, is directly related to the concentration of NO_3^- in the overlying water. In contrast, the denitrifying community have been shown to exist over a wider range of sediment depths. For instance Meyer et al. (2008) showed by increasing the concentration of NO_x^- in the overlying water, and consequently its penetration into the sediment, that denitrification activity was detected throughout the newly increased NO_3^- penetration depth, indicating that the denitrifying community can quickly extend downwards as NO_x^- saturates progressive layers. Furthermore, Neubacher et al. (2012), by artificially inducing anoxic conditions in sediment mesocosms, measured a faster response of N_2 production from denitrification than anammox. This, they suggested, was the ability of the denitrification community to rapidly switch from respiring O_2 to NO_3^- as the upper oxic zone of sediment retracted, however anammox was unable to adapt so rapidly.

Bioturbating macro fauna can alter the local structure of sediment strata through irrigation of their burrows with oxygen and nutrient rich water from the surface (Mermillod-Blondin et al., 2004). This can lead to an increase in the depth of the oxic/anoxic interface deep in the anoxic layers of sediments. Through the transport of O_2 and inorganic nitrogen into the anoxic layers, this could potentially increase NO_2^- generation through nitrification in the normally anoxic sediment, where a plentiful supply of NH_4^+ is usually available (Dollar et al., 1991, Ogilvie et al., 1997). Through bioirrigation, NO_3^- is pumped into the anoxic sediments (Mermillod-Blondin et al., 2004, Hansen and Kristensen, 1998), which increases

rates of NO_3^- reduction and denitrification (Nielsen et al., 2004, Rysgaard et al., 1995), providing additional NO_2^- in the anoxic sediments, which could potentially increase N_2 production via anammox. However as the anammox organism is slow growing, with a doubling time of 9 days at optimal temperature (Strous et al., 1999), it may not be able to swiftly adapt to rapidly changing oxygen concentrations. However Rooks et al., (2012) did observe a higher potential for anammox in the heavily bioirrigated sediments of the Medway estuary, which indicates that bioirrigation could stimulate anammox.

1.8. Detecting and quantifying anammox in aquatic sediments

It was originally thought that the anammox reaction was driven by the reduction of NO_3^-, however subsequent laboratory experiments established that it was in fact NO_2^- (van de Graaf, 1995). The production of N_2 from $NH_4^+ + NO_2^-$ has a 1:1 stoichiometry and this means ^{15}N labelled inorganic nitrogen isotopes provide an effective tool for detecting and quantifying anammox activity. Nitrogen consists of two stable isotopes ^{14}N and ^{15}N. As the natural abundance of nitrogen is 99.634% ^{14}N and only 0.366% ^{15}N, this allows ^{15}N labelled substrates to be used as a tracer for examining transformations occurring within the nitrogen cycle.

When originally surveying and proving the potential for anammox in sediments, the use of anaerobic slurries was essential and widespread (Thamdrup and Dalsgaard, 2002, Dalsgaard and Thamdrup, 2002, Trimmer et al., 2003, Nicholls and Trimmer, 2009) whereby sediment is sub-sampled into a gas-tight vial under anoxic conditions to provide the optimal anaerobic environment for anammox. By adding $^{15}NH_4^+$ +

$^{14}NO_2^-$ or its analogues $^{14}NH_4^+ + {}^{15}NO_2^-$ (equation 1.5) the subsequent production of $^{29}N_2$ would be proof positive that the anammox reaction is present in the sediment. Equally, the absence of $^{29}N_2$ when $^{15}NH_4^+$ only is used as a substrate, could be used to refute the presence of anoxic nitrification coupled to the reduction of Mn- or Fe-oxides (Luther, et al., 1997).

$$^{15}NH_4^+ + {}^{14}NO_2^- \rightarrow {}^{29}N_2 + 2H_2O \qquad (1.5)$$

Equation 1.3. The oxidation of $^{15}NH_4^+$ coupled to the reduction of $^{14}NO_2^-$. The production of $^{29}N_2$ indicates the presence of anammox.

As mentioned previously, anammox reduces NO_2^- rather than NO_3^-, however, Trimmer et al. (2005) demonstrated that NO_3^- is rapidly reduced to NO_2^- in anoxic sediment slurries and therefore no significant variation in $^{29}N_2$ production is observed if $^{15}NO_3^-$ is used instead of $^{15}NO_2^-$. Indeed, by adding $^{15}NO_3^-$ to anaerobic slurries, it is possible to determine potential rates of both anammox and denitrification for a given volume of sediment (Thamdrup and Dalsgaard, 2002, Dalsgaard and Thamdrup 2002, Trimmer et al. 2003). However it is important to remember that as the conditions have been made artificially anaerobic, and the sediment strata disturbed; using sediment slurries is therefore not representative of the *in-situ* environment, and the rates presented are only potential rates (Risgaard-Petersen et al., 2003).

In 1992, Nielsen developed a method for measuring rates of N_2 production using intact sediment cores through the addition of $^{15}NO_3^-$ (Nielsen, 1992). This method kept the strata intact so that the biogeochemical gradients within the sediment were not disturbed. However, the discovery of anammox led to a violation of one of the

underlying assumptions of Nielsen's model (Risgaard-Petersen et al., 2003). This stipulates that the isotopic labelling of the ^{15}N labelled N_2 gas must be binomially distributed in order for the ratio of $^{14}NO_3^-$ to $^{15}NO_3^-$ in the reduction zone to be estimated. With N_2 production from denitrification alone, the isotope pairing of ^{15}N labelled N_2 is binomially distributed with respect to the ratio of $^{14}NO_x^-$ and $^{15}NO_x^-$ in the pool undergoing reduction. However the additional production of $^{29}N_2$ from $^{15}NO_3^- + {}^{14}NH_4^+$ through anammox disrupts the binomial distribution leading, potentially, to an erroneous overestimation of genuine *in-situ* N_2 production (Risgaard-Petersen et al., 2003).

Risgaard-Petersen et al. (2003, 2004b) have since modified Nielsen's (1992) calculations to allow for the presence of anammox. Trimmer et al. (2006) subsequently developed a protocol whereby the binomially distributed production of ^{15}N labelled N_2O ($^{44}N_2O$,$^{45}N_2O$, $^{46}N_2O$), from denitrification, could be used to calculate the ratio of $^{14}NO_3^-$ to $^{15}NO_3^-$ (r_{14}) in the nitrate or NO_x^- reduction zone. These improvements allowed intact sediment cores to be used to more accurately estimate *in-situ* rates of anammox and denitrification. Furthermore intact sediment cores can be used to measure the rate of sediment metabolism, a proxy for the bioavailability of organic carbon (Glud, 2008). To determine the sediment metabolism in intact sediment cores, the rate of benthic oxygen consumption can be measured, and from that the variation in the bioavailability of organic carbon can be estimated seasonally or spatially at the same location.

1.9. Seasonal nitrogen cycling

Seasonal nitrogen cycling has been studied extensively in estuaries (Dong et al., 2000, Trimmer et al., 1998, Trimmer et al., 2000, Rysgaard et al., 1995, Cabrita and Brotas, 2000, Jørgensen and Sørensen, 1988, Jørgensen, 1989), however much of this work pre-dates the discovery of anammox. There are relatively few studies in estuaries specifically designed to examine seasonal variation in anammox and denitrification. Reported measurements have indicated that anammox does vary seasonally, however most such studies have used sediment slurries, so that reported rates are only potential. Zhao et al. (2013), when studying the seasonal dynamics in rivers, reported higher potential rates in the summer and autumn compared to the winter, which they attributed to an increased availability of NO_3^- in the overlying water and higher temperatures. Furthermore there was a significant correlation between the potential rates of anammox and denitrification. Teixeira et al., (2012) measured rates of anammox and denitrification in the Cavado Estuary, NW Portugal, during various seasons over a number of years and reported significant variation in potential rates of anammox, which was generally lowest in the summer months. Studies by Trimmer et al. (2005) and Risgaard-Petersen et al. (2004), while not specifically carrying out seasonal studies of anammox, did measure anammox across a range of seasons. Risgaard-Petersen et al. (2004) observed that rates of anammox declined by over 50% between April and August, whereas rates of denitrification increased. This resulted in a sharp reduction in *ra*% leading to the suggestion that the availability of organic carbon in the summer months fuelled rates of denitrification, but had little effect on anammox. Trimmer et al. (2005) however, observed a substantial increase in production of N_2 due to anammox from 0.6 nmol

of N_2 ml^{-1} of wet sediment, in June to 2.6 nmol of N_2 ml^{-1} of wet sediment in November. This suggests that, as yet, the variables which drive seasonal variation in anammox are unclear, highlighting the need for a study of anammox across all seasons.

Many studies of N_2 production have been conducted at *in-situ* NO_3^- concentration and water temperature (Dong et al. 2000, Teixeira et al., 2012), both of which vary considerably and often collinearly over a seasonal period. This makes it difficult to determine what is driving the seasonal variation; whether it is the increased temperature (Dalsgaard and Thamdrup, 2002, Rysgaard et al., 2004), variation in NO_3^- concentration (or possibly both), or whether the actual capacity for anammox within the sediment varies seasonally. Furthermore, there could be a seasonal variation of a substrate within the sediment, for instance organic carbon, which, although not directly required for anammox, could influence the supply of NO_2^- by restricting NO_3^- reducing organisms. Both temperature and NO_3^- concentration have already been shown to affect rates of anammox and its contribution to N_2 production (Dalsgaard and Thamdrup, 2002, Rysgaard et al., 2004). It is therefore important to maintain a constant experimental NO_3^- concentration and temperature in order to obtain an accurate estimation of how the anammox community, or other potential driving factors from within the sediment strata such as the bio-availability of organic carbon, vary over the year.

1.10. Availability of NO_3^- and NO_2^- and how they affect anammox

In the developed world, where nitrate pollution of aquatic environments such as estuaries is widespread, the sediments are subject to a rhythmic tidal inundation and exposure by the NO_3^- rich overlying water. Therefore sediments located at different tidal elevations on the intertidal mudflat are subject to considerably different degrees of NO_3^- exposure. Risgaard-Petersen et al. (2005) demonstrated, by varying NO_3^- concentration in water overlying sediments with a developed layer of microphytobenthos (MPB), that drastically lowering the concentration of NO_3^- reduced the penetration of NO_x^- into the sediment. This in turn severely reduced the sediments' capacity for anammox by 85%, because of a reduced production in NO_2^-. This would suggest that sediments located at lower tidal elevations, and consequently inundated for longer periods by the NO_3^- rich overlying water, would have a higher capacity for anammox.

1.11. Dissolved organic nitrogen

Dissolved organic nitrogen (DON) in river and estuarine environments has not received as much attention as the inorganic components of the nitrogen cycle, yet within estuarine sediment it can make up approximately 13% of the available nitrogen, rising to as much 83% at the surface of the ocean. (Berman and Bronk, 2003). DON compounds encompass a wide range of compounds including urea, peptides, dissolved free amino acids, methyl amides and many more (Antia et al., 1991, Berman and Bronk, 2003). Many of these DON compounds are potentially

important substrates for autotrophic and heterotrophic metabolisms (Berman and Bronk, 2003).

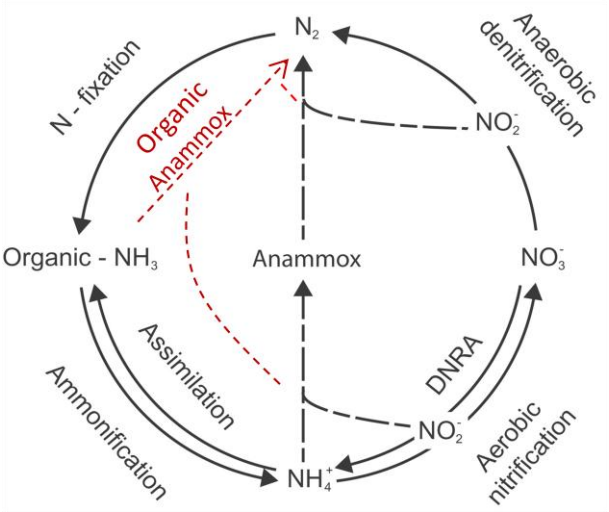


Figure 1.4: Schematic representation of the N cycle with the inclusion of the theoretical oxidation of DON coupled to the reduction of NO_2^-. (Adapted from Trimmer et. al., 2003).

More recently, Trimmer and Purdy (2012), while investigating N_2O production in the oxygen minimum zone of the Arabian Sea, inadvertently discovered evidence to suggest that allylthiourea (ATU), which was being used as an inhibitor of nitrification, was being directly oxidised to N_2 via the reduction of NO_2^-. This would suggest that some microbes have the ability to oxidize dissolved organic nitrogen (DON) directly in an anammox-like process. This would provide yet another

potential shortcut in the nitrogen cycle by removing the requirement for DON to be processed into NH_4^+ before proceeding via either nitrification-denitrification or anammox. A schematic diagram is shown in Figure 1.4.

Urea is a form of DON, and is abundant in the overlying water and within the sediments of estuaries, therefore it is possible that urea could be directly oxidized coupled to the reduction of NO_2^-. The reaction is energetically favourable at *in-situ* concentrations with a Gibbs free energy of -830 kJ mol $^{-1}$ reaction^{-1} or -415kJ mol N-^{1} (equation 1.6). Alternatively, as urea has been shown to be rapidly hydrolysed in estuarine sediment to produce NH_4^+ (Therkildsen and Lomstein, 1994) it could then be anaerobically oxidized, coupled to the reduction of NO_2^- by the anammox community.

$$CO(NH_2)_2 + 2NO_2^- + 2H^+ \rightarrow 2N_2 + CO_2 + 3H_2O \quad (1.6)$$

Equation 1.6. The oxidation of urea coupled to the reduction of nitrite.

1.12. Aims

The aims of this research are:

1) To examine how rates of anammox and denitrification vary seasonally in estuarine sediments, and what regulates it.

This will be undertaken in two ways. Firstly by keeping experimental NO_3^- concentration and temperature constant, it will be possible to determine how the

capacity for anammox within the sediment changes throughout the year. Samples will be taken from different tidal elevations monthly for a year, using intact cores to ensure the sediment strata is kept intact. Furthermore the seasonal variation in the intensity of bioirrigation will be measured to determine how it affects rates of anammox and denitrification. In addition, by measuring oxygen consumption in the cores at the same experimental temperature throughout the year, an estimation can be obtained of how sediment metabolism and organic carbon vary seasonally. Thus by examining the rates from different tidal elevations, the relationship between anammox and denitrification can be examined and how length of inundation by overlying water affects it.

2) Secondly, the effect of NO_3^- concentration and a broad range of temperature on rates of anammox, denitrification and sediment metabolism will be explored.

This will be assessed across a range of experimental NO_3^- concentrations and temperatures. Measurements will again be made seasonally to determine any seasonal effects, and whether seasonal variation in organic carbon can limit the sediments' ability to produce N_2 through anammox and denitrification, and how they respond to experimental variations in temperature and nitrate.

3) To determine whether dissolved organic nitrogen (DON) compounds can be oxidized directly to N_2 coupled to the reduction in NO_2^- in a process similar to anammox.

The investigation will be achieved by initially screening the sediments for the production of $^{29}N_2$ in a similar way to the initial anammox experiments. If positive results are determined in the screening experiments, ^{15}N and ^{13}C labelled DON will be added to sediment slurries. The ^{15}N labelling of the N_2 and the NH_4^+ pool and the ^{13}C labelled CO_2 will be examined through time to assess whether the DON is oxidized directly, coupled to the reduction in NO_2^-, or whether it is first being hydrolyzed to NH_4^+ before being utilized by the classic anammox pathway.

1.13. Overview of experimental work

Chapter 2

Anammox can contribute significantly to N_2 production in estuarine sediments, yet its seasonal characteristics are poorly understood. Previous studies with seasonal measurements have been conducted mainly in anaerobic slurries, at *in-situ* temperature and NO_3^- concentration. The object of this investigation was to measure the seasonal variation in anammox activity in estuarine sediments, using intact sediment cores, which keeps the sediment strata intact and enables estimates of both denitrification and anammox (Risgaard-Petersen et al., 2003, Risgaard-Petersen et al., 2004b).

The experimental temperature and NO_3^- concentration were kept constant throughout so that the actual sediment capacity for anammox could be measured without the influence of the seasonal variation in temperature or NO_3^- concentrations found *in-situ*. ^{15}N labelled isotopes were used in combination with state of the art mass-spectrometry to enable accurate estimations of anammox and denitrification. Additionally, as a measure of the availability of organic carbon, sediment metabolism was measured along with bioirrigation of the sediment strata to determine if either the availability of organic carbon or bioirrigation influence the sediments' capacity for anammox. Finally, measurements were also made using sediment collected from different tidal elevations to examine how length of tidal inundation by the NO_3^- rich overlying water affects the rate of anammox and its relationship with denitrification.

Chapter 3

Anammox and denitrification both respond to temperature (Dalsgaard and Thamdrup 2002, Rysgaard et al., 2004), however no previous investigations have been reported which study this in estuarine sediments. This chapter examines how temperature affects rates of anammox, denitrification and sediment metabolism across a range of temperatures at different sampling dates throughout the year. This part of the work utilized intact sediment cores to ensure that the sediment strata were kept intact, enabling both the aerobic and anaerobic processes located in the first few mm from the sediment surface to proceed naturally. Furthermore the effect of NO_3^- on rates of anammox and denitrification were studied across a range of sampling dates to assess whether both processes can respond to NO_3^- concentrations far above those found in the normal environment.

Chapter 4

Dissolved organic nitrogen (DON) can make up a considerable proportion of the total dissolved nitrogen in marine and aquatic environments (Berman and Bronk 2003). The hydrolysis of urea to ammonium is well understood in aquatic environments. Trimmer and Purdy (2012) suggested that allylthiourea (ATU) may be anaerobically oxidized via nitrite in the anoxic water column of the Arabian Sea. The aim of this section of the study was to examine whether DON in the form of urea could be anaerobically oxidized via NO_2^-, or whether it is first hydrolysed to NH_4^+ before proceeding via anammox to N_2 gas. ^{15}N and ^{13}C labelled organic nitrogen were used and the respective labelling of the N_2, NH_4^+ and CO_2 pool measured through time and used to predict N_2 production from the production of NH_4^+ from the hydrolysis of the added urea.

CHAPTER 2: SEASONAL VARIATION IN ANNAMOX IN THE MEDWAY ESTUARY

2.1. Introduction

Estuarine sediments have the potential to act as a sink for fixed nitrogen including ammonium, nitrate and nitrite (NH_4^+, NO_3^-, NO_2^- respectively) through their production of N_2 gas via denitrification (Dong et al., 2000, Rysgaard et al., 1995, Rysgaard et al., 1993, Trimmer et al., 1998) and anammox (Trimmer et al., 2003, Risgaard-Petersen et al., 2004, Meyer et al., 2005, Risgaard-Petersen et al., 2005, Trimmer et al., 2005). Fixed nitrogen is usually the limiting nutrient for primary production in coastal seas, and excess anthropogenic loading via rivers and estuaries can cause eutrophication (Howarth and Marino, 2006), which in some instances can cause hypoxia (Diaz and Rosenberg, 2008).

The discovery of anaerobic ammonium oxidation (anammox) in marine sediments (Thamdrup and Dalsgaard, 2002), redefined our understanding of how fixed nitrogen is removed from an ecosystem. Most of our knowledge to date about the biogeochemical processing of fixed nitrogen in estuaries has been based on studies of denitrification (Cabrita and Brotas, 2000, Dong et al., 2000, Ogilvie et al., 1997, Rysgaard et al., 1995, Rysgaard et al., 1993, Trimmer et al., 1998). This is not to say that the numerous long term studies into an estuary's potential to denitrify, and attenuate anthropogenic nitrogen loads, are of no value. However, they do contain an element of error because of interference in the classical ^{15}N isotope pairing technique, used to estimate the production of N_2, caused by the presence of anammox (Risgaard-Petersen et al., 2003, Trimmer et al., 2006).

Anammox is known to be widespread in estuarine sediments where appreciable concentrations of NO_3^- are often present in the overlying water (Risgaard-Petersen et al., 2004, Meyer et al., 2005, Risgaard-Petersen et al., 2005). A survey of estuaries in the S.E. of England (Nicholls and Trimmer, 2009) detected a potential for anammox at every location, unequivocally proving its widespread occurrence within estuarine sediments. Risgaard-Petersen et al. (2004) investigated the potential for anammox in two estuaries with significantly different concentrations of NO_2^- and NO_3^- (NO_x^-) in the overlying water. At one of the sites, NO_x^- disappeared completely during the summer months and the potential for anammox was absent.

Further investigation (Risgaard-Petersen et al., 2005) demonstrated that anammox activity decreased when the presence of microphytobenthos (MPB) impeded the penetration of NO_x^- into the sediment, thereby cutting off the production and supply of NO_2^- , which is essential to support an anammox community. Meyer et al. (2005) observed increased production of NO_2^- in suboxic sediments with higher concentrations of NO_x^- in the overlying water in sub-tropical mangrove sediments. They established a positive relationship between the availability of NO_2^- production and the sediments' potential for anammox. Clearly the availability of NO_x^- plays an important role in the potential for anammox. As NO_x^- increases in the overlying water, so does the production (Meyer et al., 2005, Nielsen et al., 2009) and penetration depth (Nielsen et al., 2009) of NO_2^- (Meyer et al., 2005).

Anammox bacteria, are slow-growing obligate anaerobes (Strous et al., 1999), which require a near constant supply of NO_2^- to maintain an active community

(Risgaard-Petersen, 2004).The denitrifiers however, are facultative anaerobes (Zumft, 1997) and only switch to sequentially respiring NO_x^- when the concentration of oxygen is sufficiently low, and oxidised species of nitrogen are present. Denitrification is therefore likely to be more resilient than anammox to an environment with fluctuating O_2 but plentiful NO_x^- such as the tidal mudflats of an estuary subject to the rhythmic inundation and exposure of the overlying water with anthropogenic inputs of fixed nitrogen, and therefore high concentrations of NO_3^-.

Bioirrigation and bioturbation by fauna can alter the complex stratification and substrate gradients, which influence the biogeochemical pathways found within sediments (Mermillod-Blondin et al., 2004, Hansen and Kristensen, 1998). Kristensen and Hansen (1999) demonstrated that the presence of *Nereis diversicolor* increases exchange between the overlying water and sediment through bioirrigation of their burrows. Furthermore Mermillod-Bolondin et al. (2004) showed that *N. diversicolor* could rapidly transport solutes from the surface into the sediment. The transport of NO_x^- and O_2 deep into the anoxic area of the sediment profile considerably increases the surface area of the oxic/anoxic interface. Increased oxygen penetration into the sediment would provide the conditions for aerobic oxidation of NH_4^+ to NO_3^- to occur in the normally anaerobic layers of sediment. NO_3^- could then diffuse through the oxic/anoxic interface to be reduced by the NO_3^- reducing community. Rysgaard et al. (1995) demonstrated that the density of macro fauna can increase relative rates of denitrification. Furthermore, aerobic NH_4^+ oxidation and anaerobic NO_3^- reduction, including denitrification, produce NO_2^- as an intermediary, which could be used directly by the anammox community. In

addition to the bioirrigation of burrows, sediment macro fauna can rework the sediment strata (Banta et al., 1999, Mermillod-Blondin et al., 2004) aiding in the burial of organic content deposited on the sediment surface into the sediment. This could include organic carbon which could stimulate heterotrophic NO_3^- reduction and denitrification.

Estuarine sediments often act as a source of ammonium (Dollar et al., 1991, Ogilvie et al., 1997), and ammonium in such environments would appear to be in excess, suggesting that NH_4^+ wouldn't generally be the limiting substrate in the anammox process. The NO_2^- concentration in the overlying water of estuaries is often two orders of magnitude lower than NO_3^-. However, the microbial communities within estuarine sediments have been shown to rapidly produce NO_2^- (Meyer et al., 2005, Trimmer et al., 2005). This is performed by two key heterotrophic processes. Firstly through the anaerobic reduction of NO_3^- and secondly the aerobic oxidation of NH_4^+ to NO_2^-, the first step in nitrification. However Meyer et al., (2005) demonstrated that most of the NO_2^- in the anoxic layers of sediments is a direct result of NO_3^- reduction. Therefore the availability of substrates that support the heterotrophic processes that regulate the production and availability of NO_2^- could potentially control anammox.

Both NO_3^- reduction and NH_4^+ oxidation are generally heterotrophic and therefore require the availability of bioavailable organic carbon. Rooks et al. (2012) demonstrated a clear relationship between the depth-specific potential for anammox and CO_2 production. This work suggests that the depth of sediment where

metabolism, and therefore bioavailable organic carbon is greatest (Glud, 2008), is stimulating production of NO_2^- and driving anammox activity.

A method for measuring sediment metabolism is to measure benthic oxygen uptake, which incorporates a broad range of aerobic and anaerobic respiratory pathways. Glud (2008) and Seitzinger and Giblin (1996) demonstrated that rate of N_2 production was positively correlated with oxygen uptake across a broad range of continental depths; and more recently Trimmer and Engström (2011) established a similar correlation between total N_2 production and sediment metabolism. This certainly suggests that heterotrophic denitrification is driven by increased availability of bioavailable organic carbon.

Rysgaard et al. (2004) proposed that the production of NO_2^-, as an intermediary of denitrification, could be reduced by the anammox community. This would result in a tight coupling between the two processes, and rates of both anammox and denitrification would be positively correlated. When examining a range of historic data, Trimmer and Engström (2011) demonstrated a clear correlation between rates of anammox and denitrification across a broad array of estuarine and marine sediments. Furthermore Nicholls and Trimmer (2009) demonstrated a correlation between the availability of organic carbon and anammox activity, which would indeed suggest that NO_2^- produced through heterotrophic denitrification is being used by the anammox community, and that the availability of bioavailable organic carbon could be indirectly driving rates of anammox.

Despite what we now know about anammox in estuaries, we still know relatively little about its seasonal characteristics. Risgaard-Petersen et al. (2004) and Trimmer et al. (2005), although not specifically studying the seasonal patterns of anammox in estuaries, used a broad array of sampling dates. Trimmer et al. (2005) found that anammox potential in sediment samples from the intertidal flats in the Thames estuary during November and December was considerably higher than those in February through to June.

Risgaard-Petersen et al. (2004) only covered spring and summer but found that the seasonal variation in rates of anammox was different to that exhibited by denitrification. For instance a sharp increase in denitrification between April and June, and a reduction in anammox, resulted in the ratio of N_2 production attributed to anammox (*ra*%) reducing dramatically from 25% to 5%. This would suggest that anammox and denitrification are not closely linked through the availability of a substrate, such as organic carbon in a bioavailable form, as may be expected, because anammox is a chemolithoautotrophic process (Jetten et al., 1998) and does not directly require its availability. However, as mentioned previously, the processes that generate NO_2^- for anammox are heterotrophic and so it is likely that the availability of bioavailable organic carbon does influence anammox to some degree.

Previous seasonal studies of denitrification (Jørgensen and Sørensen, 1985, Jørgensen and Sørensen, 1988, Rysgaard et al., 1995, Dong et al., 2000) have often been conducted at *in-situ* temperature and NO_3^- concentration. Seasonal variation in temperature, NO_3^- and organic carbon could vary considerably and concurrently. While this does give an indication of true rates of *in-situ* denitrification at a given

sampling date, it is impossible to isolate exactly what is driving the seasonal variation.

Previous studies have also demonstrated that both anammox and denitrification respond to changes in temperature and concentration of NO_3^- (Rysgaard et al., 2004) (Dalsgaard and Thamdrup, 2002). Therefore to fully understand the other drivers of seasonal variation of anammox, it is important to keep temperature and NO_3^- concentration constant for all experimental work. Thus in the current study, seasonal changes in the response of biogeochemical processes to availability of substrates, and in particular organic carbon within the sediment, and the effect on rates of anammox and denitrification were investigated, with experimental temperature and NO_3^- concentration kept constant throughout. Seasonal experiments were conducted at the same NO_3^- concentration, not to test the immediate response of both denitrification and anammox to NO_3^- but rather to examine how both communities change seasonally due to natural variations in organic carbon.

Much of the data reported on anammox to date was derived from the use of anoxic homogenised sediment slurries (Trimmer et al., 2003, Risgaard-Petersen et al., 2004, Risgaard-Petersen et al., 2005, Trimmer et al., 2005, Thamdrup and Dalsgaard, 2002, Dalsgaard and Thamdrup 2002). These are a good tool for surveying the potential for anammox, however, as the surface sediments are homogenised, the complex biogeochemical sediment strata are destroyed creating an entirely anaerobic environment. As mentioned previously, anammox probably requires the production of NO_2^- from both aerobic and anaerobic processes, which occur at different depths

of the sediment strata, and therefore this technique isn't an accurate reflection of what happens at *in-situ* conditions. For this, a method where the sediment strata is kept intact would provide a more accurate representation, because by keeping the O_2 gradients intact, both aerobic and anaerobic processes could proceed naturally (Nielsen, 1992, Risgaard-Petersen et al., 2003, Trimmer et al., 2006, Glud, 2008). The revised isotope pairing technique for measuring denitrification and anammox (Risgaard-Petersen et al., 2003) requires the collection of intact sediment cores so keeping the complex sediment strata intact, and allowing for far more accurate estimates of *in-situ* rates of denitrification and anammox.

The current study will investigate the seasonal variation of anammox in the Medway Estuary, Kent. Previous work at this location has demonstrated that the potential contribution of anammox to N_2 production ranges from 11% to 30% (Rooks et a., 2012, Nicholls and Trimmer, 2009), therefore anammox can account for a substantial proportion of the N_2 loss. The estuary has a tidal range of 6.7m; sediments located at lower elevations will be inundated by the NO_x^- overlying water for much longer periods than those located higher on the intertidal mudflat. As mentioned previously the potential for anammox has been shown to be higher in sediments with a greater availability of NO_x^-, therefore it may be expected that sediments at lower tidal elevations, and therefore inundated for longer periods, would have a higher potential for anammox.

Samples were taken from 3 tidal elevations in order to investigate whether the length of exposure to NO_x^- from the inundation of estuarine water affects rates of anammox, its contribution to N_2 production and its relationship with denitrification.

Bioirrigation was investigated by determining vertical O_2 profiles of sediment samples. Where bioturbating macro-fauna are present, spikes in O_2 concentrations will be observed deep in the anoxic sediment from the bioirrigation and bioturbation in burrows (Binnerup et al., 1992, Rooks et al., 2012).

The aim of this part of the study was to investigate seasonal variation in rates of denitrification and anammox in conjunction with measurement of the bioirrigation of the sediment strata in conjunction with measurement of benthic sediment metabolism, as a proxy for bioavailable carbon, as indicated by oxygen uptake, at three tidal elevations, while keeping the experimental temperature and NO_3^- concentration constant. This allowed examination of the following:

(i) How the anammox community varies seasonally in relation to denitrification.

(ii) How length of exposure to overlying estuarine water with high NO_x^- concentrations regulates the anammox community and its contribution to N_2 production.

(iii) Whether the availability of organic carbon varies seasonally and how it affects rates of anammox and denitrification.

(iv) How the relationship between anammox and denitrification varies at different tidal elevations.

(v) The influence of bioturbation on rates of anammox and denitrification measured seasonally and over different tidal elevations.

2.2. Methods

2.2.1. Study Site

Rates of anammox, denitrification, benthic oxygen uptake and oxygen penetration were measured in sediments collected at the Medway Bridge Marina [51°22'38.20"N:0°28'52.02"E], between March 2010 and March 2011. The Medway Estuary flows into the Thames estuary between the Isle of Grain and Sheerness (Figure 2.2.1). The upper catchment, 1386km ^{2} in area, (Nedwell et al., 2002), has a mixture of industrial, urban and rural land uses. Concentrations of NO_3^-, NO_2^-, NH_4^+ and O_2 saturation in the overlying water show little seasonal variation (Environment Agency, EA, Table 2.2.1). The site has a spring tidal range of 6.7m and a salinity of 6ppt at low tide and approximately 20ppt at high tide (Table 2.2.1). The site was chosen as anammox activity had previously been detected, with a *ra*% of between 11% and 30% (Nicholls and Trimmer, 2009, Rooks et al., 2012).

Table 2.2.1: Mean concentrations of NH_4^+, NO_3^-, NO_2^- and O_2 saturation between January 2000 and September 2011. Data supplied by the Environment Agency, UK, from their long term monitoring programme.

NH_4^+ (μM)	NO_3^- (μM)	NO_2^- (μM)	O_2 saturation (%)
10.5 ± 0.5 n= 110	187 ± 7 n=115	2.2 ± 0.01 n=113	81 ± 1.2 n=108

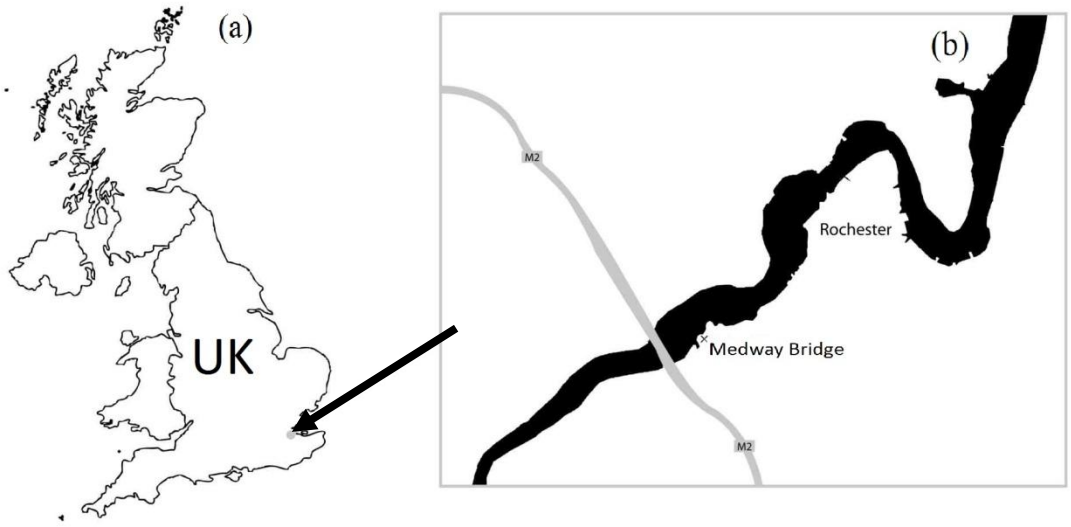
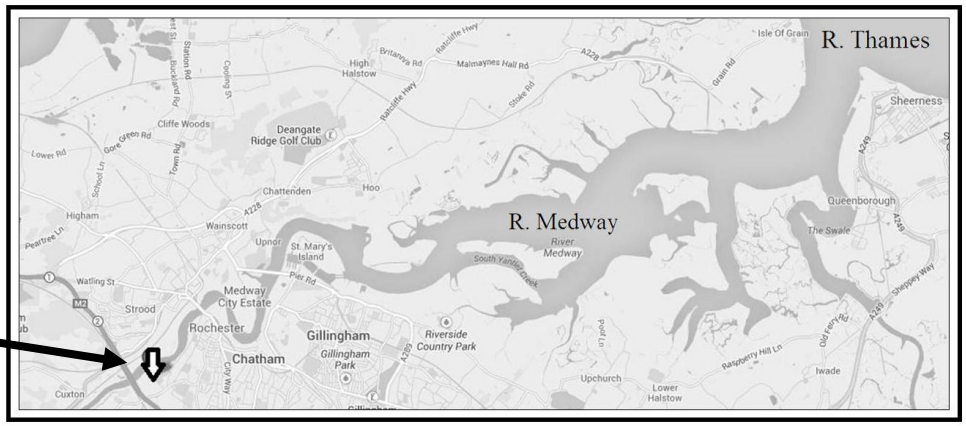


Figure 2.2.1: Map of the UK showing (a) the location of the River Medway and (b) the location of the Medway Bridge Marina on the river. (c) shows a more detailed map with the location of the marina.

(c)

Medway Bridge Marina

[51°22'38.20"N:0°28'52.02"E]



Three tidal elevations were chosen (Figure 2.2.2.) at the same site to determine whether the period of tidal inundation influenced rates of anammox and denitrification activity, and consequently the relative contribution of anammox to total N_2 production (*ra%).* Extreme low water (ELW) [51°22'41.91"N:0°28'50.23"E] was located at 0.4m above the chart datum and is covered by estuarine water for 90% of the time. Medium water (MW) [51°22'40.56"N:0°28'49.95"E] was located at 2.5m above chart datum and was representative of a mid-tide location inundated by the tide for approximately 50% of the time throughout the year. Extreme high water (EHW) [51°22'30.99"N:0°28'41.08"E] was located at 5m above chart datum which, is only inundated 10% of the time throughout the year.

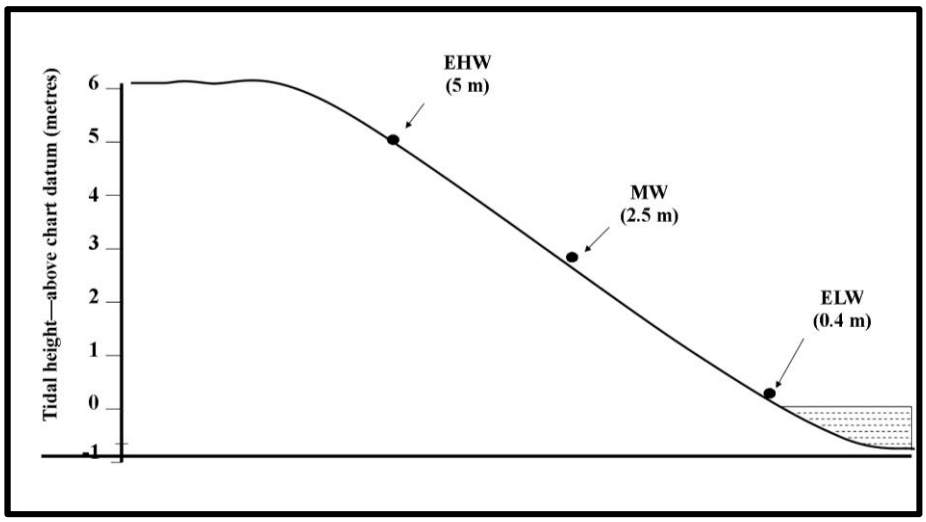


Figure 2.2.2: Diagram showing transect of the tidal elevations. Extreme Low Water (ELW), 0.4m above chart datum; Medium Water (MW), 2.5m above chart datum and Extreme High Water (EHW), 5m above chart datum.

2.2.2. Sediment sampling

At each tidal elevation, 16 Perspex tubes (3.4cm x 25cm) were each half-inserted into the sediment from above, removed with sediment and sealed with a rubber bung inserted from below. For the measurement of sediment O_2 penetration, 3 additional cores (6cm x 9cm) were collected at each elevation as described previously, and sealed with a bung. Both sets of cores were then carefully cleaned on the outside ensuring no disturbance of the sediment surface, stored vertically and transported back to the laboratory within 4 hours. Once returned to the laboratory, sea water adjusted to *in-situ* salinity (ca.6ppt) using de-ionised water was carefully introduced to the cores to avoid disturbing the sediment surface. The cores were then placed in an open tank of the same aerated water at 10^{o}C and left overnight. Surface sediments (top 2 cm) were collected using a large metal spatula and returned to the laboratory in polythene bags before refrigerating for future analysis.

2.2.3. Measurement of sediment characteristics

To measure the particle size, samples of sediment (top 5cm) were collected in 5ml truncated syringes, returned to the laboratory and left to air-dry. Once dry, the samples were placed in a mortar and gently broken up using a pestle to prevent comminution of individual particles. Sediment (5g) was placed in an empty 250ml conical flask and 5ml hydrogen peroxide (H_2O_2, 30%) was slowly added. Once the initial reaction had ceased, another 5ml H_2O_2 (30%) was added and left overnight. The following day the flask was placed on a hotplate and gently heated whilst adding 10ml H_2O_2 at a time until frothing ceased. Samples (10ml) were then

analyzed by laser diffraction (LS 13 320 Beckman Coulter Counter) to determine the grain size distribution.

2.2.4. Confirming the presence of anammox using slurries

Slurries of surface sediment (1ml) and sea water adjusted to *in-situ* salinity (ca.6ppt) using de-ionised water (1ml), were prepared in an anoxic hood, sealed in a gas-tight vial (2.5ml, Exetainer, Labco) and left to incubate overnight to ensure that any remaining oxidants (O_2, NO_3^-, NO_2^-) had been utilized (Trimmer et al., 2003, Risgaard-Petersen et al., 2004). The slurries were then injected through the septa with different combinations of concentrated stocks of ^{15}N and ^{14}N labelled inorganic N ($^{15}NH_4^+$, $^{15}NO_3^-$, $^{15}NH_4^+$+$^{14}NO_2^-$) at an initial concentration of 1000 μM and left on rollers (Spiramix, Thermo-Finnigan) at 20^{o}C overnight. Production of $^{29}N_2$ gas with the sole addition of $^{15}NH_4^+$ and $^{14}NO_2^-$ would confirm the presence of anammox. By adding $^{15}NO_3^-$ the potential production of N_2 from both anammox (equation 2.1) and denitrification (equation 2.2) could be estimated using the following equations (Thamdrup and Dalsgaard, 2002) .

$$D_{30} = P_{30} \times F_N^{-2} \quad (2.1)$$

$$A_{tot} = F_N^{-1} \times [P_{29} + 2 \times (1 - F_N^{-1}) \times P_{30}] \quad (2.2)$$

Where D_{30} is the production of $^{30}N_2$ from denitrification, P_{30} is the production of $^{30}N_2$ and F_N is the proportion of $^{15}NO_3^-$ in the NO_3^- added. Production from anammox (A_{tot}) also includes the production of $^{29}N_2$ (P_{29}).

The contribution of anammox to N_2 production (*ra*%) was determined following the calculations described by Thamdrup and Dalsgaard (2002). As these experiments were not timed, they were only useful for estimating the potential production of N_2 by anammox relative to denitrification (*ra*%) and not actual rates.

2.2.5. Measuring anammox, denitrification and sediment metabolism in intact sediment cores

Rates of anammox and denitrification were measured using the r-IPT described by Risgaard-Petersen et al., 2003) and later developed by Trimmer et al. (2006).The overlying water was enriched by adding 100µL of $Na^{15}NO_3$ (116mM [98% ^{15}N atom %], Sigma Aldrich) resulting in an initial concentration of 100µM $^{15}NO_3^-$, before the overlying water was carefully mixed. Samples were left for 30 minutes to allow the $^{15}NO_3^-$ to diffuse into the anoxic layers of sediment. Previous analysis demonstrated that there was no reduction in the ratio of $^{14}NO_3^-$ to $^{15}NO_3^-$ in the reduction zone from between 30 minutes and 4 hours. This was determined by examining the ^{15}N-N_2O produced from an intact core incubation with $^{15}NO_3^-$ added (100µL) to an initial concentration of 100µM. Measurements taken between 30 minutes and 4 hours found no variation in r_{14} with time, which demonstrated that there was a consistent mixing of $^{14}NO_3^-$ and $^{15}NO_3^-$ and that $^{15}NO_3^-$ had diffused throughout the reduction zone. This indicated that an initial mixing of 30 minutes was sufficient before

starting the experiment. After 30 minutes, the cores were sealed with a rubber bung from above with a magnetic stirrer attached, and left to incubate at 10^{o}C for 4 hours with the overlying water constantly stirred. As mentioned previously, there was no variation in r_{14} within 4 hours, and therefore a 4 hour incubation should give an accurate estimate of the rate of N_2 production.

After four hours the experiment was ended by removing the rubber bung and homogenising the overlying water and sediment using a metal spatula (Trimmer et al 2006). A sub-sample of this slurry was then transferred to a gas-tight vial (Exetainer, 12ml, Labco) and microbial activity ended by adding 100µl of formaldehyde (CH_2O, 37%) before a headspace (1ml He, analytical grade) was introduced. In addition the remaining 6 cores (2 from each site) were sacrificed with no $^{15}NO_3^-$ amendment to be used as a reference of the natural background ^{15}N-N_2 labelling.

2.2.6. Measuring benthic oxygen consumption

Oxygen uptake was measured in the same intact sediment cores used for the r-IPT. Oxygen concentration was measured using an oxygen micro-sensor (OX-50, Unisense, Denmark). Care was taken to ensure that the calibration of the oxygen sensor was performed at the same temperature (10^{o}C) as the experiment. Calibration was performed by bubbling air through water for 10 minutes to ensure 100% atmospheric O_2 saturation. Oxygen solubility was then established (Unisense, oxygen sensor manual) and an O_2 concentration of 352µM for a temperature of 10^{o}C and salinity of 6ppt was established. To measure an anoxic calibration, a 0.1M solution of sodium ascorbate was prepared. The sensor tip was inserted carefully into the aerated water to measure the top calibration value, before proceeding to the

anoxic solution to measure the lowest calibration point followed by washing thoroughly with de-ionized water. A linear calibration was then calculated between 0µM (anoxic) and 352µM (100% atmospheric O_2 saturation). To calculate the O_2 concentration in cores, the sensor tip was carefully placed into the overlying water (T_0). The cores were then sealed with a rubber bung and the r-IPT incubation started. A magnetic stirrer attached to the rubber bung continuously agitated the overlying water to ensure that a oxygen gradient did not form in the overlying water. After 4hrs (T_f) the oxygen concentration in the overlying water of the intact sediment core was again measured and consumption was determined as the difference between T_f and T_0.

2.2.7. Mass Spectrometry

To determine the concentration of N_2, a calibration was performed using an air-equilibrated (22^oC) water filled gas-tight vial (12ml Exetainer, Labco) with a 1ml headspace (He, analytical grade). A 100µl sample of the headspace from each of the gas-tight vials was injected using an auto sampler (Gerstel) into the elemental analyser (Flash-EA, Thermo-Finnigan) but bypassing the oxidation/ reduction columns so that N_2O would not be reduced to ^{15}N-N_2 gas. Gases were separated on the gas chromatographer (GC) column prior to entering the continuous flow isotope ratio mass spectrometer, where the mass-to-charge ratios for m/z 28, 29 and 30 nitrogen ($^{28}N_2$, $^{29}N_2$, $^{30}N_2$) was measured (Delta Matt Plus, Thermo-Finnigan).

For N_2O analysis, 10µl sub-samples of the headspace were taken from each sample that had previously been measured for N_2, using a gas-tight syringe (100µL SGE

Gas Tight Luer Lock syringe) into an air filled gas-tight vial (12ml, Exetainer, Labco). The gas-tight vials were then flushed with analytical grade He through a 2-way needle using an auto-sampler (as above), dried (magnesium perchlorate, ACS grade Sigma Aldrich), scrubbed of most CO_2 (Carbsorb, Sigma Aldrich) then cryo-focused twice in liquid N_2 before a final separation of N_2O from CO_2 on a PoraPLOT Q capillary column. The sample was analysed using a continuous flow isotope ratio mass spectrometer (Delta Matt Plus, Thermo Finnigan) via an interface (ConFlo III Interface, Thermo Finnigan) and the specific mass-to-charge ratios for m/z 44, 45 and 46 ($^{44}N_2O$, $^{45}N_2O$, $^{46}N_2O$) measured.

2.2.8. Calculation of r-IPT

Calculation of the r-IPT (Risgaard-Petersen et al., 2003, Risgaard-Petersen et al., 2004b) requires the ratio of $^{14}NO_3^-$ to $^{15}NO_3$ in the reduction zone (r_{14}) to be determined. Where denitrification is the sole producer of N_2 and the ratio between $^{14}NO_x^-$ and $^{15}NO_x^-$ is constant throughout the reduction zone, the three isotopic species of N_2 ($^{28}N_2$, $^{29}N_2$, $^{30}N_2$) are binomially distributed (Nielsen, 1992) however the added production of $^{29}N_2$ by the anammox community ($^{28}N_2$, $^{29}N_2$) violates this assumption and therefore isotopic analysis of the N_2 is insufficient to determine r_{14}.

When denitrification is the only source of ^{15}N labelled N_2O the production of $^{44/45/46}N_2O$ should be binomially distributed when the ratio of $^{14}NO_x^-$ to $^{15}NO_x^-$ is constant throughout the reduction zone. Trimmer et al. (2006) demonstrated that

rather than using the ^{15}N labelling of the N_2 pool, as suggested by Nielsen (1992), ^{15}N labelling of the N_2O pool could be used instead as it should be binomially distributed. The following equation allows the calculation of r_{14} (Trimmer et al., 2006).

$$r_{14} = \frac{p^{45}N_2O}{2\,.^{46}N_2O} \tag{2.3.}$$

Where r_{14} is the ratio of $^{14}NO_x^-$ to $^{15}NO_x^-$ in the reduction zone, $p^{45}N_2O$ is the production of $^{45}N_2O$ and $p^{46}N_2O$ is the production of $^{46}N_2O$.This provides a direct estimate of the $^{14}NO_x^-$ to $^{15}NO_x^-$ ratio (r_{14}) in the reduction zone, allowing the calculation of the r-IPT (Risgaard-Petersen et al., 2003) (Risgaard-Petersen et al., 2004, Trimmer et al., 2006). To calculate rates of anammox and denitrification the r-IPT method (Risgaard-Petersen et al., 2003,2004b, Trimmer et al., 2006) was used. The r-IPT procedure calculates N_2 production from both denitrification (equation 2.4.) and anammox (equation 2.5.) as follows:

$$p_{14} = 2r_{14}\,.\,(p^{29}N_2 + p^{30}N_2\,.\,(1 - r_{14})) \tag{2.4}$$

$$p_{14}AAO = 2r_{14}\,.\,(p^{29}N_2 - 2\,.\,r_{14}\,.p^{30}N_2) \tag{2.5}$$

Where p_{14} is the total production of N_2, r_{14} is the ratio of $^{14}NO_x^-$ to $^{15}NO_x^-$ in the NO_x^- reduction zone ; $p^{29}N_2$ is the total production of $^{29}N_2$, p^{30} is the total

production of $^{30}N_2$ and $p_{14}AAO$ is the production of N_2 from anammox. Denitrification is estimated as the difference between the total nitrogen production p_{14} and $p_{14}AAO$.

2.2.9. Measuring oxygen penetration in intact sediment cores

Sediment oxygen profiles were measured using Clarke-type oxygen micro-sensors (OX-50, Unisense, Denmark) attached to a motorized micro-manipulator (Unisense, Denmark). The overlying water was constantly aerated to keep the overlying water saturated with oxygen, and to ensure that there was no reduction in oxygen concentration from the water surface to the sediment. Sediment oxygen profiles were always conducted with the overlying water at 100% saturation and the oxygen sensor was calibrated as mentioned previously.

The oxygen saturation of the overlying water of the Medway Estuary close to the marina does not vary much throughout the year (EA, Table 2.2.1.) with a mean saturation of 81 ±1.2%, so that measuring at 100% saturation would not be too different from *in-situ*. However the experimental temperature was kept constant throughout the year, so changes in O_2 concentration due to temperature and the resulting variation in the position of the oxic-anoxic interface are not considered.

Oxygen concentrations were measured at 300 µm depth intervals and were recorded using a picoammeter (PA 2000, Unisense) and displayed using profiling software (Sensor trace PRO, Unisense). When bioirrigation is absent, the concentration of oxygen decreases linearly from the sediment surface, typical of diffusion exchange

(Berg et al., 1998) until the anoxic sediment is reached. When bioturbation is present, erratic spikes are measured in the sub-surface oxygen profile (Rooks et al., 2012).

The extent of bioirrigation was estimated by first calculating the coefficient of variation in the concentration of oxygen at depth intervals of 1mm throughout the oxygen profile using equation 2.6.

$$C_v = \frac{\sigma}{\mu} \tag{2.6}$$

C_v is the coefficient of variation, σ is the standard variation and μ is the mean. Larger values of the coefficient of variation indicate a greater intensity of bioirrigation at a given depth.

The degree of bioirrigation, or bio-index, for each core was then determined by plotting the coefficient of variation against depth and integrating the area, as illustrated in Figure 2.2.3. The mean was then calculated for each month.

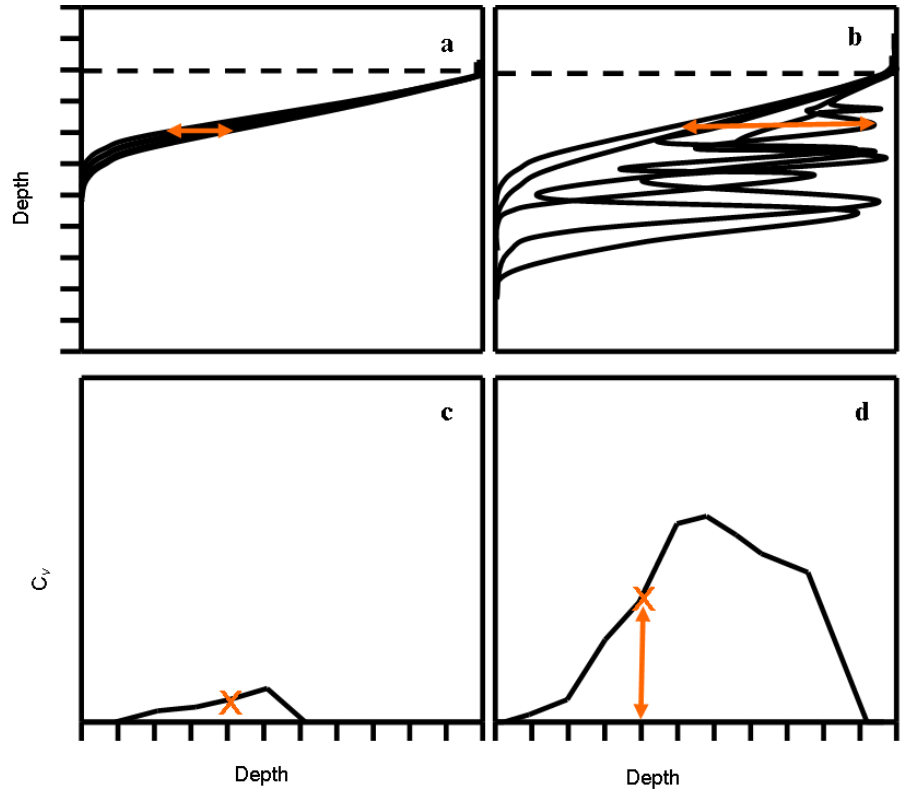


Figure 2.2.3: The bio-index is calculated by integrating the area when the coefficient of variance plotted against depth, at 1mm intervals.

Note how profiles with no bioirrigation (a) can still produce a bioindex value (c) through small variation in the oxygen profiles. However this is much smaller than profiles with intensive bioirrigation (b) which have much higher C_v values (d).

2.2.10. Statistical analysis

The effect of both tidal inundation and season on anammox, denitrification and benthic oxygen uptake data were analysed using analysis of variance (ANOVA). For seasonal variation, a model was fitted using data from all 3 sites combined, and the response of anammox, denitrification and oxygen consumption were compared for

each month. To determine the effect of tidal inundation, data from each sampling date was pooled and split into each tidal elevation; the response of anammox, denitrification and oxygen consumption was assessed at each of the 3 tidal elevations.

To determine how oxygen consumption bio-index and denitrification at the different tidal elevations affected rates of anammox, further data exploration was carried out using the protocol described in (Zuur et al., 2010). Outliers were assessed using Cleveland dot plots, and collinearly was investigated using variance inflation values (VIF). Pearson correlations and relationships between anammox and covariates were investigated using multi-panel scatter plots and co-plots.

As multiple samples were collected from each tidal elevation, linear mixed effect models were applied (Zuur, 2009). To account for any correlational structure inherent in the data, tidal elevation was nested within sampling date to produce 24 random intercepts. There were only 24 random intercepts, as a bio-index was not available for every sampling date. Based on the underlying biological questions, the following model was applied: rate of anammox was the response variable and denitrification, oxygen consumption, inundation and bio-index as main terms, and interactions between denitrification and inundation, oxygen consumption and inundation, denitrification and bio-index were included. To find the optimal model, a 10 step protocol described in Zuur (2009) was followed. Insignificant terms were stepwise backward eliminated from the initially fitted model based on the likelihood ratio test. This allows models to be compared using the ANOVA function and an assessment made whether a particular variable or interaction influenced the model

significantly. The model was fitted using the nlme package for R (Pinheiro et al., 2009). Once the model was fitted, a detailed model validation was applied to verify the assumptions (homogeneity, independence, normality). This was done by plotting histograms of the residuals versus fitted values and residuals versus each covariate in the model.

2.3. Results

2.3.1. Porosity

There was a signficant increase in the annual mean of porosity from ELW to EHW ranging from 0.65 to 0.7 respectively (Table 2.3.1). There was no seasonal pattern in porosity at any location with only a slight variation in values between sampling dates.

Table 2.3.1: Table of annual mean porosity for each of the tidal elevations: ELW (extreme low water, 0.4m above chart datum); MW (middle water, 2.5m above chart datum) ; EHW, (extreme high water, 5m above chart datum). n = number of observations.

ELW	MW	EHW
0.65 ± 0.01	0.68 ± 0.01	0.7 ± 0.02
(n=9)	(n=12)	(n=12)

2.3.2. Sediment grain size

The grain size distribution varied markedly between tidal elevations with a larger percentage of coarser particles between 63 and 1000μm at the lower tidal elevation. The grain size got progressively smaller as tidal elevation increased, with few

particles with a grain size >63µm at MW and EHW respectively. Table 2.3.2 shows the particle size distribution for each tidal elevation.

Table 2.3.2: Sediment particle size distribution (%) at each tidal elevation.

	PARTICLE SIZE									
Site	**<2 (µm)**	**2-4 (µm)**	**4-8 (µm)**	**8-16 (µm)**	**16-31 (µm)**	**31-63 (µm)**	**63-125 (µm)**	**125-250 (µm)**	**250-500 (µm)**	**500-1000 (µm)**
ELW (%)	14.1	8.2	11.1	12.4	7.6	16.7	19.2	4.8	4.5	1.4
MW (%)	22.7	8.5	11.3	16.7	15.4	17.2	7.4	0.8	0	0
EHW (%)	29.9	15.9	19.8	18.3	7.2	7.3	1.5	0	0	0

2.3.3. Confirming the presense of anammox in slurries

Anammox was present at every location throughout the year in sediment slurries as evidenced by the production of $^{29}N_2$ gas with the addition of $^{15}NH_4^+$ and $^{14}NO_3^-$ and the absence of $^{15}N\text{-}N_2$ in sediment slurries ammended with the sole addition of $^{15}NH_4^+$ (data not shown). At no point throughout the year was any $^{15}N\text{-}N_2$ detected in sediment slurries ammended with the sole addition of $^{15}NH_4^+$. There was a range in relative contribution (ra%) between 3% and 45%, and the average was 19%. Table 2.3.3. shows the relative contribution (*ra*%) of anammox to N_2 production in sediment slurries with the addition of $^{15}NO_3^-$. Generally, the relative contribution of anammox was lowest at ELW increasing to EHW, however there was no significant relationship between mean relative contribution of anammox and tidal elevation.

Table 2.3.3: The mean monthly relative contributions of N_2 produced by anammox to N_2 production (*ra*%), in anaerobic sediment slurries with the addition of $^{15}NO_3^-$ for each tidal elevation EMW, MW and EHW, from March to December.

Month	ELW (*ra*%) (monthly mean)	MW (*ra*%) (monthly mean)	EHW (*ra*%) (monthly mean)
March	14	20.5	30
April	12	18	16
May	13	16	25
June	12	13	25
July	17	15	17
August	44	22	25
September	45	24	43
October	-	10	15
November	11	8	15
December	3	7	14

2.3.4. Seasonal variation in anammox, denitrification and sediment metabolism at the three tidal elevations.

Using the data from the intact core experiments, measurements of anammox, denitrification and benthic oxygen uptake from each tidal elevation were pooled, and the mean rate for each month determined. There was significant ($p<0.05$, ANOVA) seasonal variation in anammox, denitrification and oxygen uptake. Despite all of the laboratory incubations being performed at a constant 10°C, all three processes showed a clear seasonal pattern, with maximum rates occurring in the spring and summer compared to autumn and winter (Figure 2.3.1.). Average monthly rates of anammox (Figure 2.3.1a) ranged from a minimum of 1.3 µmol N m^{-2} h^{-1} in October, to a maximum of 22 µmol N m^{-2} h^{-1} in April. Rates of denitrification (Figure 2.3.1b) ranged from a minimum of 10µmol N m^{-2} h^{-1}, in October, to a maximum of 135µmol N m^{-2} h^{-1} in June. Although the rates of oxygen consumption demonstrated a significant seasonal pattern (Figure 2.3.1c), the activity did not fluctuate as greatly as that for denitrification or anammox, which both varied by an order of magnitude. Mean monthly rates of oxygen consumption ranged from a minimum of 677 µmol O_2 m^{-2} h^{-1} in September, to a maximum of 2590 µmol O_2 m^{-2} h^{-1} in June.

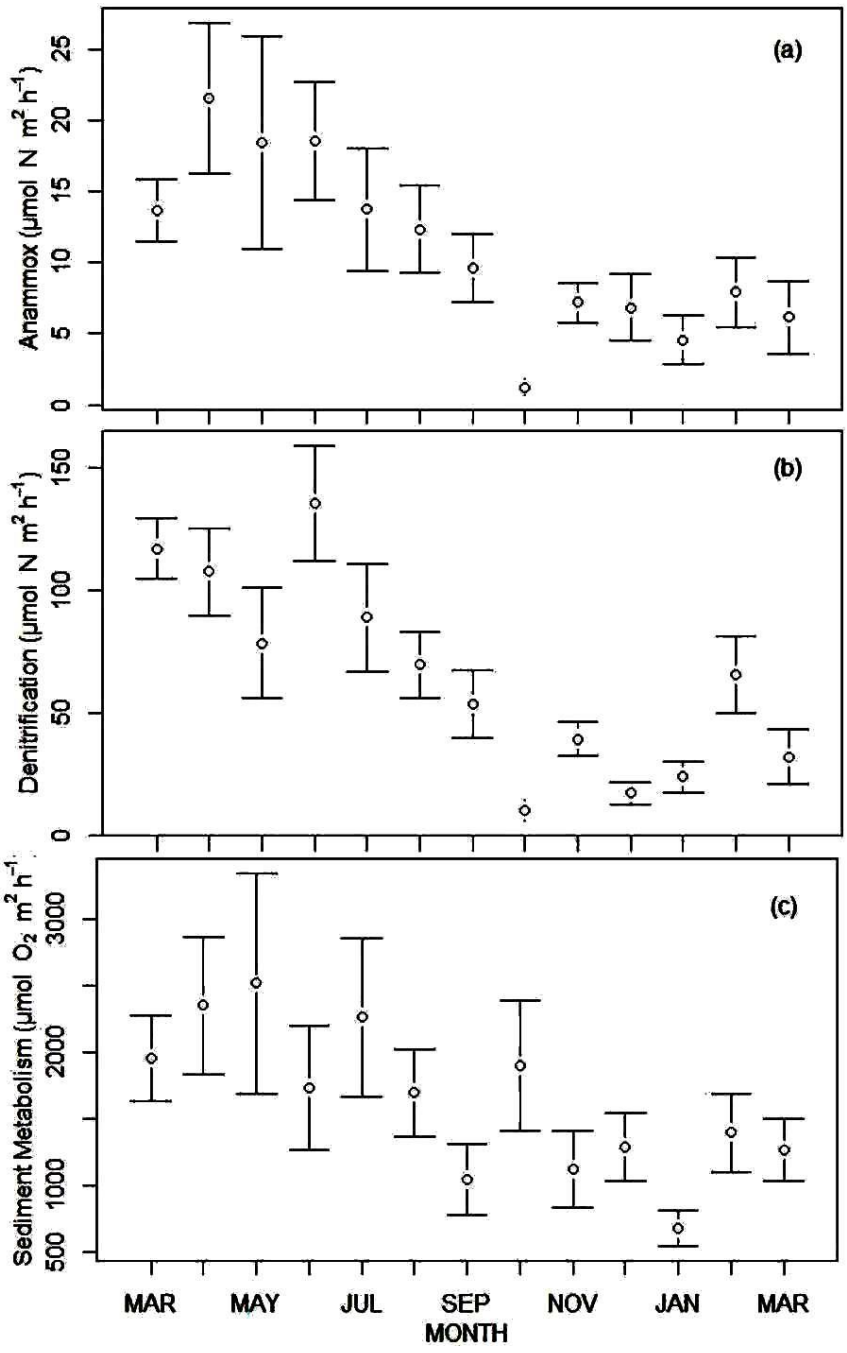


Figure 2.3.1: Means for the monthly pooled data for all 3 tidal elevations showing (a) rates of anammox (µmol N m^{-2} h^{-1}), (b) rates of denitrification (µmol N m^{-2} h^{-1}) and (c) sediment metabolism (µmol O_2 m^{-2} h^{-1}). Error bars represent the 95% confidence interval.

2.3.5. Seasonal variation in oxygen penetration and bioirrigation in sediment cores

There was a significant increase in O_2 penetration depth from ELW to EHW with a mean annual depth of 6.5mm at ELW to 9.9mm at EHW (data not shown). Figure 2.3.2. shows examples of sediment oxygen profiles from the three tidal elevations (May 2010). Spikes of oxygen away from the diffusional gradient clearly illustrates how bioirrigation affects the diffusion of oxygen from the overlying water into the anoxic layers of sediment and how there is an increase in intensity of spikes as tidal elevation is increased.

There was a clear seasonal and tidal elevation effect on oxygen penetration within the seasonal data, illustrated by Figure 2.3.3. There was a greater intensity of bioirrigation in spring and summer compared to autumn and winter. Bio-indices, calculated by integrating the variation under the oxygen profiles, are shown for each of the tidal elevations in Table 2.3.4., and clearly demonstrate variation in the intensity of bioirrigation both seasonally and over the three tidal elevations, with bio-index at EHW showing a much bigger range than either of the other elevations, where the maximum values of bio-index were almost three times higher than the maximum value at either of the other two sites.

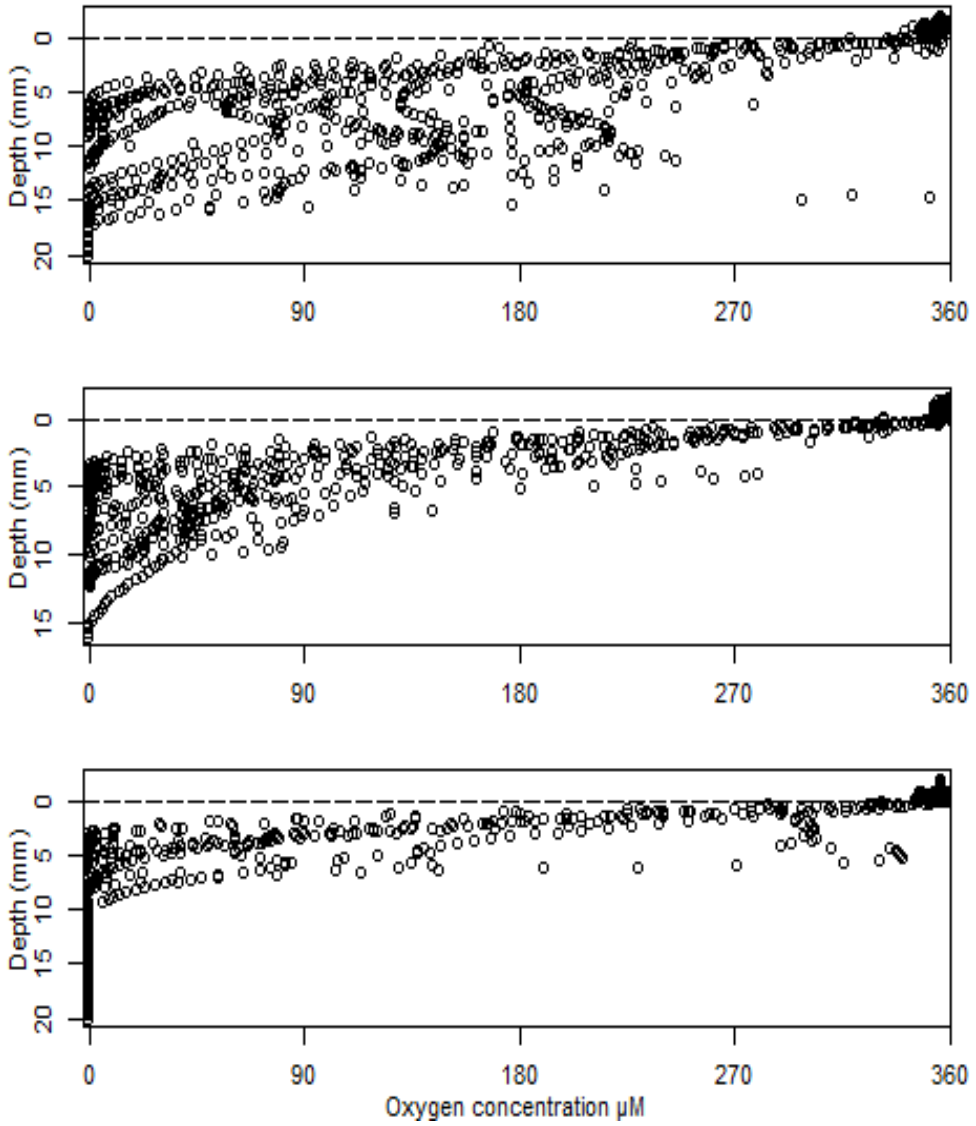


Figure 2.3.2: Examples of sediment oxygen profiles measured in May 2010 for (a) EHW (b) MW and (c) ELW. Spikes in the oxygen profiles show where bioirrigation has occurred.

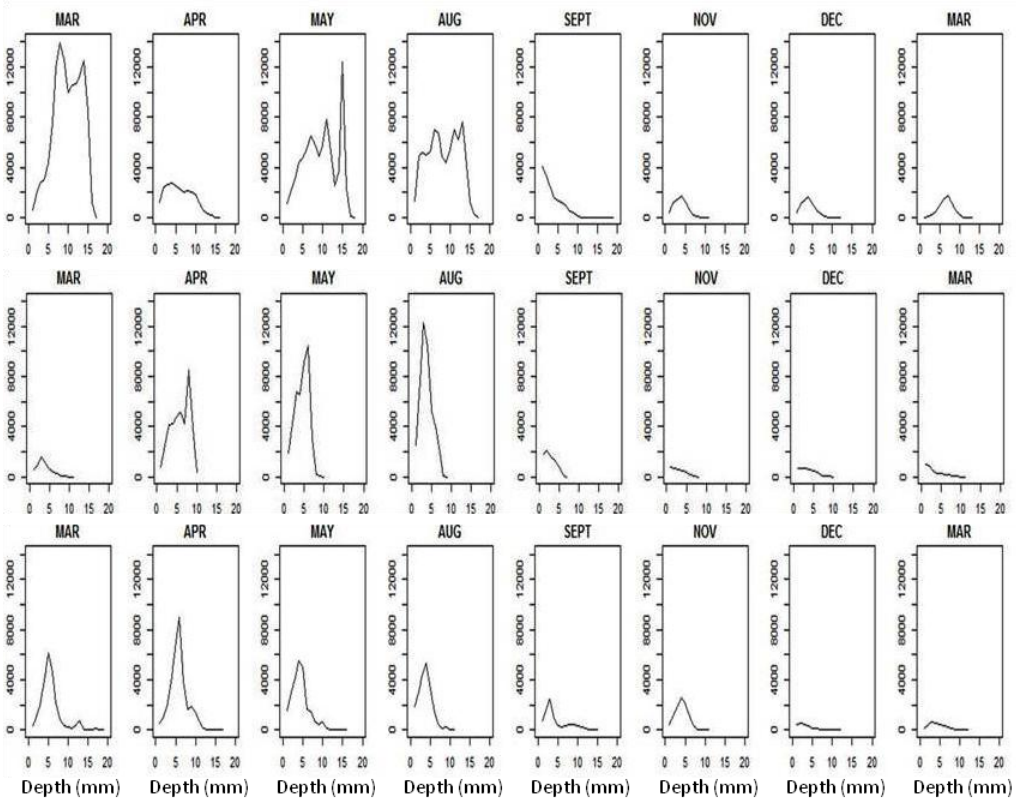


Figure 2.3.3: Variation in oxygen profiles between the depths of 0mm and 20mm, measured in intact sediment cores, where 0 is the sediment surface. EHW (top) MW (middle) and ELW (bottom row). To determine bio-index values the area under the variation was integrated (Table 2.3.4. and Figure 2.2.3.)

Table 2.3.4: Bio-indices derived from the variation in the O_2 profiles for each month calculated by integrating the area under variation.

	BIO-INDEX		
Month	**ELW**	**MW**	**EHW**
March	23000	5000	123000
April	33000	38000	24000
May	23000	41000	78000
August	19000	43000	77000
September	8000	7000	14000
November	9000	3000	7000
December	2000	3000	6000
January	3000	1000	7000

2.3.6. The influence of tidal elevation on anammox, denitrification and oxygen consumption

To examine the effect of tidal elevation on rates of anammox, denitrification and oxygen consumption, data for all months were combined and averaged to determine the overall response of anammox, denitrification and oxygen consumption to tidal elevation. For anammox, there was an overall significant (p<0.05) increase in the rate with tidal elevation from ELW to EHW (Figure 2.3.4a), from an annual mean of 8 µmol N m^{-2} h^{-1} at ELW to 14 µmol N m^{-2} h^{-1} at EHW. There was, however, no significant relationship between tidal elevation and the rate of denitrification (Figure 2.3.4b). Annual mean rates of denitrification ranged from 60 µmol N m^{-2} h^{-1} at ELW to 80 µmol N m^{-2} h^{-1} at MW, with a slight decrease from MW to EHW with a rate of 74 µmol N m^{-2} h^{-1}. Although there did appear to be an increase in oxygen consumption with tidal elevation, it was not significant, with rates ranging from 1480 µmol O_2 m^{-2} h^{-1} at ELW to 1744 µmol O_2 m^{-2} h^{-1} at EHW (Figure 2.3.4c).

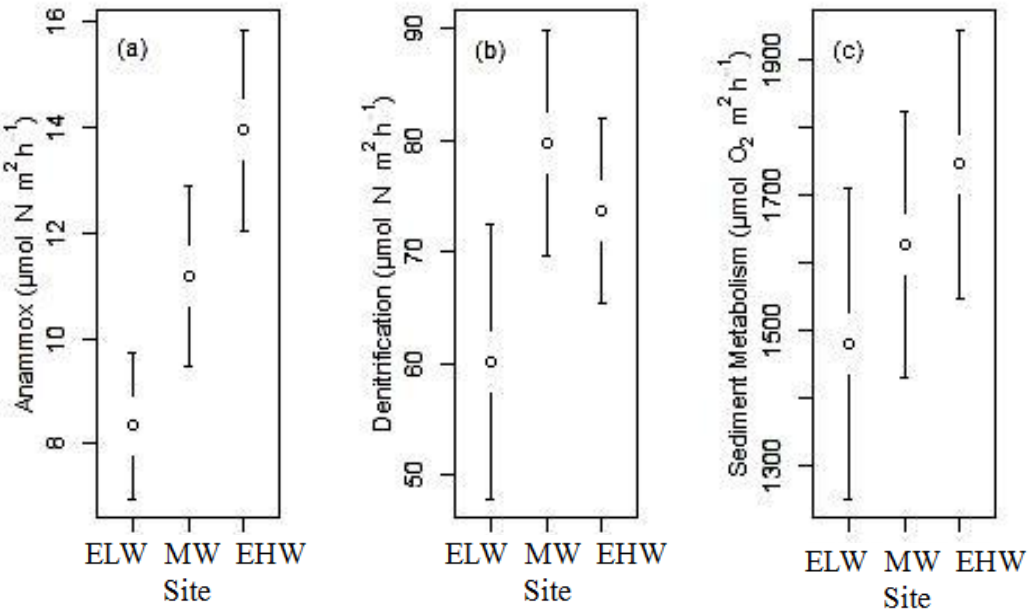


Figure 2.3.4: Annual averages for rates of (a)anammox, (b) rates of denitrification and (c) sediment metabolism for ELW, MW and EHW (all units µmol m $^{-2}$ h^{-1}). Error bars represent standard error of the mean.

2.3.7. Mixed effects model to examine how anammox responds to inundation by overlying estuarine water, bioirrigation, oxygen consumption and denitrification.

In order to explore the combined effects of bioirrigation, sediment metabolism (as represented by oxygen consumption) and denitrification on anammox at different tidal inundations, a mixed effect model was used. For the modelling, instead of tidal elevation, inundation was used: from 90% (0.9) at ELW to 50% (0.5) at MW and 10% (0.1) at EHW.

Data exploration revealed no obvious outliers in rates of denitrification, oxygen consumption or bio-index and no collinearity between the continuous covariates and month. Multi panel scatter plots (Figure 2.3.5.) indicated that the relationship between anammox and denitrification changed for different values of inundation, clearly shown in Figure 2.3.6. Using likelihood ratio tests (see protocol, Zuur et al., 2009) the following covariates were dropped: bio-index × inundation interaction (L-ratio=0.09, DF=1, p=0.76); rate of denitrification × bio-index (L-ratio= 0.44, DF=9, p=0.51); bio-index (L-ratio=1.24 ,DF=8 , p=0.27); sediment metabolism × inundation of overlying estuarine water (L-ratio=3.53 , DF=7, p=0.60) and sediment metabolism (L-ratio=1.04, DF=6 , p=0.31).

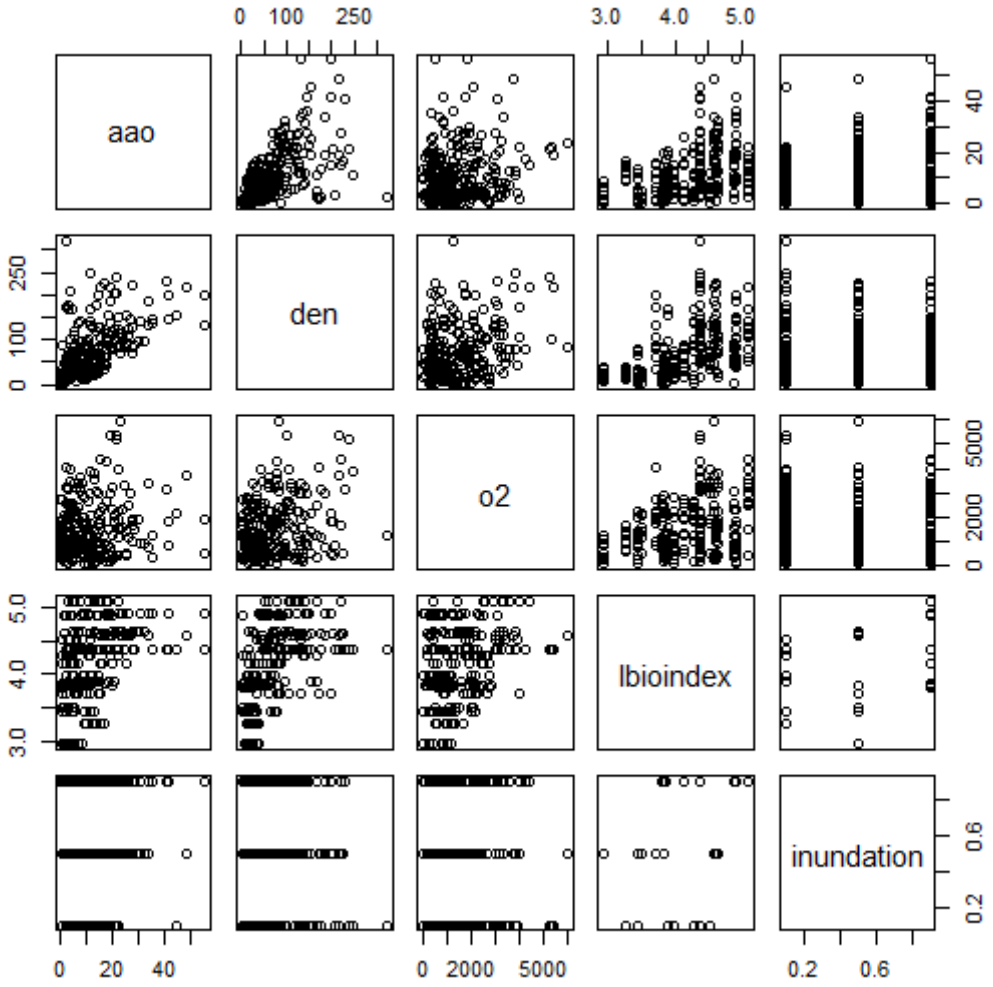


Figure 2.3.5: Multi panel scatter plot of the rates of anammox (aao, µmol N m^{-2} h^{-1}) against the explanatory covariates: denitrification (den, µmol N m^{-2} h^{-1}), sediment metabolism (O_2, µmols O_2 m^{-2} h^{-1}), bioturbation as indicated by bio-index (log transformed bio-index, lbioindex) and inundation (inundation).

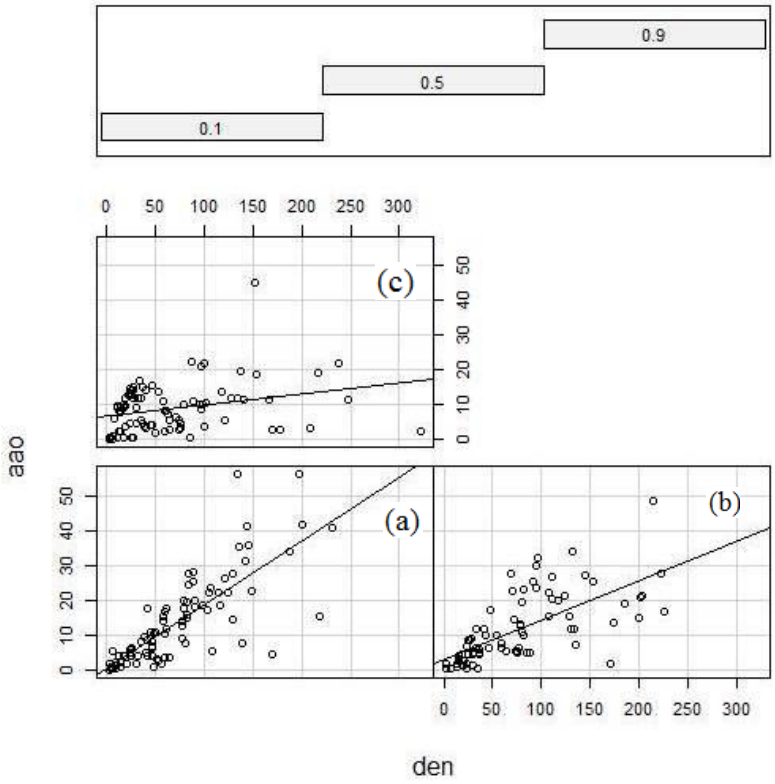


Figure 2.3.6: Co-plot for rate of anammox (aao, µmol N m^{-2} h^{-1}) against denitrification (den; µmol N m^{-2} h^{-1}) for different levels of tidal inundation (a) 0.1 (EHW) (b) 0.5 (MW) and (c) 0.9 (ELW).

The optimal model (Figure 2.3.7.) contained the main term of rate of anammox as a function of rate of denitrification and percentage of time inundated (%) and also the interaction between denitrification and inundation. Estimated parameters standard errors, *t*-values and *p*-values are given in Table 2.3.5. Bio-index was included in this model

Table 2.3.5: Table of the fitted mixed effects model parameters for the dataset with the bio-index covariate included.

	Value	Standard error	DF	t-value	p-value
(Intercept)	0.46	2.17	233	0.22	0.83
Denitrification	0.19	0.02	233	11.2	0.000
% inundation	7.58	3.50	22	2.17	0.0416
Denitrification × inundation	-0.19	0.03	233	-7.22	0.000

The graphical representation of the model (Figure 2.3.7) illustrates a clear variation in the relationships between anammox and denitrification for the three tidal elevations, inundated for different periods of time. The relationships between anammox and denitrification steepened as inundation decreased at the higher tidal elevations. As a consequence of the application of the mixed effects model, samples from the same site are correlated with a value of 0.38 (interclass correlation).

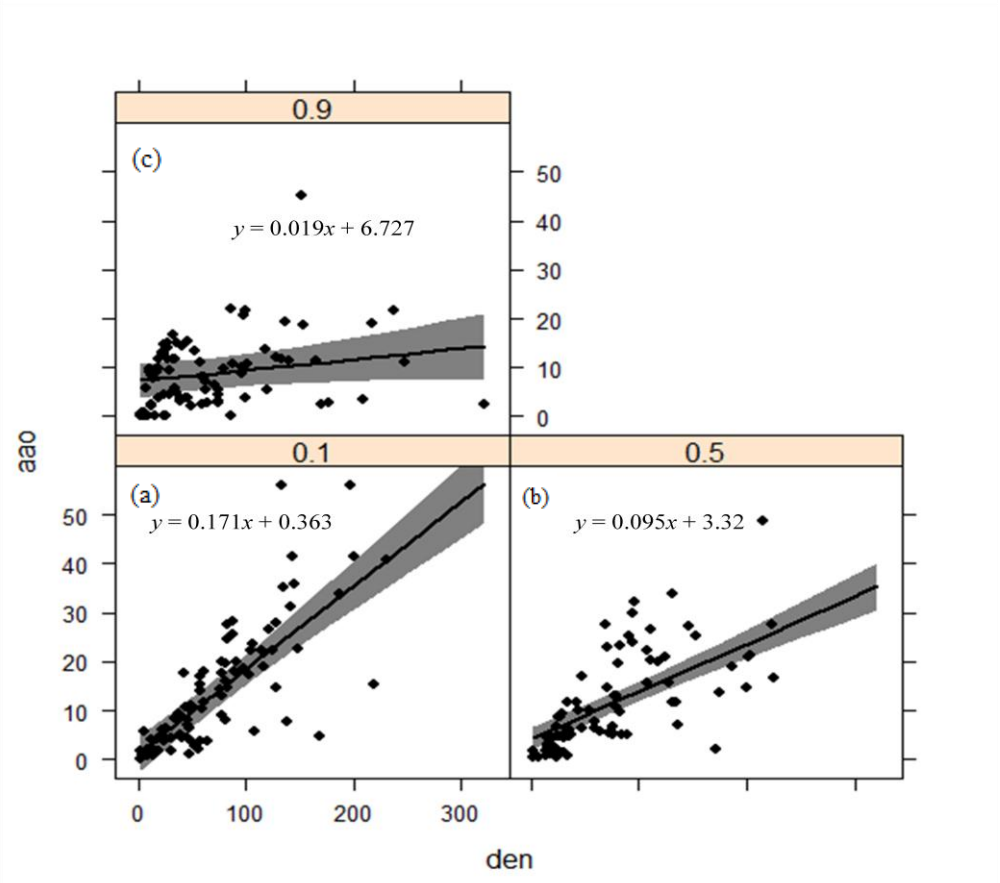


Figure 2.3.7: Scatter plots of rates of anammox (aao µmol N m^{-2} h^{-}) against rates of denitrification (den µmol N m^{-2} h^{-1}) for varying inundations (a) 0.1 (10%, EHW), (b) 0.5 (50%, MW) and (c) 0.9 (90%, ELW) Bio-index covariate was included in this mixed effect model. See table 2.3.5. for parameters of the fitted mixed effects model for the dataset with the bio-index covariate included.

As a bio-index was not available for every sampling date, the previously modelled dataset was a reduced set, therefore to complete the analysis, the larger data set, but excluding bio-index, was used. The number of random intercepts increased from 24 to 35 as more sampling dates were included and the main term and interactions including bio-index were removed. This analysis confirmed the previous results, and the model contained the same terms. Estimated parameters, standard errors, *t*-values and *p*-values are given in Table 2.3.6., and a graphical representation is given in

Figure 2.3.8. In a similar way to the previous model, there are clear relationships between anammox and denitrification, which increased as tidal inundation decreased. The slope for the model at ELW, inundated for 90% of the time, was steeper when all data was considered.

Table: 2.3.6: Table of the fitted mixed effects model parameters for the whole seasonal dataset.

	Value	**Standard error**	**DF**	**t-value**	**p-value**
(Intercept)	1.33	1.94	354	0.69	0.4936
Denitrification	0.17	0.02	354	10.8	0.0000
% inundation	3.81	3.26	34	1.17	0.25
Denitrification × inundation	-0.12	0.03	354	-4.64	0.0000

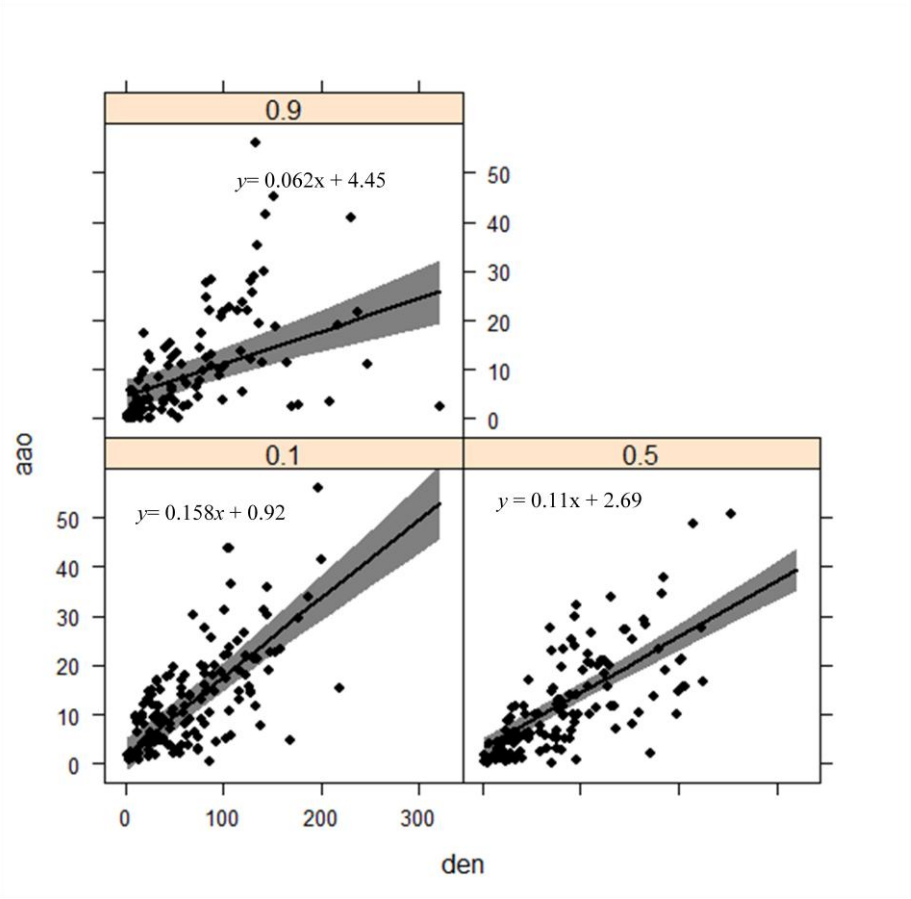


Figure 2.3.8: Scatter plots of rates of anammox (aao, µmol N m^{-2} h^{-}) against rate of denitrification (den µmol N m^{-2} h^{-1}) for varying exposure rates: 0.1 (10%) EHW, 0.5 (50%) MW and 0.9 (90%) ELW, with fitted models for each inundation for the whole dataset. The covariate bio-index was removed for this mixed effect model and the entire dataset used. (For parameters see Table 2.3.6).

2.4. Discussion

Anammox was present in the estuarine sediments of the River Medway throughout the year, with a mean rate of 12 μmol N m^{-2} h^{-1} compared to 72 μmol N m^{-2} h^{-1} for denitrification. There was large variation in the rates of anammox, denitrification and oxygen consumption throughout the year, all following a similar seasonal pattern with greatest rates in the spring and summer (Figure 2.3.1). Mixed effect modelling showed a clear relationship between anammox and denitrification. Furthermore the slope of the relationship became steeper at the higher tidal elevations.

Seasonal studies

Rates of anammox followed a clear seasonal pattern with greatest rates in the spring and summer and a mean annual rate of 12 μmol N m^{-2} h^{-1}, which is in the range reported by the limited number of studies that have been carried out using intact estuarine sediments cores (Trimmer et al., 2006, Risgaard-Petersen et al. 2004b). Previously Rooks et al., (2012) had reported a *ra*% of up to 37% using anaerobic slurries from the same location. The mean annual relative contribution of 19% reported in the current study is considerably lower, however it is higher than the results for the majority of estuarine sediments to have been reported (Nicholls and Trimmer, 2009, Risgaard-Petersen et al., 2004, Risgaard-Petersen et al., 2005, Trimmer et al, 2003, Rysgaard et al., 2004, Trimmer et al., 2003).

When the *ra*% from the intact sediment cores and anaerobic slurries were compared, no significant relationship was found ($p < 0.005$) between the relative contributions of anammox to N_2 production. This indicates that using anaerobic sediment slurries

to measure rates of anammox and denitrification isn't comparable to using sediment cores. This is probably the consequence of a range of factors.

Firstly, sediment slurries destroy the natural stratification and substrate gradients within estuarine sediments. Anammox relies on external sources of NO_2^- which are produced throughout the upper sediment layers including in both the oxic and anoxic zones. By homogenising and creating an anoxic environment it is likely to create disparity between sediment cores and slurries through the removal of sources of NO_2^- for anammox. Secondly, sediment slurries were incubated at a higher temperature (20^{o}C) compared to the intact sediment cores (10^{o}C). Previous studies (Rysgaard et al. 2004, Dalsgaard and Thamdrup 2002) demonstrated that temperature has an effect on ra% with different optimum temperatures for both anammox and denitrification. If the optimum temperatures were to change seasonally for either process this could influence the relative contribution of anammox to N_2 production due to the experimental temperature difference between slurries and intact cores. Thirdly as the sediment is sampled by hand an element of human error could be introduced. Slight variation in the volume of sediment collected between dates, or a slight increase in the depth of anoxic sediments collected, would have an effect, as much of the biogeochemical processing of N_2 occurs over a fine scale (mm). In particular, the anammox community may only inhabit a thin layer of sediment (Risgaard-Petersen et al. 2004, Meyer et al. 2005). Small errors in collection volume could lead to large variation in ra%.

The current work further confirms that sediment slurries are strictly used for assessing the potential of anammox in sediments, with intact sediment cores being

used for quantifying *in-situ* rates, as they are a far more controlled method of sampling, by keeping the sediment strata intact.

As experimental temperature and NO_3^- were kept constant throughout the year, the seasonal variation in anammox activity indicates that the capacity of the sediment for anammox varied seasonally. Previous studies that have included seasonal measurements in estuaries have not provided a clear explanation as to why anammox varies seasonally. Most previous seasonal studies have been conducted at *in-situ* temperature and NO_3^- concentration, using anaerobic sediment slurries making it is impossible to isolate what is driving seasonal variation. In studies of some European estuaries, anammox activity has been reported to be greatest in the winter and spring months (Trimmer et al., 2005 Risgaard et al., 2004, Teixeiria et al., 2012). In contrast, when studying the freshwater sediments of a Chinese river, Zhao et al. (2013) reported anammox activity to be highest during the summer months. Previous studies have suggested that the availability of NO_3^- can regulate anammox, with greatest rates when *in-situ* NO_3^- concentration is highest (Zhou et al., 2013, Teixeira et al, 2012). As NO_3^- concentration is relatively constant throughout the year in the Medway Estuary (Environment Agency, UK) it is unlikely that the seasonal availability of NO_3^- is controlling the capacity for anammox within the sediments of the Medway Estuary.

Despite our lack of seasonal anammox studies there is a wealth of information as to the seasonal dynamics of nitrogen cycling in general, and, in particular, denitrification (Jørgensen and Sørensen, 1985, Jørgensen, 1989, Dong et al., 2000, Jaeschke et al., 2009, Trimmer et al., 2000, Berg et al., 1998, Rysgaard et al., 1995).

In the present study, rates of denitrification were greatest in the spring and summer following the same seasonal pattern as anammox. Other studies when investigating seasonal variation have measured a similar seasonal pattern in rates of denitrification with greater rates in the summer months (Rysgaard et al., 1995). In contrast, Cabrita and Brotas (2000) and Hietanen and Kuparinen (2008) reported maximum rates of denitrification in the estuarine sediments from a Portuguese estuary in Autumn and Winter. Dong et al., (2000), when studying denitrification in the sediments of the Colne estuary over several years did not establish a seasonal pattern that repeated in consecutive years. Again, these studies were conducted at *in-situ* NO_3^- concentration and temperature, so it is unclear whether the actual capacity for denitrification varied as was reported here, or whether it was stimulated by higher temperatures and a greater availability of NO_3^-.

In the present study, the maximum rates of oxygen uptake and denitrification were measured in spring and summer indicates an increase in total sediment metabolism and therefore an overall increase in the bioavailability of organic carbon (Glud, 2008). Furthermore the strong seasonal pattern in sediment metabolism mirrored anammox and denitrification activity. It is unsurprising that rates of denitrification appeared to increase at times with a greater bioavailability of organic carbon as denitrification is generally considered to be a heterotrophic process, requiring a supply of organic carbon. Risgaard-Petersen et al. (2004) observed an increase in denitrification during spring and summer. Whereas anammox activity in their study reduced which resulted in a lower relative contribution of anammox to N_2 production. This was attributed to an increase in organic carbon being utilised by the

heterotrophic denitrifying bacteria which, in effect, "drowned out" the *ra*%, suggesting that an increase in organic carbon was stimulating denitrification but not anammox. However as the activity of anammox and denitrification at the Medway estuary both followed a similar seasonal pattern, this clearly was not the case here.

To date, anammox has only been detected as a chemolithoautotrophic process and therefore does not directly require a source of organic carbon (Jetten et al. 1998). However as anammox is coupled to an external source of NO_2^- and NH_4^+, whose production is heterotrophically mediated in estuarine sediments, it is highly likely that anammox is regulated by the bioavailability of organic carbon, at least to some extent, as previously suggested in other studies (Engström et al., 2005, Nicholls and Trimmer, 2009, Rooks et al., 2012, Trimmer and Engström, 2011, Trimmer et al., 2003). The similar seasonal patterns in anammox, denitrification and sediment metabolism in the current study do indeed suggest that anammox is regulated by the bioavailability of organic carbon.

In estuarine sediments, NH_4^+ is generally in excess (Dollar et al., 1991, Ogilvie et al., 1997). However the availability of NO_2^- in the Medway Estuary was two orders of magnitude lower (Environment Agency, UK). The majority of NO_2^- production in anoxic layers of estuarine sediments is a direct result of heterotrophic NO_3^- reduction (Meyer et al., 2005), and sediment consumption has been shown to balance production in the nearby Thames Estuary (Trimmer et al., 2003). In the sediments from the Medway, Rooks et al. (2012) measured CO_2 production over a range of sediment depths which positively correlated with anammox activity, suggesting that anammox was reducing heterotrophically produced NO_2^-. This

would indicate that anammox is to some extent regulated by the bioavailability of organic carbon, through its reliance on heterotrophic NO_3^- reduction to provide NO_2^-. In contrast, Zhao et al. (2013) did not establish a significant relationship between dissolved organic carbon (DOC) content and anammox when measuring seasonal variation in anammox. However they did not measure sediment metabolism. And while organic carbon may have been highest in the winter months, when anammox activity was lowest, the DOC may not have been in a bioavailable form which the microbial heterotrophic community could utilize.

In the current study there was a clear seasonal pattern in bioirrigation, which, broadly followed rates of denitrification, anammox and oxygen uptake. The method used provided a good estimation of bioirrigation with much higher bio-index values calculated during months with the highest bioirrigation and values orders of magnitude lower when bioirrigation was absent. However, although the method for quantifying the extent of bioirrigation was useful, there were some limitations, as mentioned previously (Figure 2.2.3). Slight variation between oxygen profiles in un-bioirrigated sediments did indeed indicate that bioirrigation was present during the winter months when it wasn't. Furthermore when sediments were heavily bioirrigated, spikes in oxygen from different individual oxygen profiles had similarly high oxygen concentrations at similar depths which would have therefore underestimate the coefficient of variation.

The seasonal variation in bioirrigation, with a greater intensity over the summer months, was similar to that reported by Rysgaard et al. (1995), where increased

bioturbation was observed between May and November. This has the potential to increase anammox and denitrification in two main ways. Firstly, through bioturbation, the sediment can be reworked distributing organic carbon throughout the anoxic and oxic layers and stimulating the heterotrophic processes such as NO_3^- reduction and aerobic NH_4^+ oxidation increasing NO_2^- production (Mermillod-Blondin et al., 2004). In addition, the bioirrigation of burrows with NO_3^- and O_2 rich surface water would extend the penetration of oxygen and NO_3^- into the anoxic sediment layers (Henriksen et al., 1980, Mermillod-Blondin et al., 2004, Hansen and Kristensen, 1998).

This was illustrated in the current study at the Medway Bridge Marina, with rapid increases in oxygen concentration deep into the anoxic sediment when profiling the sediment strata. This could potentially increase anammox through an increase in the supply of NO_2^- through either aerobic oxidation of ammonia by providing oxygen to the usually anoxic layers of sediment where NH_4^+ concentrations are high and also NO_3^- reduction through an increased supply of NO_3^- into the anoxic layers of sediment. Nielsen (2004) reported an increase in NO_3^- reduction of as much as 82% as the direct result of microbial activity at the oxic / anoxic interface in the burrow walls. Furthermore Rysgaard et al., (1995) demonstrated that the presence of macro-fauna rapidly increased rates of coupled nitrification-denitrification, which could potentially be utilized by the anammox community, driving the seasonal variation.

With regard to overall seasonal effects, there was a significant seasonal pattern in anammox, denitrification, sediment metabolism and bioirrigation, all of which varied collinearly. It is likely that the availability of organic carbon would appear to

be driving NO_3^- reduction, which includes denitrification, providing a supply of NO_2^- to anammox. The presence of bioturbation and bioirrigation during the summer months will likely aid both nitrification and denitrification through the reworking of organic carbon into the sediment and providing a supply of electron donors (O_2, NO_2^-, NO_3^-) for both heterotrophic processes such as denitrification and chemolithoautotrophic anammox.

Tidal elevations

Elevation determines an array of biogeochemical and physical parameters from the amount of time sediment is inundated with the NO_3^- rich overlying water, which was maximal at the lowest tidal elevation, to the degree of bioirrigation, which increased significantly as tidal inundation decreased at the higher tidal elevations. Sediment characteristics also varied with tidal elevation, with a greater porosity as well as smaller grain size distribution at the higher tidal elevations.

There was no significant variation in the annual mean rate of denitrification between the three sites. This would suggest that the denitrifying community is not affected by the length of exposure to NO_3^-. Denitrification is an organotrophic process (Zumft, 1997), preferentially using O_2 as its electron donor and switching to NO_3^- when concentration of O_2 becomes low. Therefore the denitrifying community can maintain an active metabolism when exposed from the overlying water by respiring O_2 and switching to NO_3^- when inundated.

There was an increased mean annual rate of anammox at the higher tidal elevations, which are inundated with the NO_3^- rich water for progressively shorter periods of time. Anammox, unlike denitrification, cannot use an alternative electron donor and is therefore reliant on a source of NO_2^-, which is likely to become substantially less available as a large proportion of NO_2^- in the anoxic sediments is derived from the reduction of NO_3^- (Meyer et al. 2005). Risgaard-Petersen et al. (2004) observed that NO_x^- must be present in the overlying estuarine water for an anammox community to be present and, furthermore, that a reduction in the NO_3^- concentration of the overlying water coupled to the presence of MPB lowered the NO_x^- sufficiently to reduce the capacity for anammox within a sediment (Risgaard-Petersen et al., 2005). However, as mentioned previously, the current study suggested the opposite, with an increased mean annual rate of anammox at the higher tidal elevations, which have considerably less access to the NO_3^- from the overlying water and therefore probably a much lower production of NO_2^- in the anoxic sediments (Meyer et al., 2005).

Mixed effect models

Mixed effects modelling indicated there was a highly significant positive relationship between anammox and denitrification activity at each tidal elevation, which suggests that the relative contribution of anammox to N_2 production (*ra*%) at each site does not vary a great deal throughout the year. Rysgaard et al. (2004) proposed a coupling between the two processes, suggesting that denitrification could in fact supply NO_2^- to the anammox community and hypothesized that a correlation between rates would be observed. Trimmer and Engström (2011) compiled a set of anammox and denitrification data from sediments located around the world and did

indeed notice a significant correlation, suggesting that denitrification supplies at least some NO_2^- to anammox. More recently, Zhao et al., (2013) established a similar relationship in riverine sediments. However, not only did the present study establish a positive relationship at each tidal elevation, but also as tidal elevation increased, the dependence on denitrification to supply anammox with NO_2^- increased considerably. The increasing relationship between anammox and denitrification was observed in both the reduced data set that included bioirrigation data (bio-index) and the larger data set. The fitted models for each tidal elevation can be observed in the table below (Table 2.4.1).

Table 2.4.1: Model parameters for determining the response of anammox to denitrification at the 3 tidal elevations for the data including bio-irrigation and the whole data-set.

Tidal Elevation	Inundation	Bio-irrigation data included	Whole data-set
EHW	0.1	y = 0.171x + 0.363	y = 0.158x + 1.711
MW	0.5	y = 0.095x + 3.315	y = 0.11x + 3.235
ELW	0.9	y = 0.019x + 6.727	y = 0.062x + 4.759

The sediments at the different tidal elevations investigated at the Medway Bridge Marina will have very distinct NO_x^- regimes. For instance, sediments located at ELW will have a near constant supply of NO_3^- supplied when inundated by the overlying estuarine water. Therefore most of the supply of NO_2^- to the anoxic

sediments will be provided from the reduction of NO_3^- by the NO_3^- reducing community (Meyer et al., 2005, Steif and De Beer, 2002). NO_3^- concentration does not vary greatly throughout the year at the Medway estuary (Environment Agency, UK), with permanently high concentrations providing a near constant pool of NO_2^-, located over a very consistent range of sediment depths regulated by the stable NO_3^- concentration of the overlying water. And as there was considerably less bioirrigation at the lower elevation, this would result in a more uniform biogeochemical and microbial profile.

In estuarine sediments, nitrification is often coupled to denitrification (Jenkins and Kemp, 1984, Sebilo et al., 2006, Zumft, 1997). As tidal inundation decreases it is likely that denitrification must rely on NO_3^- produced from the aerobic oxidation of ammonia. With this supply of NO_3^-, the denitrifiers could in turn, through the reduction of NO_3^-, provide a supply of NO_2^- for the anammox community. Additionally aerobic NH_4^+ oxidation could provide the NO_2^- for anammox as it produces NO_2^- as an intermediary before fully oxidizing the NO_2^- to NO_3^-. Both these scenarios could produce the positive correlations between anammox and denitrification observed in this study. As tidal inundation decreases, and anammox and denitrification rely more heavily on the aerobic oxidation of ammonia to provide NO_3^- and NO_2^-, the relationship would become stronger resulting in a steepening of the relationship. Figure 2.4.1 illustrates a simplified theoretical coupling between the three processes across the oxic/anoxic interface. The borderline significant interaction between O_2 uptake and inundation, with higher rates of oxygen consumption at EHW, would suggest that, if anything, the bioavailability of organic

carbon increased with tidal elevation. This may suggest that the availability of organic carbon is stimulating coupled nitrification-denitrification which could in turn provide NO_2^- to anammox.

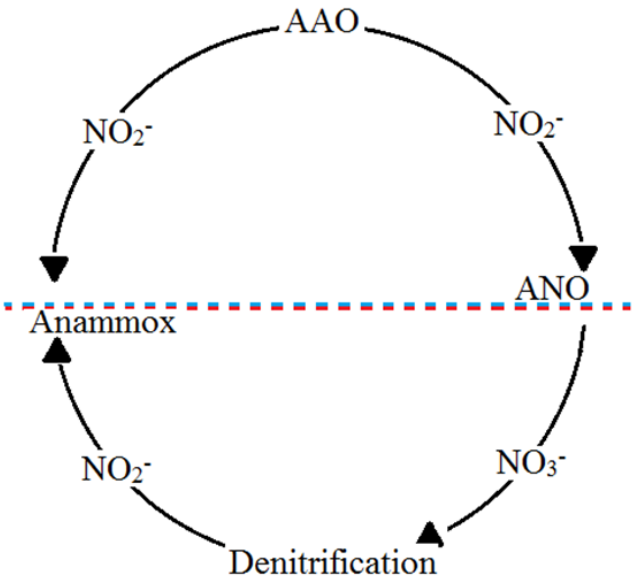


Figure 2.4.1: A simplified theoretical three-way coupling between the two-step process nitrification which involves aerobic ammonium oxidation (AAO) to NO_2^- followed by aerobic nitrite oxidation (ANO) , denitrification and anammox, which occur both vertically in the sediment profile and across the anoxic/oxic interface in the burrows of bioirrigating fauna. The blue and red dotted line represents the oxic /anoxic interface.

There was a significant increase in bioirrigation with increased tidal height, from an average annual bio-index of 15000 at ELW to 45000 EHW. As mentioned previously bioturbation and bioirrigation can enhance a sediments ability to process fixed nitrogen (Mermillod-Blondin et al., 2004). Additionally, the bioirrigation of burrows is intermittent (Mermillod-Blondin et al., 2004) which creates a dynamic O_2

regime within the sediment. For instance, when the *N. Diversicolor* irrigates its burrow, O_2 is pumped into the sediment allowing nitrification to proceed. When bio-irrigation stops, the sediment quickly becomes anaerobic, (Kristensen, 1984, Mermillod-Blondin et al., 2004), which would allow anammox and denitrification to utilize the remaining NO_x^-. Through the increased bioirrigation, and therefore nitrification, which provides NO_3^- to the anoxic sediments a larger denitrifying community is maintained than may be expected. As anammox is tightly coupled to the denitrifiers through the NO_2^- they provide, bioirrigation, in turn, further increases the relationship between the two processes. As mentioned previously Nielsen (2004) reported a huge increase in NO_3^- reduction in bioturbated sediments. NH_4^+ oxidation could provide a supply of nitrate to the NO_3^- reducing community, in turn producing NO_2^-, supporting an increased anammox community throughout the sediment strata and therefore producing N_2 at higher rates than the lowest elevation when inundated by the overlying water.

To summarise, the increasing relationship between anammox and denitrification is likely to be due to the reliance of both anammox and denitrification on aerobic ammonia oxidation to supply NO_x^- either through an intermediary in NO_3^- reduction via denitrification or directly through the aerobic oxidation of ammonia. This essentially couples the three processes together both spatially and through a symbiotic sharing of substrates whereby it would be impossible for both denitrification and anammox to occur without the presence of an NH_4^+ oxidizing community. Bioirrigation, which is far more intense at the higher tidal elevations,

increases rates of both nitrification and denitrification, which could, in turn, increase rates of anammox, and therefore increase the relationship between the processes.

This study was the first in depth seasonal study using sediment cores to examine the capacity for anammox and denitrification in estuarine sediments. It confirmed the presence of an anammox community throughout the entire year and demonstrated that there is significant seasonal variation in rates of anammox, denitrification and benthic oxygen consumption. It is likely that increased rates of heterotrophic denitrification are driven by the availability of bioavailable organic carbon as both were greatest in the spring and summer months compared to the rest of the year. Furthermore the increased heterotrophic NO_3^- reduction is supporting a larger capacity for anammox in the spring and summer.

Investigating anammox activity at different tidal elevations, and consequently inundation from the NO_3^- rich overlying water established that, surprisingly, the anammox capacity of the sediment slightly increased. Furthermore tidal elevation increased the coupling between anammox and denitrification as length of inundation from the overlying estuarine water decreased. This may suggest a three way coupling between nitrification, denitrification and anammox, as aerobic NH_4^+ oxidation is likely to be the only source of NO_x^- at times when the sediment is not inundated by the overlying water. Additionally bioirrigation and bioturbation enhance this capacity by transporting substrates to areas of the sediment where it is required, increasing anammox capacity and its coupling with denitrification.

CHAPTER 3: FACTORS THAT AFFECT THE RATE OF ANAMMOX

3.1. Introduction

The second chapter demonstrated that rates of anammox varied seasonally with greater rates in the spring and summer compared to the rest of the year. Rates of denitrification, oxygen consumption and the extent and intensity of bioirrigation all followed a similar seasonal pattern. This suggested that an increase in the availability of organic carbon during the spring and summer stimulated heterotrophic NO_3^- reduction which in turn provided NO_2^- for the anammox community. Surprisingly rates of anammox increased from the lower tidal elevation (ELW) to the highest (EHW). There was a significant relationship between rates of anammox and denitrification. Furthermore tidal elevation had a significant effect on the relationship between anammox and denitrification with a steeper slope between the relationship between anammox and denitrification as tidal elevation increased.

NO_x^- must be available in the overlying estuarine water for an anammox community to be present (Risgaard-Petersen et al., 2004). Furthermore it has been demonstrated that concentration of NO_x^- in the overlying water directly affects the sediments potential for anammox (Meyer et al., 2005, Risgaard-Petersen et al., 2004, Risgaard-Petersen et al., 2005). Teixeira et al. (2012) demonstrated increased potential rates of anammox with higher concentrations of NO_3^-, however, these seasonal measurements were conducted at different experimental temperatures, which could confound any potential relationship with the NO_3^- concentration.

As mentioned previously, it is NO_2^- and not NO_3^- that anammox reduces (van de Graaf et al., 1997, Mulder et al., 1995), therefore any NO_3^- present in the overlying water must first be reduced to NO_2^- by the NO_3^- reducing community. For anammox to take advantage of an increase in the NO_3^- in the overlying water, the NO_3^- reducers must be able to increase reduction rates so that an increased concentration of NO_2^- is reflected within the anoxic layers of sediment. As NO_3^- concentration of the overlying water increases, so does the penetration depth (Meyer et al., 2008). This will extend the zone of NO_2^- production deeper into the layers of sediment (Meyer et al., 2005). As the penetration of NO_3^- increases, so does the depth over which denitrification occurs, as denitrifying bacteria begin to respire NO_3^-, producing NO_2^- as an intermediary (Meyer et al., 2008, Neubacher et al., 2012). It is, however, unknown whether the anammox community can take full advantage of the greater availability of NO_2^- as they have been shown to be slow growing (Strous et al., 1999) and would take time to expand into the new NO_2^- producing region.

The main seasonal study (Chapter 2) was conducted throughout the year at a constant experimental temperature (10^oC), and therefore could not demonstrate how seasonal temperature variation could potentially affect rates of both anammox and denitrification. Previous studies have demonstrated that both anammox and denitrification respond to temperature with rates rising until their respective optimum temperatures are reached, which have been reported to be lower for anammox than denitrification (Rysgaard et al. 2004, Dalsgaard & Thamdrup 2002). In arctic marine sediments, Rysgaard et al. (2004) demonstrated that rates of

anammox peaked at the lower temperature of 12^{o}C, whereas the optimum for denitrification was 25^{o}C, which was much higher than the bottom water temperature the sediment would normally be subjected to. Similar observations were made using the marine sediments of the Skagerrak (Dalsgaard & Thamdrup 2002), with the optimum temperature for anammox being slightly higher at approximately 15^{o}C. Optimum rates of denitrification rose rapidly until 15^{o}C with no further change until the temperature reached approximately 35^{o}C where rates declined sharply suggesting that the optimal activity of denitrification occurred across a broad range of temperature.

Temperature data for anammox in estuarine sediments is sparse. Teixeira et al., (2012) demonstrated a rise in rates of anammox up to approximately 15^{o}C suggesting that the optimum temperature for anammox would appear to be in a similar range to that from the colder marine sediments of the Arctic and deep Skagerrak. However, this was reported from seasonal measurements at the actual *in-situ* temperature rather than from a controlled experiment to specifically examine the influence of temperature.

The previously mentioned temperature optima were, however, much lower than those found in wastewater plants reported to be as high as 37^{o}C (Strous et al. 1999; Kuenen & Jetten, 2001). More recent work has detected the potential for anammox at 60^{0}C and 85^{o}C from samples collected at hydrothermal vents from the Mid-Atlantic ridge, which would suggest that an active anammox community can operate under much higher temperatures than those reported for marine and estuarine sediments.

The apparent activation energy reported by Rysgaard et al. (2004) for anammox measured between -1.8°C and 13°C was 0.53eV. However the activation energy for denitrification was higher at 0.62eV, but measured over a wider temperature range between -1.8°C and 25°C. These activation energies are similar to those measured in wastewater reactors (Strous et al., 1999) and sediments from the Skagerrak (Dalsgaard and Thamdrup 2002). This would suggest that similar activation energies might be observed for both anammox and denitrification in a temperate estuary to those reported in marine sediment and wastewater plants.

Yvon-Durocher et al., (2012), calculated an average activation energy of 0.62eV with a range of between 0.57eV and 0.69eV for short term measurements across all aquatic environments for whole ecosystem respiration. Denitrification is a form of respiration which preferentially respires O_2 in aerobic conditions, switching to NO_3^- when O_2 is not present (Zumft, 1997) and therefore the potential apparent activation energy for denitrification should be similar to that of whole system respiration. It was suggested by Yvon-Durocher et al. (2012) that activation energies would vary between sites due to the availability of organic carbon, which supports production of biomass and therefore rates of sediment metabolism. It may therefore be sensible to assume that the apparent activation energies will be affected temporally at a single site by the availability of organic carbon.

The previous chapter demonstrated that rates of benthic oxygen consumption, and therefore bioavailable organic carbon, changed seasonally, with highest rates occurring in spring and summer. By measuring rates of denitrification and anammox across a broad array of temperatures over a seasonal period, variation in the response

of anammox and denitrification to temperature can be measured. Furthermore, by measuring sediment metabolism through benthic oxygen consumption the variation in the availability of organic carbon can also be estimated and whether the temperature response varies seasonally. This will indicate whether N_2 production via denitrification, and in particular anammox, is related to the availability of organic carbon as previously suggested (Chapter 2, Trimmer et al. 2003, Trimmer et al. 2009). In addition, the response of anammox and denitrification to variation in NO_3^- concentration will also be investigated.

This part of the study aims firstly to examine how rates of anammox, denitrification and oxygen consumption will respond to temperature. As the water temperature varied between 4 and 21^oC during the seasonal study, anammox, denitrification and oxygen consumption will be measured across a range of experimental temperatures from 5 to 25^oC. Furthermore, the apparent activation energies will be calculated and any seasonal pattern observed. Secondly the immediate response of both anammox and denitrification to variation in experimental NO_3^- concentration will be measured. Both the temperature and NO_3^- concentration experiments will use intact sediment cores in order to maintain the *in-situ* sediment strata. This should therefore be representative of how the complex array of aerobic and anaerobic processes within the sediment react to changing concentrations of NO_3^- and temperature. This is particularly important as anammox will rely on the reduction of NO_3^- in the anoxic sediments to provide NO_2^- and increased temperature and NO_3^- is likely to affect its production. Finally, rates of anammox, denitrification and oxygen consumption will

be measured over more elevations than in chapter 2 to examine the response to tidal elevation over a finer scale.

3.2. Methods

3.2.1. Sampling site and sediment collection

Samples were collected between July 2010 and January 2011 from the mudflats of the Medway Estuary at the Medway Bridge Marina, UK (Figure 2.2.1). The Medway Estuary is hypernutrified and flows into the Thames Estuary with a spring tidal range of around 6m, and consistently high NO_3^- concentrations with a mean annual NO_3^- concentration of 187µM ±7 (Table 2.2.1., Environment Agency, UK) and with a temperature range between 4^oC in winter and 21^oC in summer (Table 2.2.1., Environment Agency, UK). All samples were collected during low tide. The sediment samples for the temperature and NO_3^- concentration experiments were all collected from the vicinity of the MW tidal elevation. To measure the effect of tidal elevation on rates of anammox, denitrification, benthic oxygen consumption and porosity, samples were taken at 1m intervals from 0.3m above the chart datum.

The number of sediment cores varied for each experiment, and is described in the respective sections. Intact sediment cores were collected for use with the revised isotope pairing technique (Risgaard-Petersen et al., 2003, Risgaard-Petersen et al., 2004b, Trimmer et al., 2006) following the protocol described in Chapter 2, and returned to the laboratory within 4 hrs before being stored in a holding tank filled with seawater adjusted to *in-situ* salinity at 10^oC. Additionally the surface sediment

(top ~2cm) was collected and stored in polythene bags before returning to the laboratory and stored in a refrigerated room at 5^{o}C.

3.2.2. Temperature experiments

To determine how temperature affects rates of anammox and denitrification both anaerobic sediment slurries and intact sediment cores were used. The sediment slurries were used to assess how temperature would affect the potential rates of anammox and denitrification under simple and controlled conditions. Once it was determined that both anammox and denitrification respond to temperature, intact sediment cores were used to provide a more accurate estimate of how rates of anammox and denitrification respond to temperature.

Sediment slurries were prepared by adding 1ml of homogenised surface sediment with low nutrient sea water (1ml) adjusted to *in-situ* salinity (de-ionized water, 6ppt) into gas tight vials (2.5ml Exetainer, Labco). Slurries were prepared in an anaerobic hood to ensure low levels of O_2 and left on rollers overnight so any oxidants remaining (O_2, NO_2^-, NO_3^-) were utilized (Risgaard-Petersen et al., 2004).

Five sediment slurries for each temperature were placed in three temperature controlled rooms (5^{o}C, 10^{o}C, 22^{o}C) and placed on rollers to ensure constant mixing, and left to reach ambient temperature for 1 hour, with a further 5 left unamended to use as references. A concentrated stock of $^{15}NO_3^-$ ($Na^{15}NO_3$ (116mM [98% ^{15}N atom %], Sigma Aldrich) was injected through the septum resulting in an initial

concentration of 800µM. After four hours the experiment was ended by the addition of 100µL 40% CH_2O.

Analysis of the ^{15}N labelling of the N_2 and estimates of the potential rates of anammox and denitrification were calculated (Chapter 2.2.4., equation 2.1 and 2.2). A detailed description of how the ^{15}N labelling of the N_2 pool was analyzed on the mass spectrometer is presented in Chapter 2 (2.2.7), however calibration of the sediment slurries used to determine N_2 concentration was performed using analytical grade N_2 (100%). The calculations to estimate potential rates of anammox and denitrification are described in Chapter 2 (2.2.8).

Cores, were initially stored at 10^oC in a holding tank filled with seawater adjusted to *in-situ* salinity and constantly aerated to maintain a steady oxygen concentration. Five separate experimental temperatures were used (5^oC, 10^oC, 15^oC, 20^oC, 25^oC) each consisting of 10 intact sediment cores. 5 additional intact sediment cores were left unamended with $^{15}NO_3^-$ to be used as references. One hour before starting the experiment for each temperature, the cores were transferred to a second water bath connected to a temperature controlled recirculation chiller (Grant RC350G), which maintained the water temperature for the four hour incubation. The initial concentration of $^{15}NO_3^-$ in the overlying water for all temperature experiments was 100µM. Cores were treated according to the detailed protocol described in Chapter 2 (2.2.5) and rates of anammox and denitrification were estimated using the r-IPT (2.2.8), (Risgaard-Petersen et al., 2003,2004b). To measure the effect of temperature on oxygen consumption, the oxygen concentration was measured before and after the cores were sealed for the 4hr incubation using an O_2 micro sensor

(Unisense, OX 50). Care was taken to ensure that the O_2 sensor was calibrated and kept at the experimental temperature. The O_2 concentration for each temperature was calculated as previously (Chapter 2, 2.2.6).

The apparent activation energies of anammox, denitrification and sediment metabolism were estimated using the temperature data from the sediment cores experiment, however only the apparent activation energies of anammox and denitrification were measured in sediment slurries. The range of data used varied between the different processes, as only data calculated up to the optimum temperature could be used. The activation energy was estimated by using the natural log of the rate of the process plotted against 1/*k*T where *k*=Boltzmann constant= 8.62×10^{-5} eV K^{-1} and T= absolute temperature in Kelvin. The slope of the linear regression is equal to the activation energy (eV) where 1eV =96.49 kJ mol^{-1}. Q^{10} was also determined, which is the factor by which a rate increases over a 10^{o}C rise in temperature and can be calculated using equation 3.1.

$$Q^{10} = \left(\frac{R_2}{R_1}\right)^{10/(T_2 - T_1)} \quad (3.1)$$

Where R_2 is the rate of the respective process at the higher temperature, R_1 is the rate at the initial temperature and T_2 and T_1 are the higher and lower temperatures respectively. Q^{10} is the factor by which the rate of a biological or biogeochemical process, in this case anammox, denitrification or sediment metabolism, increases for every 10^{o}C rise in temperature.

3.2.3. NO_3^- concentration experiments

Intact sediment cores were used to investigate the effect of NO_3^- concentration. For this experiment, starting concentrations were 50, 100, 200, 400 and 800µM $^{15}NO_3^-$. Cores were incubated for four hours at 10^{o}C. Six cores were used for each concentration with a remaining 5 left unamended for use as a reference.

The aim of this experiment was to determine the effects of varying NO_3^- concentrations; however p_{14} from the revised isotope pairing technique, used for all previous core experiments (Chapter 2) (Risgaard-Petersen et al., 2003, Risgaard-Petersen et al., 2004b) is independent of r_{14} so is unaffected by variation in the concentration of $^{15}NO_3^-$. For this the following equations, which are based on equations devised by Rysgaard et al., (2003) during the development of the r-IPT for estimating N_2 production from anammox (A_{15}) and denitrification (D_{15}), were used.

$$D_{29} = 2r_{14} \cdot p^{30}N_2 \quad (3.2)$$

Where D_{29} is the production of $^{29}N_2$ attributed to denitrification, r_{14} is the ratio of $^{14}NO_3^-$ to $^{15}NO_3^-$ in the reduction zone determined from the ratio of $^{45}N_2O$ to $^{46}N_2O$, as suggested by Trimmer (2005) and described in Chapter 2 (2.2.8) and $p^{30}N_2$ is the production of $^{30}N_2$.

$$D_{30} = p_{30} \quad (3.3)$$

The production of $^{30}N_2$ attributed to denitrification (D_{30}) equals the production of $^{30}N_2$ (p_{30}) D_{15} is calculated using the following equation (equation 3.4).

$$D_{15} = 2D_{30} + D_{29} \quad (3.4)$$

A_{29} is the production of $^{29}N_2$ produced by anammox, which equates to the total anammox (A_{total}) and A_{15} is equal to A_{29}

$$A_{15} = A_{29} = p^{29}N_2 - 2 \cdot r_{14} \cdot p^{30}N_2 \quad (3.5)$$

The ratio of $^{15}NO_3^-$ to $^{14}NO_3^-$ (q) can be calculated using equation 3.6 which involves the calculation of r_{14}. The calculation of r_{14} is described in chapter 2 (2.2.8., equation 2.3).

$$q = \frac{1}{r_{14} + 1} \quad (3.6)$$

Using q, and knowing the starting concentration of $^{15}NO_3^-$ in the overlying water of the core, the initial concentration of $^{14}NO_3^-$ can be estimated.

3.2.4. Tidal elevation transect

Porosity was determined for each site from the difference between wet and dry weight of the top 2ml of surface sediment. Potential rates of anammox and denitrification were first estimated using anoxic sediment slurries. Five sediment slurries were prepared for each site in accordance to the protocol described in Chapter 2 (2.2.4). A concentrated stock of $^{15}NO_3^-$ ($Na^{15}NO_3$ (116mM [98% ^{15}N atom %], Sigma Aldrich) was injected through the septum resulting in an initial concentration of 250µM $^{15}NO_3^-$. The experiment was ended after two hours using ZnCl (50 µl, 50%) before analysis on a mass spectrometer following the protocol described in chapter 2 (2.2.7). Potential rates of anammox and denitrification were calculated following the calculations described in Chapter 2 (2.2.4, equations 2.1 and 2.2).

Six intact sediment cores from each elevation were then used to estimate *in-situ* rates of anammox, denitrification and benthic oxygen consumption following the protocol described in Chapter 2 (2.2.6). Mass spectrometric analysis was performed as described previously, and estimated rates of anammox and denitrification were calculated using the r-IPT (Risgaard-Petersen et al., 2003,2004b) following the detailed procedure described in Chapter 2 (2.2.8).

3.2.5. Statistical Analysis

Statistical analysis was carried out using R statistical software (R. Development. Core. 2006). Data exploration was undertaken using the protocol described by Zuur et al (2009). Analysis of variance (ANOVA) was used to determine variation in rates between sampling dates for both the temperature and NO_3^- concentration

experiments. Analysis of covariance (ANCOVA) was used to determine whether there was a significant interaction of sampling date with the relationships between experimental temperature and rates of anammox, denitrification and sediment metabolism. Furthermore ANCOVA was used to determine whether the activation energy for these three processes varied significantly between sampling dates.

3.3. Results

3.3.1. Tidal elevation transect

There was no significant relationship between tidal elevation and potential rates of anammox, denitrification or the contribution of anammox to N_2 production (*ra*%) in either August or September data measured in sediment slurries (Figure 3.3.1). Similarly in intact sediment cores there was no significant relationship between tidal height and the rate of anammox (Figure 3.3.2A) or *ra*% (Figure 3.3.2B). It is important to remember that this is not representative of the relationship between anammox and denitrification but rather the rates of anammox at each elevation. There was however significant ($p<0.001$) variation in rates of oxygen consumption measured in intact sediment cores including a sudden step up in oxygen consumption from a tidal elevation of 2.3m upwards.(Figure 3.3.2C) with rates almost quadrupling to a maximum rate of 2180 μmol O_2 m^2 h^{-1} at a tidal elevation of 4.3m.

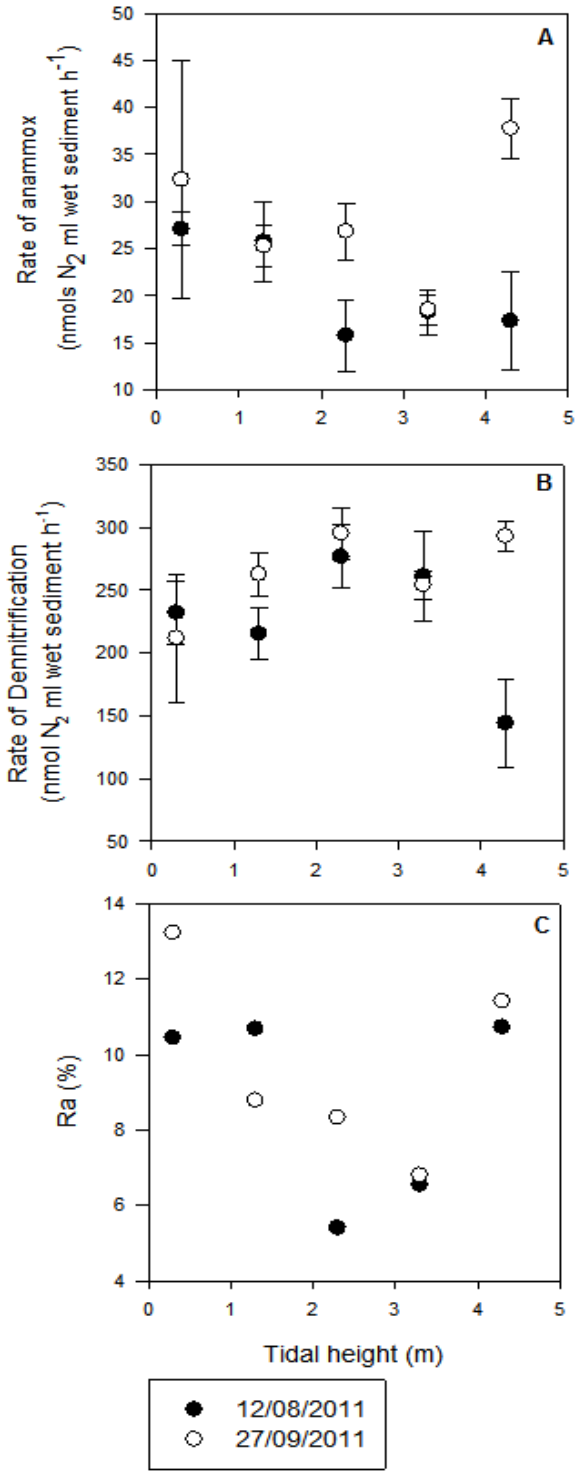


Figure 3.3.1: Rates of (A) anammox (B) denitrification and (C) the relative importance of anammox to N_2 production (*ra*%) in anaerobic sediment slurries over a range of intertidal heights.

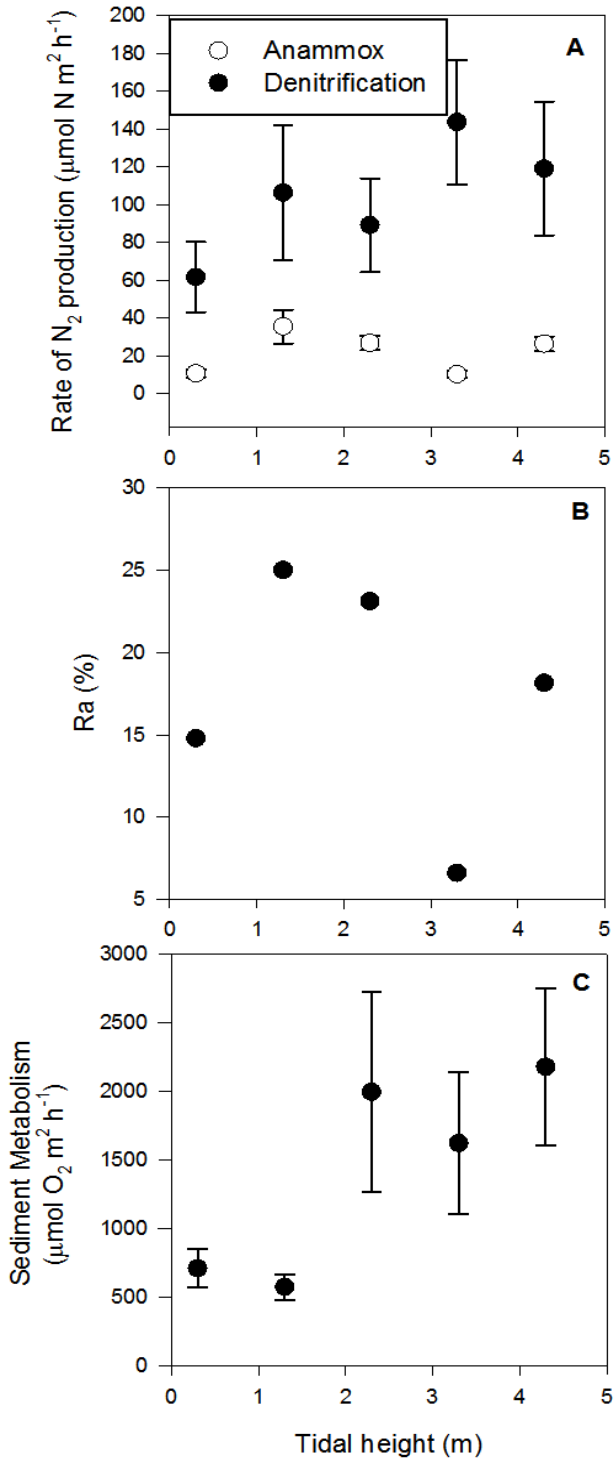


Figure 3.3.2: Rates of (A) anammox and denitrification (B) the relative contribution of anammox to N_2 production (*ra*%) and (C) sediment metabolism in intact sediment cores over a range of intertidal heights.

3.3.2. The effect of NO_3^- Concentration on rates of anammox and denitrification

The mean ratio of $^{15}NO_x^-$ to $^{14}NO_x^-$ (q) across all sampling dates for the different $^{15}NO_3^-$ concentrations measured was 0.66, which, when considering the mean concentration of 310 μM $^{15}NO_3^-$, would indicate an average $^{14}NO_x^-$ background concentration of 109 μM $^{14}NO_x^-$. Figure 3.3.3 is compiled from all monthly data recorded for each NO_3^- concentration for anammox, denitrification and the relative contribution of anammox to N_2 production.

There was a significant ($p<0.001$) increase in rates of anammox as $^{15}NO_3^-$ increased in the water overlying the intact sediment cores from an initial concentration of 50 μM up to 200 μM $^{15}NO_3^-$; however as the concentration rose further there was no significant increase in the rate of anammox (Figure 3.3.3a). Figure 3.3.3a clearly illustrates the plateau reached in N_2 production from anammox when a concentration of 200 μM $^{15}NO_3^-$ is reached. Unlike anammox there was a highly significant ($p<0.001$) relationship between denitrification and the initial NO_3^- concentration over the complete range of NO_3^- concentration (50 μM to 800 μM) (Fig. 3.3.3b). Furthermore there was significant variation in the mean rate of anammox and denitrification across all NO_3^- concentrations for each sampling date with higher average rates observed in July and September compared to November and January (Table 3.3.1).

The relative contribution of anammox to N_2 production was higher in November and January compared to the other months. Additionally there was a significant non-linear relationship between the relative contribution of anammox to N_2 production (*ra%*) and NO_3^- concentration with a higher contribution of anammox at lower NO_3^-

concentrations for all sampling dates. Figure 3.3.3c illustrates this non-linear relationship with the average *ra*% from all months for each NO_3^- concentration.

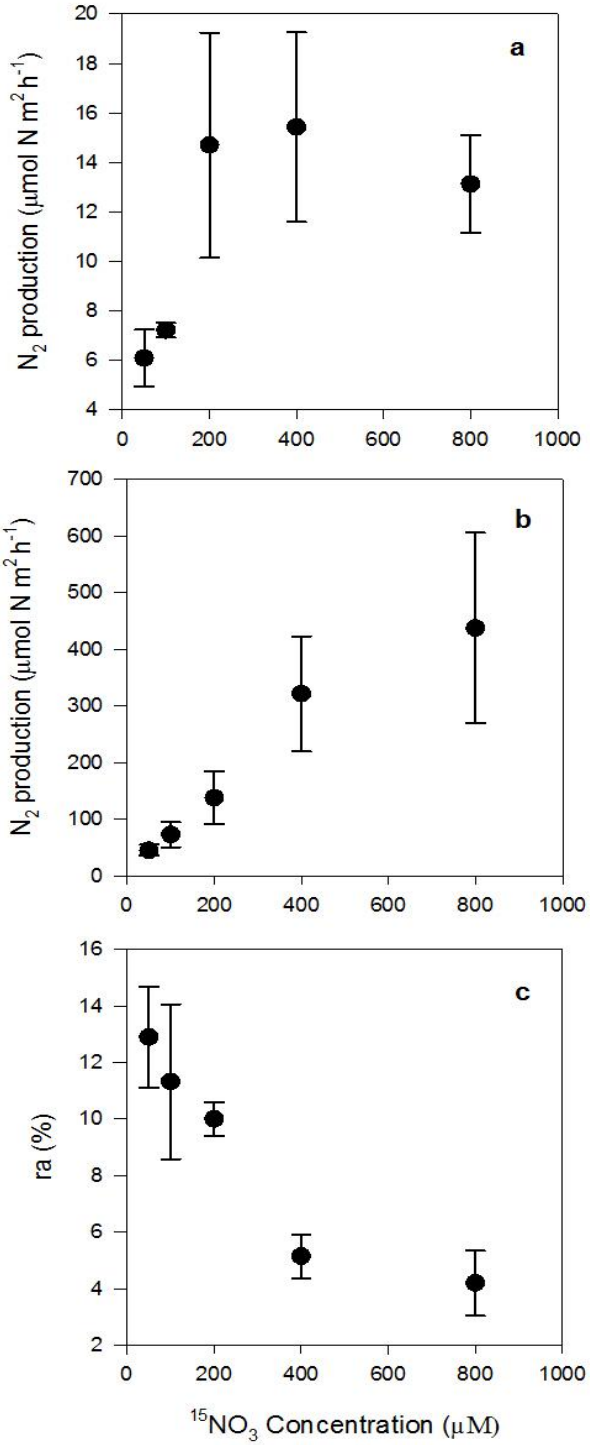


Figure 3.3.3: The response of (a) anammox as A_{15}, (b) denitrification as D_{15} and (c) the relative contribution of anammox to N_2 production (*ra*%) to varying concentration of NO_3^- between 50µM and 800µM in intact sediment cores. Values are mean rates at each $^{15}NO_3^-$ concentration, and error bars are standard error of the mean.

Table 3.3.1: The mean rates of anammox, denitrification and relative contribution of anammox (*ra*%) in intact sediment cores from the NO_3^- concentration experiment. Standard error of the mean is included.

Month	Mean rate of anammox (μmol N_2 m^2 h^{-1})	Mean rate of denitrification (μmol N_2 m^2 h^{-1})	Mean *ra* (%)
July	16 ± 3.8	348± 138	6.7 ± 1.7
Sept	14.3 ± 2.8	291 ± 114	7.2 ± 1.9
Nov	7.45 ± 1.4	91 ± 34	10.7 ± 2.5
Jan	7.5 ± 0.8	79 ± 20	10 ± 1.8

3.3.3. Response of anammox and denitrification to temperature in slurries

There was a significant linear increase in potential anammox activity with increasing temperature in the anoxic slurries, from 14 nmol N_2 ml^{-1} wet sediment h^{-1}, on average, at 5°C, to 62 nmol N_2 ml^{-1} wet sediment h^{-1} at 20°C (Figure 3.3.4). Similarly there was a significant linear relationship between potential rates of denitrification and increasing temperature from an average of 71 nmol N_2 ml^{-1} wet sediment h^{-1} at 5°C to 198 nmol N_2 ml^{-1} wet sediment h^{-1} at 20°C (Figure 3.3.4). There was a significant ($p<0.05$) increase in the relative importance of anammox to N_2 production (*ra*%) from 16 to 23%. The apparent activation energy for anammox was 0.5eV with a Q^{10} of 2.95, and was considerably lower for denitrification with an apparent activation energy of 0.35eV and a Q^{10} of 1.98.

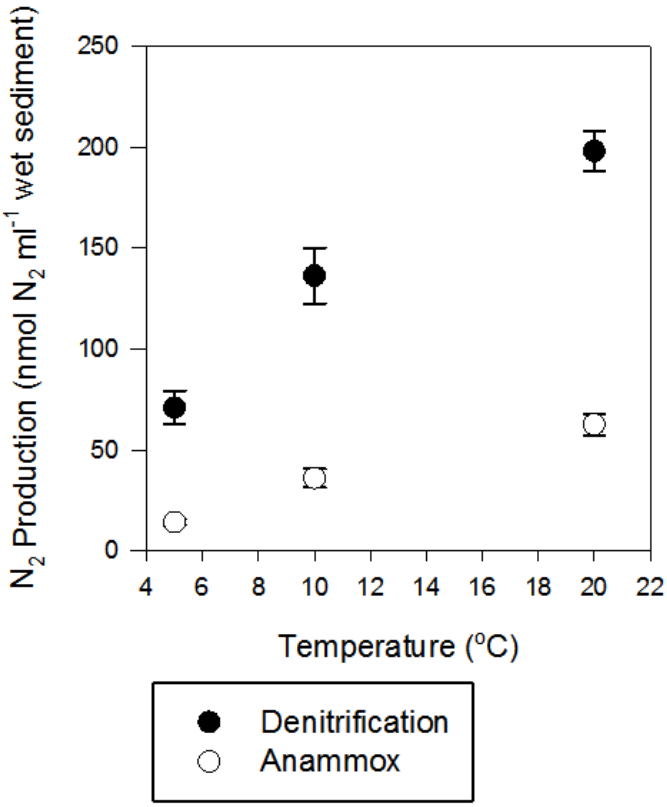


Figure 3.3.4: Response of anammox and denitrification to temperature between 5°C and 20°C in anaerobic sediment slurries.

3.3.4. The response of anammox, denitrification and oxygen consumption to temperature in intact sediment cores

Analysis of covariance indicated there was a significant ($p<0.001$) increase in rates of anammox as temperature increased for all sampling dates. Analysis of covariance (ANCOVA) indicated there was a significant increase in the slope of anammox when correlated with temperature between 5^{o}C and 20^{o}C. This resulted in a significantly steeper slope in July compared to the remaining months. Anammox data for all sampling dates is illustrated in Figure 3.3.5A.

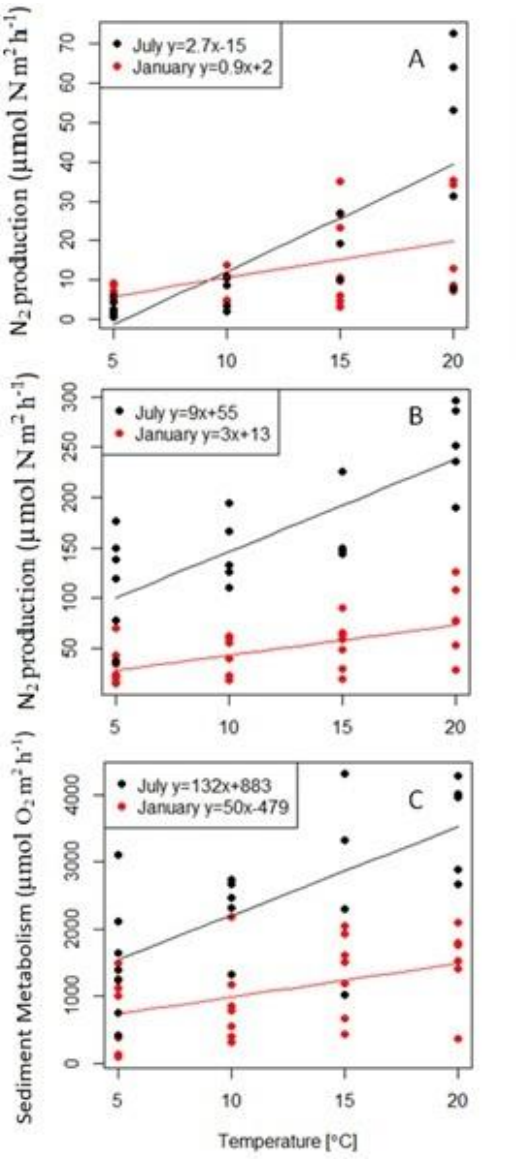


Figure 3.3.5: The effect of temperature on (A) rates of anammox (µmol N m^{-2} h^{-1}) (B) denitrification (µmol N m^{-2} h^{-1}) and (C) oxygen consumption in July and January.

Figure 3.3.5A clearly illustrates the variation in slope between July and January, with an increased slope in July.

There was a highly significant positive linear increase between rates of denitrification and temperature between 5^{o}C and 25^{o}C for all sampling dates (Figure 3.3.6B). The response of denitrification to temperature in January was less steep than the other 3 months, which were similar and are clearly illustrated in Figure 3.3.6B. Analysis of covariance indicated that July had a significantly greater intercept than the other three months, which indicated higher rates of denitrification across all temperatures. Generally the relative contribution of anammox to N_2 production (*ra*%) rose with temperature between 5 and 20^{o}C (Fig. 3.3.6D) when the optimum rate of anammox was reached. From there onwards, *ra*% declined as rates of anammox steadied or began to fall, whilst rates of denitrification increased. The average rates of anammox, denitrification and oxygen consumption for each sampling date are described in table 3.3.2 and the optimum temperatures for anammox are presented in table 3.3.3.

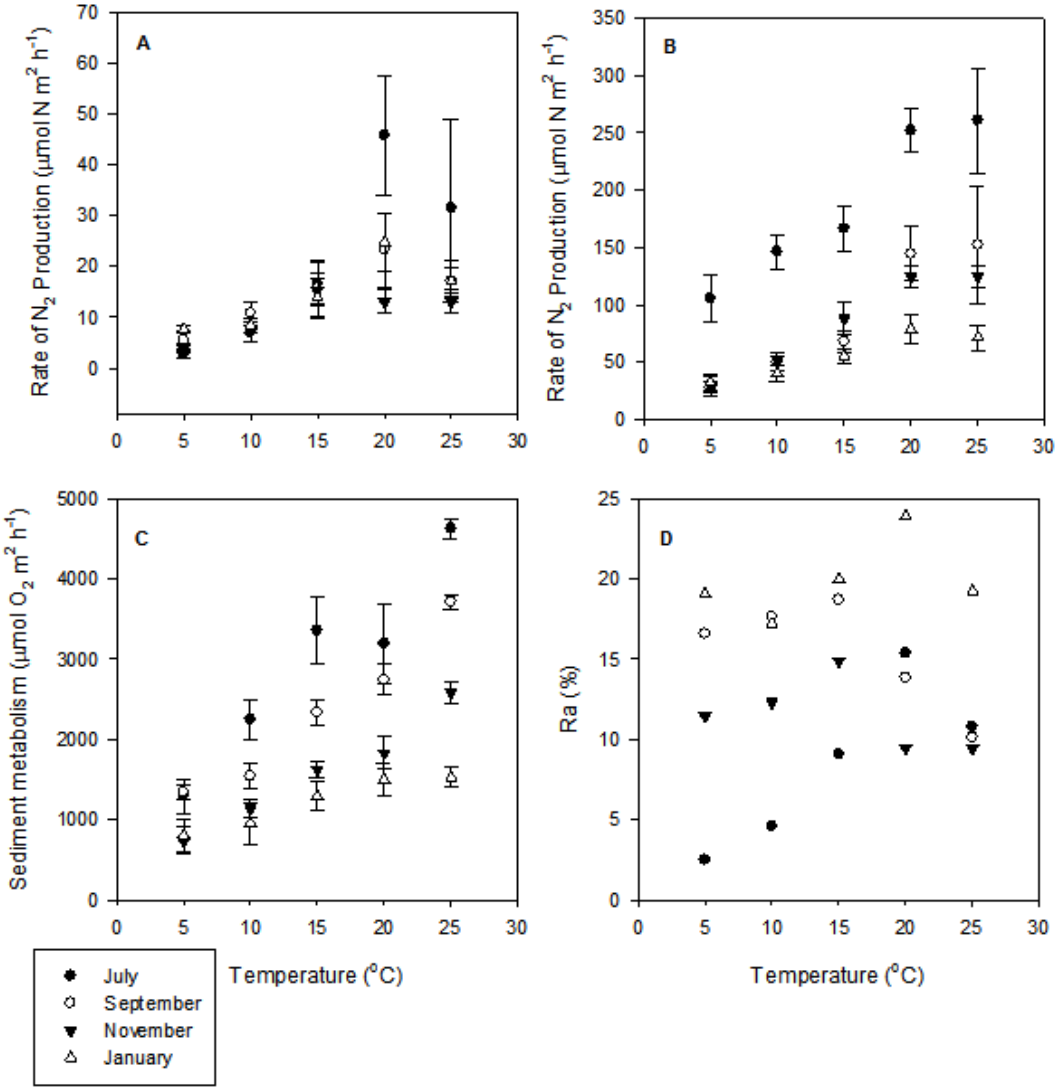


Figure 3.3.6: The temperature response of (A) anammox (B) denitrification (C) sediment metabolism and (D) the relative contribution to N_2 production (*ra*%) between 5°C and 25°C in intact sediment cores at each of the four sampling dates.

Table 3.3.2 Average rates of anammox, denitrification, sediment metabolism and the relative contribution of anammox to N_2 production in the intact sediment core temperature experiments. Data from across all temperatures is pooled to calculate an average for each sampling date.

Month	Mean rate of anammox (μmol N_2 m^{-2} h^{-1})	Mean rate of denitrification (μmol N_2 m^{-2} h^{-1})	Mean rate of sediment metabolism (μmol O_2 m^{-2} h^{-1})	Mean *ra* (%)
July	21 ± 8	186 ± 30	4427 ± 42	8
Sept	14 ± 3	88 ± 25	2969 ± 620	15
Nov	10 ± 2	84 ± 19	1763 ± 352	12
Jan	14 ± 3	56 ± 9	1214 ± 145	19

Table 3.3.3: Optimum temperature for anammox and the associated rates of anammox, denitrification and relative contribution of anammox to N_2 production (*ra*%). Standard errors of the mean are included for rates of anammox and denitrification.

Month	Optimum temp (^oC)	Optimum rate of anammox (μmol N_2 m^{-2} h^{-1})	Rate of denitrification (μmol N_2 m^{-2} h^{-1})	*ra* %
July	20	45 ± 12	251 ± 19	15
Sept	20	23 ± 7	144 ± 24	13
Nov	15	15 ± 3	88± 9.9	14
Jan	20	24 ± 5.6	78 ± 12	23

3.3.5. Oxygen Consumption

There was a significant rise in the rate of oxygen consumption between 5^{o}C and 15^{o}C for all months (Fig 3.3.6C), however, in July and September, between 15^{o}C and 25^{o}C, there was no significant increase in the rate of oxygen consumption. This was because of severely reduced oxygen concentration at 25^{o}C with a reduction of 90% in July and 50% in September after 4hrs, so is not representative of true oxygen consumption. Analysis of covariance indicated there was significant variation in the slope of the response of oxygen consumption to temperature seasonally with a steeper response between 5 and 15^{o}C in July and September compared to the other two months, which were similar. Figure 3.3.6c illustrates the variation in the slope between July and January.

3.3.6. Activation energy

Analysis of covariance indicated that there was a significant ($p<0.001$) difference in the apparent activation energy of anammox between sampling dates (Figure 3.3.7A), from a maximum of 0.6~~4~~eV in July, to a minimum of 0.23eV in January, with a mean activation energy of 0.35eV. Similarly the Q^{10} varied massively between sampling dates with a much larger Q^{10} of 6.1 in July compared to the other months (Table 3.3.4) with a mean Q^{10} of 3.7 ± 1.1.

The activation energy was calculated between 5 and 15^{o}C for oxygen consumption and between 5^{o}C and 20^{o}C for denitrification and anammox, except during July where the rates obtained from 20^{o}C were excluded from the calculations as the oxygen concentration was highly depleted.

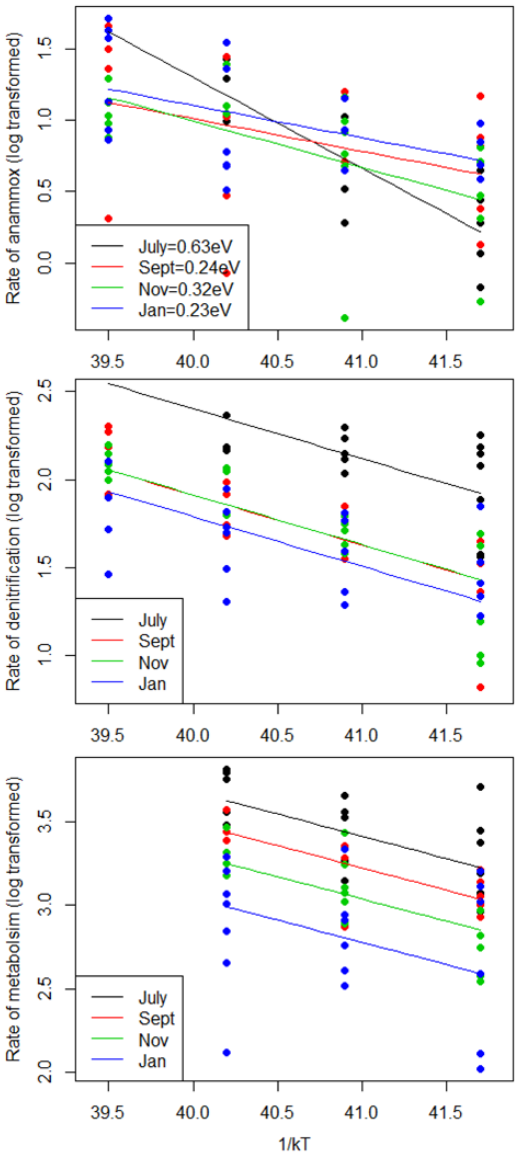


Figure 3.3.7: Temperature dependence of (A) anammox (B) Denitrification and (C) sediment metabolism. Slopes were determined by plotting the logarithm of the rate of the process against 1/kT and the slope of the temperature response equates to the activation energy for each process. To determine whether there was significant variation between sampling dates analysis of covariates (ANCOVA) was used with an interaction term between log(rate) × month. The only significant interaction was for anammox (A) which indicated significantly different apparent activation energies between sampling dates observed in the variation in the slope.

There were no significant differences in apparent activation energies for either denitrification (Fig. 3.3.7B) or sediment metabolism(Fig. 3.3.7C) seasonally, with mean a mean apparent activation energy of 0.27eV and 0.28eV respectively. Furthermore there was little seasonal variation in the Q^{10} of oxygen consumption with a mean of 1.82. The Q^{10} for denitrification was considerably higher with a mean annual Q^{10} of and 2.16 . (Table 3.3.4).

Table 3.3.4: The seasonal activation energies and Q^{10} for anammox, denitrification and O_2 consumption with the temperatures ranges for which they were calculated.

	July	**September**	**November**	**January**
Activation Energy (eV)	0.64	0.24	0.32	0.23
Anammox	5-15^{o}C	5-20^{o}C	5-20^{o}C	5-20^{o}C
Activation Energy (eV)	0.20	0.37	0.36	0.15
Denitrification	5-15^{o}C	5-20^{o}C	5-20^{o}C	5-20^{o}C
Activation Energy (eV)	0.32	0.25	0.38	0.15
O2 consumption	5-15^{o}C	5-15^{o}C	5-15^{o}C	5-15^{o}C
Q^{10}	6.1	2.84	3.33	1.81
Anammox	5-15^{o}C	5-15^{o}C	5-15^{o}C	5-15^{o}C
Q^{10}	1.58	2.45	2.54	1.7
Denitrification	5-15^{o}C	5-15^{o}C	5-15^{o}C	5-15^{o}C
Q^{10}	1.8	1.66	1.74	1.6
O_2 Consumption	5-15^{o}C	5-15^{o}C	5-15^{o}C	5-15^{o}C

3.4. Discussion

A clear seasonal pattern in the capacity of estuarine sediment to produce N_2 from both anammox and denitrification was measured in Chapter 2. However as the experimental temperature and NO_3^- concentration were fixed for all samples throughout the year, there was no indication of how anammox and denitrification would respond to changing NO_3^- concentration and temperature. The current study demonstrated that increasing the NO_3^- concentration of the overlying water stimulated rates of denitrification between 50µM and 800µM $^{15}NO_3^-$, however there was no response in rates of anammox to NO_3^- concentration above 200µM. There was a clear correlation between rates of anammox, denitrification and oxygen consumption measured with temperature on each sampling date measured in intact sediment cores, however the responses varied with a larger temperature response in July and September compared to the remaining months.

There was no relationship between tidal elevation and either the potential rates of anammox or denitrification in anaerobic sediment slurries or estimated rates measured in intact sediment cores. Similarly there was no relationship between the relative contribution of anammox to N_2 production (*ra*% in either experiment. This confirms previous findings (Chapter 2), which suggested that sediment at the lowest tidal elevations, which are inundated by the NO_3^- rich overlying water for longer periods of time, did not have a higher capacity for anammox or denitrification. There was however a considerable increase in oxygen consumption measured in intact sediment cores from elevations of 2.3m upwards which would suggest a greater availability of bioavailable organic carbon at the three higher tidal heights

(Glud, 2008). Risgaard-Petersen et al., (2004) suggested that the availability of organic carbon could increase rates of denitrification and have little effect on the anammox community. This would have resulted in lowering of the relative contribution of anammox to N_2 production at the higher tidal elevations, where oxygen consumption increased, however in the current study, no decrease occurred.

3.4.1. The response of anammox and denitrification to variation in nitrate concentration and temperature

Nitrate concentration

There was a strong positive relationship between denitrification and NO_3^- concentration between 50 and 800 μM in intact sediment cores. In contrast, anammox activity only increased up to a concentration of 200 μM $^{15}NO_3^-$. This resulted in a non-linear relationship between *ra*% and NO_3^- concentration with anammox contributing far less to total N_2 production at higher NO_3^- concentrations. This would suggest that the denitrifying community has the ability to process far higher concentrations of NO_3^- than is generally observed in the *in-situ* overlying water, which has a mean concentration of 187μM (Environment Agency, UK). Denitrifying bacteria encompasses a much broader group of bacteria, which respire NO_3^- when available and O_2 concentrations are low (Zumft, 1997). Meyer et al.(2008) demonstrated that with a ten-fold increase in the concentration (500 μM) of NO_3^- in the overlying water of estuarine sediments, the depth of NO_3^- penetration increased from 4mm to 10mm. However not only did the NO_3^- penetration increase, the depth of NO_2^- and N_2O production also increased, both associated with denitrification . This would suggest that the depth of sediment where denitrification

occurs can increase downwards as more layers of sediment become saturated with NO_3^- therefore increasing N_2 production through denitrification.

Rates of anammox increased rapidly from 50 μM to 200 μM $^{15}NO_3^-$. There was however a background concentration of approximately 100μM $^{14}NO_3^-$. From 200μM $^{15}NO_3^-$ there was no increase in the rate of anammox, which would suggest that the anammox community was saturated between 200μM and 300μM of total NO_3^- ($^{14}NO_3^- + {}^{15}NO_3^-$). This is similar to the average concentration of NO_3^- (187μM) in the overlying water at Medway Bridge Marina (Environment Agency, UK). Unlike denitrification, anammox may be better suited to a much more specific and therefore narrower area of anoxic sediment (Risgaard-Petersen et al. 2005, Meyer et al., 2005).

Sediments with a relatively stable concentration of NO_3^- in the overlying water similar to the Medway Estuary are likely to have a relatively consistent depth which NO_3^- diffuses down to, and therefore the area of NO_2^- production is likely to be stable at a similar range of depths throughout the year. Risgaard-Petersen et al. (2005) suggested that anammox bacteria require a supply of NO_2^- to maintain an active enzyme apparatus, and therefore will only be located in an area of sediment where NO_2^- is constantly available. Considering anammox bacteria are relatively slow-growing (Strous et al. 1999; Kuenen & Jetten, 2001) it is likely that they will be constrained to areas of sediment with these favourable conditions.

However more recent studies (Rooks et al., 2012) have suggested that the potential capacity for anammox has been detected across a broader range of depths including the oxic layers and well into the anoxic sediments. There were, however, sediment

depths where anammox bacteria numbers were highest which could have been saturated at higher NO_3^-. When sediment depths with the maximum concentrations of anammox bacteria numbers become saturated with NO_3^-, it is likely that progressive exposure of the lower sediment depths, only sparsely populated with anammox bacteria, to higher experimental concentrations of NO_3^- will result in only small increases in N_2 production from anammox. In addition, the relatively slow-growing anammox bacteria (Strous et al, 1999, Kuenen & Jetten, 2001) would be unable to rapidly respond to an increase in No_2^-.

Neubacher et al. (2012), demonstrated that over a period of time (70 days) in hypoxic mesocosms, rates of denitrification responded more quickly than anammox to the artificially hypoxic conditions. It was suggested that the denitrifiers, which initially respire O_2 in the oxic layers quickly switched to NO_3^- when the oxic layer retracted due to hypoxia. They suggested that rates of anammox increased linearly as the NO_2^- production zone increased upwards through increased denitrification and NO_3^- reduction, however it took a much longer period than the 4hr incubations in this study. This would suggest that the anammox community's response to an increase in NO_3^- concentration would probably increase over time as the NO_2^- production zone grew, however it is unable to take advantage of rapid spikes in NO_3^- concentration.

Temperature

Rates of anammox and denitrification both generally increased with temperature in both anaerobic sediment slurries and intact sediment cores. In intact sediment cores the optimum temperature of 20°C for anammox was slightly higher than those reported previously for marine sediments (Dalsgaard and Thamdrup, 2002, Rysgaard et al., 2004), however it was much lower than wastewater treatment plants with optimum reported temperature of 37^{o}C (Strous et al., 1999, Kuenen & Jetten, 2011). The optimum temperature for denitrification was higher throughout, in agreement with previous reported findings (Dalsgaard & Thamdrup, 2002, Rysgaard et al., 2004).

Both anammox and denitrification had a significantly steeper slope in their response to temperature in July compared to January between 5 and 20^{o}C when measured in intact sediment cores. This would suggest that the sediments' capacity to produce N_2 via denitrification and anammox from NO_3^- may have been inhibited in January by the lack of a substrate. Bioavailable organic carbon is required for heterotrophic NO_3^- reduction (Zehr and Ward, 2002), therefore this limitation in November and January may have been inhibiting the response to temperature of denitrification directly, but also anammox indirectly by limiting the availability of NO_2^-. Furthermore the lower rates of oxygen consumption in November and July indicates a lower rate of sediment metabolism, and therefore availability of bioavailable organic carbon (Glud et al., 2008).

As previously mentioned the anammox community reduces NO_2^- (Mulder et al., 1995) rather than NO_3^-. This requirement can either be met through the reduction of NO_3^- to NO_2^- in the reduction zone of the sediment, or the aerobic oxidation of ammonium to NO_2^- in the oxic layers of sediment, both of which are heterotrophic (Zehr and Ward, 2002). When the only addition of $^{15}NO_x^-$ to the overlying water of intact sediment cores is $^{15}NO_3^-$, anammox must ultimately rely on $^{15}NO_3^-$ reduction to provide $^{15}NO_2^-$ and therefore must be affected by how temperature controls the NO_3^- reducing community, in addition to how temperature directly effects the anammox community. The reduction of rates of the heterotrophic processes (denitrification and O_2 consumption) in the winter months was clearly reflected in a reduction in anammox activity, which would suggest carbon limitation of NO_3^- reduction, which produces most of the NO_2^- in anoxic sediments.

Synthesis of results from Chapters 2 and 3

Seasonal data from chapter 2 was re-examined by using model coefficients from the linear relationship between temperature and anammox, denitrification and sediment metabolism from the different seasonal temperature experiments (section 3.3.4). Model coefficients are presented in table 3.4.1. Rates at *in-situ* temperature were predicted using rates measured at 10^{o}C from Section 2.3.5 and presented in Figure 3.4.1.

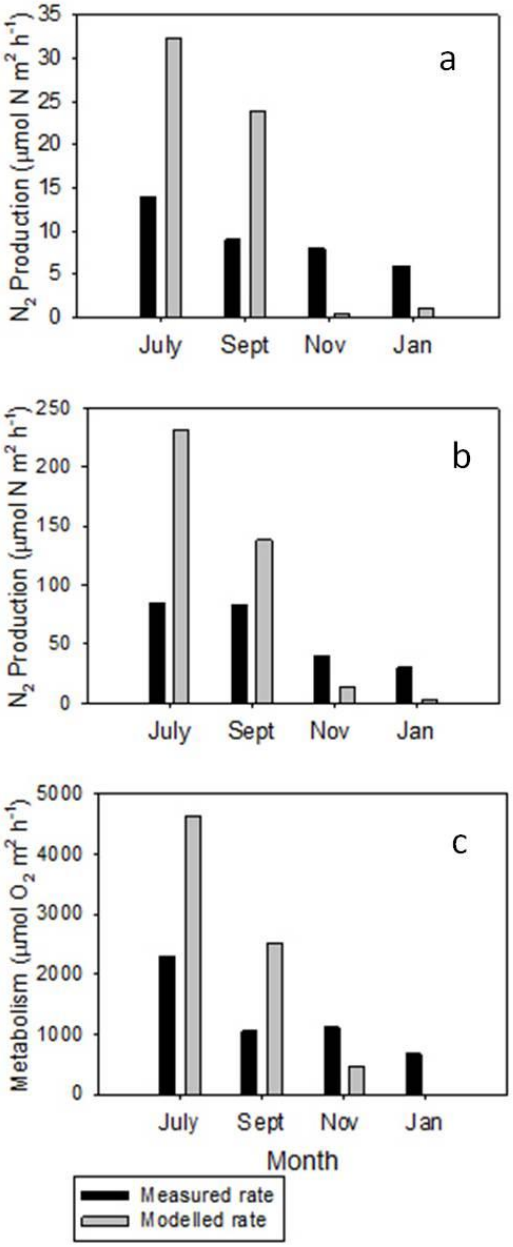


Figure 3.4.1: Modelled and measured rates of (a) anammox (b) denitrification and (c) sediment metabolism using measured rates from section 2.3.4 at 10°C and seasonally varying temperature coefficients for the 3 processes from section 3.3.4.

Table 3.4.1: The *in-situ* temperature and model coefficients for the response of anammox, denitrification and sediment metabolism measured in July, September, November and January.

Month	In-situ Temp (^{o}C)	Temp Difference	Model coefficient Anammox	Model coefficient Denitrification	Model coefficient Metabolism
July	20	+10	y = 2.39x - 5.6	y = 9.35x + 53	y = 199x+ 339
September	13	+3	y = 1.07x + 7.3	y = 6.29x - 11.3	y = 99x + 783
November	7	-3	y = 0.66x + 5.6	y = 6.5x - 6.9	y = 73x + 435
January	6	-4	y = 1.02x - 0.2	y = 2.98x + 14.8	y = 48x + 547

The predicted rates demonstrated the substantial effect that *in-situ* temperature can have, whereby increases in *in-situ* temperature from the 10^{o}C experimental temperature were greatly under-estimated in July and September and in November and January with lower temperatures than the experimental temperature (10^{o}C) rates were over estimated.

3.4.2. Activation energy

The activation energy in anoxic sediment slurries for anammox of 0.5eV measured between 5 ^{o}C and 10^{o}C is in the range of those previously reported for marine sediments (Rysgaard et al., 2004, Dalsgaard and Thamdrup, 2002). Denitrification, however, was considerably lower with an activation energy of 0.35eV compared to 0.53eV and 0.63eV reported by Rysgaard et al. (2004) and Dalsgaard and Thamdrup (2002) respectively.

The current study was the first to examine how temperature affects both anammox and denitrification in intact sediment cores and was therefore the first to report the activation energies of anammox most comparable to *in-situ* conditions. There was significant variation in the activation energy of anammox (ANCOVA) with a higher activation energy in July (0.64eV) compared to the other three months. This activation energy agrees with those previously reported in marine sediments (Thamdrup & Dalsgaard, 2002, Rysgaard et al., 2004) and wastewater plants (Strous et al. 1999). However the activation energy for the other months was considerably lower than any previously reported.

The activation energy for sediment metabolism and denitrification was much lower than expected at 0.28eV and 0.27eV respectively compared to Yvon-Durocher et al. (2012) who reported a mean activation energy of sediment metabolism across a broad range of estuaries of 0.59eV, which is more than twice the activation energy reported here. The sediment core incubations were started soon after the cores had reached the intended temperature, however previous work (Thamdrup, in conversation, 2013) indicated that microbial processes can, in fact, take much longer

(>20hrs) to reach an optimum rate at a given temperature when subjected to either an increased or decreased temperature. This would suggest that cores across the temperatures may in fact have not reached optimum N_2 production in the time-frame of the incubation. This would have a more profound effect on the cores at the higher temperatures, which are furthest away from the 10^oC that the cores were stored at, therefore dulling the response to increased temperature. This would decrease the slope of N_2 production from anammox and denitrification to temperature and therefore lessen the apparent activation energy.

Statistical analysis indicated that there was no significant variation in the slope of the activation energy for either process between the different sampling months, which indicated no significant variation in activation energy for either process. This would suggest that although rates of oxygen consumption and denitrification were higher during the summer months, the energy required to activate the process did not vary seasonally. It was suggested that variation in organic carbon between sampling locations may be a controlling factor in determining the apparent activation energies of heterotrophic processes (Yvon-Durocher et al., 2012). The increased rates of sediment oxygen consumption and denitrification in September and July would indicate an increase in organic carbon, however this did not appear to significantly affect the activation energy of either process.

The mean annual Q^{10} reported in this study for both anammox and denitrification of 3.52 and 2.06 respectively are within the range of those measured in marine sediments by Rysgaard et al. (2004) who reported Q^{10} values of 2.2 and 2.4 for anammox and denitrification respectively; however in contrast, the current study

reports that the Q^{10} value for denitrification is lower than that of anammox. Rysgaard et al. (2004) suggested that the Q^{10} of anammox was lower as rates of anammox would be relatively higher at lower temperatures. In the current study rates of both anammox and denitrification generally rose in line with each other between 5 and 20^{o}C only, whereas Rysgaard et al. (2004) reported that rates of anammox dropped from temperatures of approximately 12^{o}C upwards while rates of denitrification continued to rise, which lowered the relative contribution of anammox to N_2 production.

Evaluation of the isotope pairing technique taking account of the effect of the natural (background) nitrate pool.

The revised isotope pairing technique was used extensively throughout Chapters 2 and 3. The technique's rationale stipulates that the production of N_2 from anammox and denitrification is independent of the ratio of $^{15}NO_3^-$ and $^{14}NO_3^-$ in the reduction zone (r_{14}) (Risgaard-Petersen et al., 2003). By using the natural background concentration of $^{14}NO_3^-$ from within the core and the careful addition of $^{15}NO_3^-$ in increments as completed in section 3.3.2 the validity of the r-IPT can be evaluated over a range of $^{15}NO_3^-$ concentrations. Using q, the proportion of $^{15}NO_3^-$ to $^{14}NO_3^-$ in the reduction zone is determined using equation 3.6 and is often used for statistical analysis as it is constrained between 0 and 1 (Trimmer, et al., 2005).

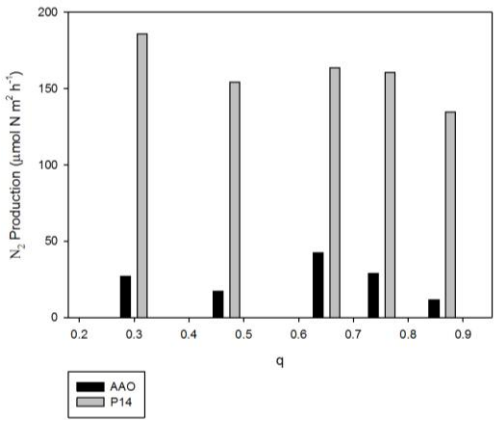


Figure 3.4.2: The estimated rates of anammox (AAO) and denitrification ($_{P14}$), predicted using the revised isotope pairing technique across a range of $^{15}NO_3^-$ concentrations. q is an estimation of the proportion of $^{15}NO_3^-$ to $^{14}NO_3^-$ in the reduction zone with a lower q indicating a lower proportion of $^{15}NO_3^-$.

Figure 3.4.2 illustrates that there is no pattern in rates of either anammox or denitrification across a large range of $^{15}NO_3^-$ concentrations, indicating that the r-IPT is valid across a big differences in r_{14} and therefore q.

In conclusion, previous research (Chapter 2, Trimmer et al., 2003, Nicholls & Trimmer, 2009), suggested that the availability of organic carbon may be regulating NO_3^- and, in particular, denitrification, which could, in turn, be providing an excess of NO_2^- for the anammox community. Rates of anammox, denitrification and sediment metabolism were all at their highest in the summer months, which would suggest that rates of anammox were greatest when there was a greater bioavailability of organic carbon. Furthermore the steepness of the response of anammox to temperature were greatest in July and September compared to the remaining months, suggesting that the availability of organic carbon in the summer months increased rates of anammox and denitrification whereas carbon limitation in the winter restrained the response of anammox to temperature. This supports the findings from

the previous chapter that suggested a lack of bio-available organic carbon is limiting rates of anammox in the autumn and winter months.

Furthermore this study indicated that a rapid increase in NO_3^-concentration can lower the relative contribution of anammox to N_2 production. When NO_3^- concentrations far exceeded the average annual NO_3^- concentration, rates of anammox did not continue increasing, probably as they are confined to an area within the sediment with a constant supply of NO_2^- and low O_2 concentrations, which, in the Medway sediment with a fairly constant supply of NO_3^-, will be fairly stable. In contrast it would appear, that the area within the sediment that denitrification can occur may be able to penetrate downwards as the denitrifying community begins to respire the NO_3^-, which penetrates deeper into the sediment as NO_3^- concentration in the overlying water increases and therefore positively affect the rates of denitrification.

The current study thus indicated that at Medway Bridge Marina, anammox contributes greatest to total N_2 production at or around the average *in-situ* concentration of NO_3^- and between 15 ^oC and 20 ^oC. Furthermore it has been shown that when NO_x^- in the overlying water rapidly increases, the denitrifying community can increase N_2 production, helping to attenuate NO_x^- reaching coastal waters. Future studies examining the relative contribution of anammox to N_2 production either seasonally or spatially should carefully consider how *in-situ* NO_3^- concentration and temperature can affect its relative contribution, as both can have a substantial effect on its contribution. In particular, an *in-situ* spike in NO_3^- concentration could lead to the conclusion that anammox is contributing far less than usual to N_2 production.

CHAPTER 4: THE CYCLING OF ORGANIC NITROGEN AND ITS POTENTIAL FOR OXIDATION COUPLED TO THE REDUCTION OF NITRITE IN ESTUARINE SEDIMENTS

4.1. Introduction

The previous chapters have focussed on how anammox and denitrification activity within estuarine sediments vary seasonally. In chapter 2, by maintaining a constant experimental temperature and NO_3^- concentration for all samples collected throughout the year, we were able to measure how the sediments' capacity to produce N_2 through denitrification and anammox varied seasonally and across a series of elevations. In chapter 3, the experimental temperature and NO_3^- concentration were varied when conducting anammox measurements on seasonally collected samples. Both chapters indicated that anammox was probably indirectly driven by the seasonal bio-availability of organic carbon through its reliance on heterotrophic NO_3^- reduction and NH_4^+ oxidation to provide a supply of NO_2^-. This was supported by a reduction in rates of anammox and its inability to respond as effectively as denitrification to increases in temperature in the winter months. This chapter will focus on whether anammox has the ability to oxidize dissolved organic nitrogen compounds (DON) as well as NH_4^+, and how DON is hydrolysed in estuarine sediments and incorporated into the NH_4^+ pool.

To date it is understood that fixed nitrogen can be removed from an ecosystem as N_2 either through the two-step process of aerobic nitrification, whereby ammonium is first oxidised to nitrate, followed by anaerobic denitrification, or directly via the anaerobic oxidation of ammonia (anammox) (Figure 4.2.1) (Thamdrup and Dalsgaard, 2002b, Dalsgaard and Thamdrup, 2002, Trimmer et al., 2003).

For organic forms of nitrogen to be removed from an ecosystem as N_2, it must first be broken down into an inorganic form of nitrogen, namely NH_4^+ (Berman et al., 1999), before proceeding via one of the two previously mentioned processes. DON make up a significant proportion of the total dissolved nitrogen pool in the aquatic and marine environments (Antia et al., 1991, Bronk 2002, Berman and Bronk 2003) and has been recognised as a major component of both the N and C cycles (Antia et al., 1991).

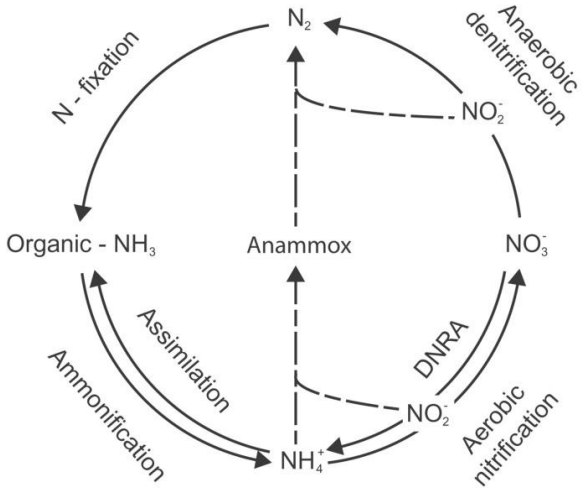


Figure 4.1.1: The Nitrogen cycle, which illustrates how anammox shortcuts the requirement for both aerobic nitrification and anaerobic denitrification for removal of inorganic nitrogen from the environment by coupling the oxidation of ammonia to the reduction of NO_2^- (Trimmer et al., 2003).

Much of the composition of DON cannot be characterized in terms of known substances such as urea, amino acids or aliphatic amines (Sharp, 1983, Lee, 1988, Antia et al., 2001). There are many ways in which DON can be released into aquatic and marine environments, including actively growing phytoplankton (Bronk and Ward, 1999, Diaz and Raimbault, 2000), viral lysis and autolysis of bacteria (Fuhrman, 1999). Furthermore elevated concentrations of DON have also been measured in blooms of the cyanobacteria *Trichodesmium*, which is among the most important primary nitrogen fixers in the ocean (Capone et al., 1994, Vidal et al., 1999). In addition to these autochthonous sources of DON, much of an aquatic system's DON can be supplied from allochthonous sources, for example terrestrial run-off from fields fertilized with DON, or through discharges from sewage treatment works (Glibert et al., 2006). The composition of the DON within an aquatic, estuarine or coastal system can therefore be heavily affected by the anthropogenic activity within its catchment (Seitzinger and Sanders, 1999).

Many organisms excrete DON in the form of urea (Shand et al., 2002, Visek, 1972), which has two amine groups and the chemical formula $CO(NH_2)_2$. For instance, all mammals excrete urea in their urine, which contains a concentration of approximately 0.5M urea (Griffith et al., 1976). Since the breakthrough discovery of the Wohler process in 1828, whereby the inorganic compounds silver cyanate and ammonia were reacted to produce urea, the Bosch-Meiser process of 1922 enabled ammonia to be reacted directly with CO_2, allowing industrial scale synthesis. By

now, urea production has reached nearly 70 million tons yr^{-1} (Berman and Bronk, 2003, Glibert et al., 2006). Much of this is used as an agricultural fertilizer throughout the world (Smil, 2002, Howarth et al., 2000, Galloway and Cowling, 2002) and can leach into aquatic ecosystems.

Many planktonic communities utilize urea during primary production and rates of uptake can be higher than inorganic forms of nitrogen (McCarthy and Whitledge, 1972, Glibert et al., 1991, Berg et al., 1997, Kudela and Cochlan, 2000, Berman and Bronk, 2003). Alternatively urea can be biogeochemically hydrolysed with the aid of the enzyme urease to CO_2 and NH_4^+ (Glibert et al., 2006), of which the latter can subsequently be removed from the ecosystem via either nitrification-denitrification or anammox (Dalsgaard and Thamdrup, 2002, Dalsgaard and Thamdrup, 2002).

Trimmer and Purdy (2012) made a surprising observation when using allylthiourea (ATU) as a nitrification inhibitor in anoxic water samples from the Arabian Sea. They found that ATU was being anaerobically oxidized, coupled to the reduction of NO_2^-, indicating that a biogeochemical process similar to anammox was present but capable of using ATU rather than NH_4^+. This would bypass entirely the requirement for organic nitrogen to first be converted into NH_4^+ in order to be removed from an aquatic ecosystem. Figure 4.1.2 illustrates how organic anammox (organammox) could potentially provide a further shortcut in the nitrogen cycle by coupling the oxidation of organic nitrogen to the reduction of NO_2^-.

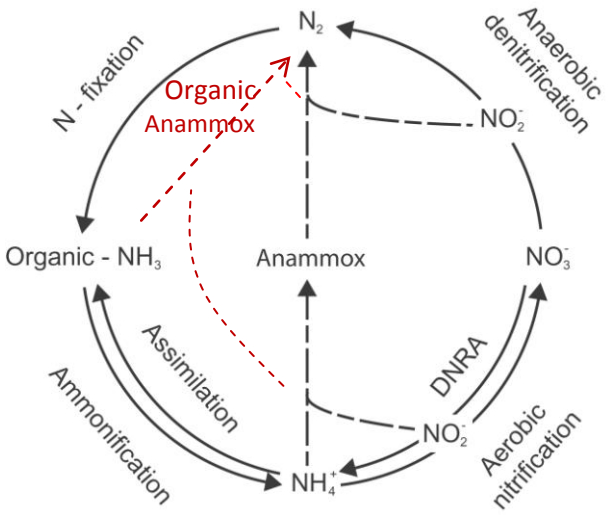


Figure 4.1.2: Illustrates how organic anammox could provide a further short cut in the nitrogen cycle by coupling the oxidation of organic N heterotrophically to the reduction of NO_2^- (modified from Trimmer et al., 2003.)

Sliekers et al. (2004) demonstrated that the anammox community located in activated sludge from a wastewater plant was unable to hydrolyse urea directly. This would suggest that another member of the microbial community would be required to hydrolyse urea to NH_4^+ before it could oxidized via anammox in the environment. However if a form of direct organic nitrogen oxidation, similar to that observed in the Arabian sea samples, exists within the estuarine sediments of the Medway estuary, urea could be one of the sources of organic nitrogen. When urea oxidation is coupled to NO_2^- reduction, the reaction is energetically favourable with a Gibbs free energy of -830 kJ mol^{-1}, at *in-situ* conditions, which equates to -415 kJ mol^{-1} per N atom, compared to -240 kJ mol^{-1} for NH_4^+ oxidation via anammox. From a chemical perspective, this makes organanammox using urea a more favourable process than

anammox but this may not be the case *in-vivo*, where the kinetics is governed by the availability of organisms with suitable enzymes.

^{15}N-labelled nitrogen has been used extensively for studying the aquatic nitrogen cycle; for instance it was vital for proving the existence of anammox (Thamdrup and Dalsgaard, 2002) by showing the oxidation of $^{15}NH_4^+$ was coupled to the reduction of $^{14}NO_2^-$ by measuring $^{29}N_2$ (equation 4.1).

$$^{15}NH_4^+ + {}^{14}NO_2^- \rightarrow {}^{29}N_2 + 2H_2O \qquad (4.1)$$

A control experiment with the sole addition of $^{15}NH_4^+$ is used to assess whether any of the $^{15}NH_4^+$ was anaerobically nitrified to NO_3^-, coupled to the reduction of Mn- or Fe- oxides in the sediment (Luther et al., 1997).

In order to determine whether organic nitrogen could be anaerobically oxidized, a similar approach to that used to detect anammox could be utilized by ^{15}N labelling the urea ($^{13}CO(^{15}NH_2)_2$). Following addition of $^{14}NO_2^-$, the production of $^{29}N_2$ would indicate that either the $^{13}CO(^{15}NH_2)_2$ has been hydrolysed to NH_4^+ followed by anammox oxidation or that it has been directly oxidized via NO_2^-. Both urea hydrolysis to NH_4^+ (equations 4.2 and 4.3) followed by anammox (equation 4.1) and organammox (equation 4.4) produce $^{29}N_2$ so they could not be distinguished by this method. Furthermore the two processes cannot be distinguished by ^{13}C labelling the urea as $^{45}CO_2$ is produced in each case (equations 4.2and 4.4).

$$^{13}CO(^{15}NH_2)_2 + H_2O \xrightarrow{urease} {}^{15}NH_3 + H_2{}^{15}NCOOH \rightarrow 2^{15}NH_3 + {}^{45}CO_2 \quad (4.2)$$

$$2^{15}NH_3 + 2H_2O \rightarrow 2^{15}NH_4^+ + 2OH^- \quad (4.3)$$

$$^{13}CO(^{15}NH_2)_2 + 2^{14}NO_2^- + 2H^+ \rightarrow 2^{29}N_2 + {}^{45}CO_2 + 3H_2O \quad (4.4)$$

The above reactions can be used as screening test for the identification of hydrolysis/anammox and/or organammox, but more sophisticated timed experiments will be required if the two processes are to be distinguished.

The aims of this chapter can therefore be divided into two parts. Firstly to explore if the dissolved organic nitrogen compound (DON), urea, can be oxidized anaerobically via the reduction of NO_2^-. This will be done in a similar way to the initial anammox screening experiments (Thamdrup and Dalsgaard, 2002) whereby $^{14}NO_2^-$ will be added to samples of slurries, but amended with $CO(^{15}NH_2)_2$, rather than $^{15}NH_4^+$ and the production of $^{29}N_2$ monitored.

Secondly a series of timed experiments will be carried out. This will be done by monitoring $^{29}N_2$ production over time in slurries amended with $^{15}NO_2^-$ and $^{15}NH_4^+$ + $^{14}NO_2^-$ and estimating the total NH_4^+ pool available to the anammox community. Slurries will also be amended with $^{13}CO(^{15}NH_2)_2$ + $^{14}NO_2^-$ and the production of $^{15}NH_4^+$, $^{29}N_2$ and $^{45}CO_2$ measured over time . By measuring the production of $^{15}NH_4^+$ and estimating the background NH_4^+, the labelling of the $^{15}NH_4^+$ pool can be predicted. The production of $^{29}N_2$ can then be modelled for the ^{15}N labelling of the NH_4^+ pool resulting from the hydrolysis of $^{13}CO(^{15}NH_2)_2$ using the rate of $^{29}N_2$

production from a slurry amended with $^{15}NO_2^-$. If the rate of $^{29}N_2$ production from $^{13}CO(^{15}NH_2)_2$ + $^{14}NO_2^-$ is clearly higher than that predicated from anammox alone, the presence of organammox would be indicated.

4.2. Methods

Samples were collected from tidal elevation ELW located at the Medway Bridge Marina. Sampling was carried out between May 2012 and October 2012 where surface sediment from the oxic and suboxic layers (from 0-2cm) were collected during low tide, stored in polythene bags and returned to the laboratory within 2hrs before being stored in a refrigerated room at 10^{o}C.

4.2.1. Screening

A series of experiments using various combinations of labelled nitrogen compounds (Table 4.2.1) were designed either to measure the capacity for anammox (Table 4.2.1 amendments 3 and 5) or to examine whether the microbial community within the sediment had the ability to oxidize organic nitrogen coupled to the reduction of NO_2^- (or hydrolysis + anammox). It is important to remember that the production of $^{29}N_2$ is not conclusive evidence for organammox as the $^{13}CO(^{15}NH_2)_2$ may have been initially hydrolysed to $^{15}NH_4^+$. However it would allow an assessment to be made as to whether carrying on with the next set of experiments would be fruitful. All experiments were conducted using equimolar concentrations of ^{15}N from the oxidizable nitrogen substrate so that the initial concentration of $^{15}NH_4^+$ was always double to that of $^{13}CO(^{15}NH_2)_2$.

Table 4.2.1: The amendments to the ^{15}N labelled anoxic slurries, and the biogeochemical processes that the production of $^{29}N_2$ and $^{30}N_2$ would indicate.

No.	Amendment	$^{15}N_2$ labelling Produced	Biogeochemical process
1	1000µM $^{15}NH_4^+$	None	
2	500µM $^{13}CO(^{15}NH_2)_2$	None	
3	1000µM $^{15}NH_4^+$ 1000µM $^{14}NO_2^-$	$^{29}N_2$	Anammox if no $^{29}N_2$ is measured in the slurry with sole addition of $^{15}NH_4^+$
4	500µM $^{13}CO(^{15}NH_2)_2$ + 1000µM $^{14}NO_2^-$	$^{29}N_2$	Oxidation of urea via nitrite or hydrolysis of urea to ammonium followed by anammox
5	1000µM $^{15}NO_2^-$	$^{29}N_2$ $^{30}N_2$	Anammox via $^{15}NO_2^-$ Denitrification of $^{15}NO_2^-$

Sediment slurries were prepared with 3 ml of surface sediment and 3 ml of artificial sea water (sea salt, Tropic Marin) amended to *in-situ* salinity (6ppt) in gas-tight vials (12.5ml Exetainer, Labco) in an anoxic hood (N_2) before incubating overnight on rollers (Spiramix, Thermo-Finnigan) to remove any residual oxidants (O_2, NO_3^-, NO_2^-) (Risgaard-Petersen et al., 2004, Trimmer et al. 2003). As set out in table 4.2.1., slurries were amended (5 replicates) with various combinations of concentrated stocks of $^{14}NO_2^-$ (72 mM $Na^{14}NO_2$), $^{15}NO_2^-$ (72 mM $Na^{15}NO_2$ [98 atom % ^{15}N]), $^{15}NH_4^+$ (80 mM, $^{15}NH_4Cl$ [98 atom % ^{15}N]), $^{13}CO(^{15}NH_2)_2$ (80 mM [99 atom % ^{13}C, 98 atom % ^{15}N]). These were injected through the septum (100µl gas-tight micro-light, Hamilton, Bonaduz, Switzerland) and the slurries returned to the rollers for 3hrs, at which point the experiment was terminated by the injection of 100 µl of CH_2O (37%).

4.2.2. Time series experiments

Having established production of $^{29}N_2$ in the screening experiments (amendments 3,4 and 5 of table 4.2.1), time-series experiments were conducted in July, August and October 2012 to determine whether the organic nitrogen in urea was being oxidized directly, or whether it was first hydrolysed to NH_4^+ and CO_2. The production of $^{15}NH_4^+$, $^{29}N_2$, $^{45}CO_2$ was measured over time after the addition of $CO(^{15}NH_2)_2$ + $^{14}NO_2^-$ (amendment 4, table 4.2.1). Also the production of $^{29}N_2$ via the well characterised anammox process from the addition of $^{15}NH_4^+$ + $^{14}NO_2^-$ (amendment 3, table 4.2.1) and from a $^{15}NO_2^-$ (amendment 5, table 4.2.1) were also measured over time.

A total of 80 sediment slurries were prepared following the protocol described previously (4.2.1). Ten were used as references to check for background oxidants, of which 5 were used with $^{15}NH_4^+$ and 5 with $^{13}CO(^{15}NH_2)_2$. A total of 4 timed experiments were prepared, each consisting of a set of 15 anaerobic slurries. Two sets of 15 were amended with ^{15}Urea + $^{14}NO_2^-$ following the protocol described previously (4.2.1). The initial NO_2^- concentration was 1000µM and $^{13}CO(^{15}NH_2)_2$ concentration 500 µM . One of the sets was used to measure the production of $^{29}N_2$ and $^{45}CO_2$ in the headspace over time and the second set to measure the production of $^{15}NH_4^+$ in the pore-water over time. The two remaining sets of 15 slurries were used to examine the sediments' capacity for anammox: one using $^{15}NH_4^+$ + $^{14}NO_2^-$ and the other, using $^{15}NO_2^-$, all of which had an initial concentration of 1000µM in the slurry pore-water.

Once amended with the respective combinations or inorganic and organic nitrogen substrates, a slurry from each set was sacrificed every 10 minutes until completion (150 minutes). For N_2 and CO_2 analysis, slurries were sacrificed using CH_2O (100µl, 37%), and stored for later analysis on the mass spectrometer. To examine the ^{15}N labelling of the NH_4^+ pool, samples were sacrificed every 10 minutes, centrifuged (ALC, Annita PK131R) before a sample of the pore water was withdrawn (1ml), filtered (0.2µM Minisart Plus filter, Sartorius UK Ltd.) into a gas-tight vial (Exetainer 2.5ml) and frozen for later analysis. 10 slurries were left unamended, 5 of which were sacrificed (100µl, 37% CH_2O) for use as references for background ^{15}N and ^{13}C of the N_2 and CO_2 respectively. The remaining 5 were used as a

reference of the background ^{15}N labelling of the NH_4^+ pore-water following the protocol described previously.

^{15}N enrichment of the pore water with NH_4^+ was determined using the micro-diffusion and hypobromite oxidation assay (Risgaard-Petersen et al., 1995). The time-series pore-water samples were defrosted at 22^oC before a reaction cap, made from an injection needle, flattened to stop any hypobromite iodine leaking through into the sample, was placed into the gas-tight vial. Hypobromite iodine was then transferred into the reaction needle (50µl). The pH in the vial was increased to >12 by adding 0.1ml 10N NaOH through the septum with a syringe to convert NH_4^+ to volatile NH_3. The gas-tight vials were then left for 24 hours at 20^oC on a shaker and gently agitated in order to allow the NH_3 to diffuse into the headspace and react with the hypobromite iodine solution in the needle cap, which oxidizes any NH_3 to N_2.

4.2.3. Analysis

To measure production of $^{29}N_2$, a 50µl sample from the headspace of each anaerobic slurry was injected into a continuous-flow isotope ratio mass spectrometer, following the protocol described in Chapter 2 (2.2.7). Calibration was performed using analytical grade N_2 (100%). Following the ^{15}N-N_2 analysis, a 500µl sample from the same headspace was measured for m/z 44 and 45 for CO_2 ($^{44}CO_2$, $^{45}CO_2$ Calibration was performed using a mixed standard containing a concentration of 2700ppm CO_2.

To measure $^{29}N_2$ and $^{30}N_2$ in the $^{15}NH_4^+$ production experiment, the 2.5ml gas-tight vials were carefully placed on to the mass spectrometer autosampler (Gerstel), ensuring that no hypobromite spilled from the reaction cap into the pore-water sample. Mass spectrometer analysis was performed following the protocol described in chapter 2. Calibration was performed with air filled Exetainers (2.5ml).

4.2.4. Modelling $^{29}N_2$ production from the hydrolysis of $^{13}CO(^{15}NH_2)_2$ to $^{15}NH_4^+$

To determine whether the rate of $^{29}N_2$ production from slurries amended with $^{13}CO(^{15}NH_2)_2 + {}^{14}NO_2^-$ is higher than would be expected from the $^{15}NH_4^+$ produced from the hydrolysis the following equations were used:

A_{29} is the rate of production of $^{29}N_2$ in slurries amended with $^{15}NH_4^+ + {}^{14}NO_2^-$. A_{total} is the rate of $^{29}N_2$ production by anammox in slurries amended with $^{15}NO_2^-$ and can determined using the equation described in chapter 2 (section 2.4 equation 2.2). s is the ratio of $^{15}NH_4$+ to $^{14}NH_4^+$ in the anaerobic slurry and is related to A_{29} and A_{total} by the following equation (equation 4.5).

$$\frac{A_{29}}{A_{total}} = s \tag{4.5}$$

The following equation (equation 4.6) is used to estimate the combined concentration of $^{15}NH_4^+$ and $^{14}NH_4^+$ ($^{total}NH_4^+$), where the concentration of $^{15}NH_4^+$ is taken as its initial concentration of 1000μM.

$$\frac{^{15}NH_4^+}{s} = {}^{total}NH_4^+ \tag{4.6}$$

To estimate the concentration of $^{14}NH_4^+$ in the NH_4^+ pool, the initial $^{15}NH_4^+$ concentration is subtracted from the total, $^{total}NH_4^+$ (equation 4.7).

$$^{total}NH_4^+ - {}^{15}NH_4^+ = {}^{14}NH_4^+ \quad (4.7)$$

The next phase of the calculation uses the estimated turnover of $^{15}NH_4^+$ from urea in the NH_4^+ pool. For this $^{15}NH_{4org}^+$, the average concentration of $^{15}NH_4^+$ produced between 60 and 150 minutes was calculated for each sampling date [see results]. To estimate S_{org}, which is the proportion of the $^{15}NH_4^+$ from the hydrolysis of $^{13}CO(^{15}NH_2)_2$, in the total NH_4^+ pool ($^{total}NH_4^+$) the concentration of $^{15}NH_4^+$ from the hydrolysis of urea ($^{15}NH_{4org}^+$) and the background $^{14}NH_4^+$ concentration were used (equation 4.8.).

$$\frac{^{15}NH_{4org}^+}{^{15}NH_{4org}^+ + {}^{14}NH_4^+} = S_{org} \quad (4.8)$$

Finally the rate of $^{29}N_2$ production through anammox of $^{15}NH_4^+$ generated from the hydrolysis of $^{13}CO(^{15}NH_2)_2$, p_{org}^{29}, is estimated from S_{org} and A_{total}, according to the following equation (equation 4.9).

$$S_{org} \times A_{total} = p_{org}^{29} \quad (4.9)$$

This method assumes that the rate of N_2 production is independent of the NH_4^+ concentration and that the anammox community will oxidize both the $^{14}NO_3^-$ and $^{15}NO_3^-$ in the $^{13}CO(^{15}NH_2)_2$ amendment at the same rate.

4.3. Results

4.3.1. Screening of sediment for organic anammox

Table 4.3.1 shows the total production $^{29}N_2$ and $^{30}N_2$ (nmol ml^{-1} wet sediment) obtained from the sediment slurry incubations after 3 hours. There was no ^{15}N enrichment of the N_2 for any of the sampling dates with sole additions of either $^{15}NH_4^+$ or $^{13}CO(^{15}NH_2)_2$ (amendments 1 and 2, table 4.2.1), and therefore no evidence of direct or indirect oxidation of NH_4^+ or $CO(NH_2)_2$ with residual NO_x^- or for anoxic nitrification coupled to the reduction of Mn- or Fe- oxides (Luther et al., 1997). Screening using $^{15}NH_4^+ + {}^{14}NO_2^-$ and $^{15}NO_2^-$ (amendments 3 and 5) indicated that anammox was present at all dates.

The screening results demonstrated that organisms within the sediment can utilize nitrogen from urea so that it is oxidized to $^{29}N_2$, either via hydrolysis followed by anammox or directly through organammox, albeit at a lower level than classic anammox starting with NH_4^+ There was significant variation between the sampling dates in $^{29}N_2$ production in the $^{15}NO_2^-$ amendment, with a much higher total production of $^{29}N_2$ in May compared to June and July**.** Interestingly, $^{29}N_2$ production in slurries amended with $^{13}CO(^{15}NH_2)_2 + {}^{14}NO_2^-$ showed the reverse trend, starting with a minimal 1.9 nmol $^{29}N_2$ ml^{-1} (wet sediment) in May and climbing to over 6 nmol $^{29}N_2$ ml^{-1} (wet sediment) in June and July.

Table 4.3.1: Total $^{29}N_2$ and $^{30}N_2$ produced after 3 hours during screening using various amendments of ^{15}N labelled reagents. Standard error of the mean is shown.

Amend -ment No.	Treatment	$^{29}N_2$ *or* $^{30}N_2$	May (nmol ml^{-} wet sediment)	June (nmol ml^{-} wet sediment)	July (nmol ml^{-} wet sediment)
1	$^{15}NH_4^+$	$^{29}N_2$	0	0	0
2	$^{13}CO(^{15}NH_2)_2$	$^{29}N_2$	0	0	0
3	$^{15}NH_4^+ + {}^{14}NO_2^-$	$^{29}N_2$	24 ± 2	20 ± 1	11.5 ± 0.5
4	$^{13}CO(^{15}NH_2)_2 + {}^{14}NO_2^-$	$^{29}N_2$	1.9 ± 0.4	6.8 ± 1	6.5 ± 1
5	$^{15}NO_2^-$	$^{29}N_2$	90 ± 6	50 ± 2	54 ± 4
		$^{30}N_2$	650 ± 36	350 ± 16	347± 21

4.3.2. Time series experiments

Production of $^{15}NH_4^+$ from urea climbed rapidly in samples collected at all sampling dates, reaching a plateau after approximately 60 minutes (Figure 4.3.1a). As the data showed considerable scatter, it was not feasible to calculate a rate of $^{15}NH_4^+$ production. It was therefore estimated from the average production of $^{15}NH_4^+$ from 60 minutes onwards, which equated to 559, 535 and 337 nmol NH_4^+ ml^{-1} (wet sediment), a percentage turnover of the urea spike of 41%, 40% and 25% for July, August and October, respectively (Figure 4.3.1b).

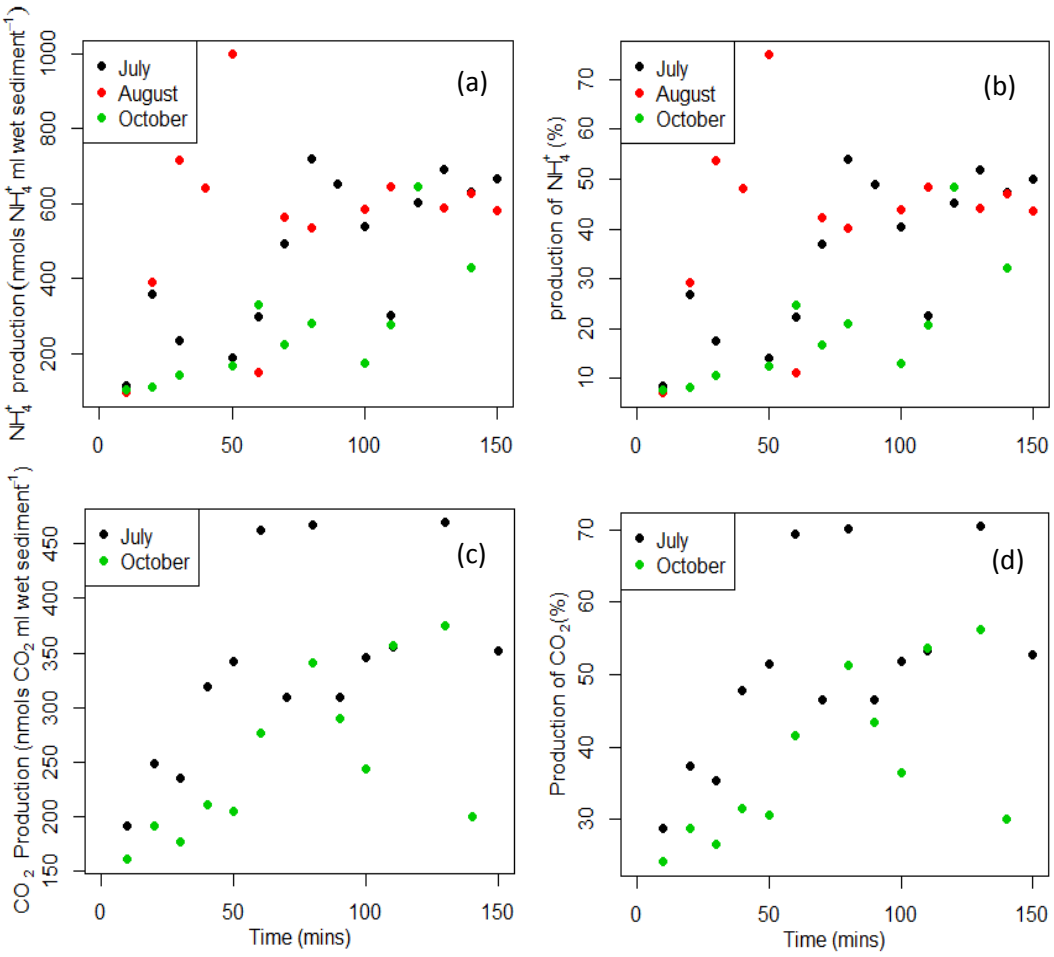


Figure 4.3.1: The production through time of (a) $^{15}NH_4^+$ (b) percentage of $^{13}CO(^{15}NH_2)_2$ recovered as $^{15}NH_4^+$ (c) $^{45}CO_2$ (d) percentage of $^{13}CO(^{15}NH_2)_2$ added recovered as $^{45}CO_2$.

The production of $^{45}CO_2$ rose rapidly before reaching a plateau (Figure 4.3.1c), generally after approximately 1 hr. Again the scatter of the points made it difficult to determine an accurate rate of production therefore the mean production of $^{45}CO_2$ between 60 and 150 minutes was calculated, which equated to 383 nmol (July) and 298 nmol (October) $^{45}CO_2$ ml^{-1} wet sediment, a turnover of the added urea to CO_2 of between 57% and 45% for July and October respectively (Figure 4.3.1d). Table 4.3.2 presents the production of $^{45}CO_2$ and $^{15}NH_4^+$.

Table 4.3.2: Production of $^{15}NH_4^+$ and $^{45}CO_2$ in anaerobic sediment slurries; the percentage turnover of the initial $^{13}CO(^{15}NH_2)_2$ spike recovered as either $^{15}NH_4^+$ or $^{45}CO_2$. Values are averages of measurements obtained between 60 and 150 minutes for each process.

Month	$^{15}NH_4^+$ (nmol ml^{-1} wet sediment)	NH_4^+ (%) [a]	$^{15}NH_{4org}^+$ (µM)	$^{45}CO_2$ (nmol ml^{-1} wet sediment)	$^{45}CO_2$ (%) [b]
July	559 ± 48	41 ± 3	410	384 ± 25	58 ± 4
August	589 ± 14	44 ± 1	440	----	----
October	300 ± 14	23 ± 1	230	297 ± 24	44 ± 4

[a]Proportion of added $^{13}CO(^{15}NH_2)_2$ recovered as $^{15}NH_4^+$
[b]Proportion of added $^{13}CO(^{15}NH_2)_2$ recovered as $^{45}CO^2$

As in the initial screening experiments, there was no production of $^{29}N_2$ during the time series experiments with the sole addition of $^{15}NH_4^+$ or $^{13}CO(^{15}NH_2)_2$. There was a highly significant (p<0.001) linear increase in $^{29}N_2$ production within 10 minutes of $^{15}NH_4^+$ and $^{14}NO_2^-$ addition for all sampling dates (Figure 4.3.2b). Furthermore, there was significant (p<0.001) variation in the rate of $^{29}N_2$ production between sampling dates, with both July and August being significantly higher than October. Figures 4.3.2c illustrates the rapid growth in the production of $^{29}N_2$ and $^{15}NO_2^-$ and analysis of covariance indicated that the rate of $^{29}N_2$ and $^{30}N_2$ production varied significantly from month to month (p<0.001).

Figure 4.3.2a illustrates the production of $^{29}N_2$ from the $^{13}CO(^{15}NH_2)_2$ + $^{14}NO_2^-$ amended slurry. A delay is observed in $^{29}N_2$ production, which varies between 30 minutes in July and August to 50 minutes in October, however once detected, production of $^{29}N_2$ rose linearly at each sampling date. Analysis of covariance indicates a significant variation between sampling date of $^{29}N_2$ production rate, with a faster rate in August compared to July and October.

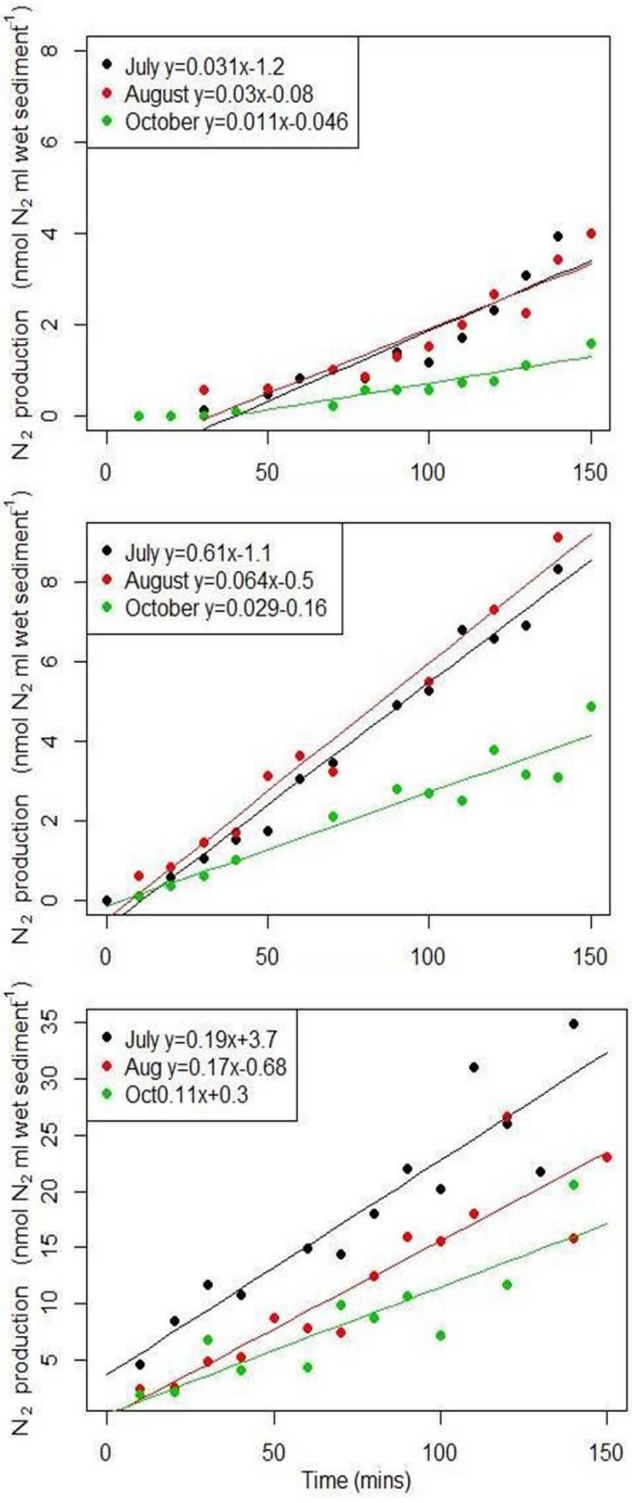


Figure 4.3.2: The production of $^{29}N_2$ over time from the addition of (a) $^{13}CO(^{15}NH_2)_2$+ $^{14}NO_2^-$, (b) $^{15}NH_4^+$ + $^{14}NO_2^-$, (A_{29}) and (c) $^{15}NO_2^-$ (A_{total}) in anaerobic sediment slurries for the months of July (black), August (red) and October (green).

4.3.3. Modelling rates of anammox from hydrolysis of urea

The predicted production of $^{29}N_2$ was modelled using estimates of the production of $^{15}NH_4^+$ from the hydrolysis of $^{13}CO(^{15}NH_2)_2$ to $^{15}NH_4^+$ and calculation described previously. Table 4.3.3 describes the predicted values and the variables input into the model. In July, the predicted and measured rates were identical, whereas in August the predicted value was slightly higher than that measured, and in October the predicted value was slightly lower than that measured.

Table 4.3.3: The predicted and measured rates of $^{29}N_2$ production from the hydrolysis of $^{13}CO(^{15}NH_2)_2$ to $^{15}NH_4^+$ in anaerobic slurries. Model input parameters are also presented.

Month	A_{total} (nmol m^{-2} h^{-1})	A_{29} (nmol m^{-2} h^{-1})	s (nmol m^{-2} h^{-1})	$^{13}CO(^{15}NH_2)_2$ turnover (%)	$^{15}NH_{4org}^+$ (μM)	S_{org}	Predicted rate of $^{29}N_2$ (p_{org}^{29}) (nmol m^{-2} h^{-1})	Measured rate of $^{29}N_2$ (nmol m^{-2} h^{-1})
July	11.5	3.9	0.34	41	410	0.172	1.86	1.86
August	10.1	3.84	0.39	44	440	0.2	1.98	1.74
October	6.6	1.8	0.26	23	230	0.073	0.54	0.66

4.4. Discussion

The initial screening experiments using $^{15}NO_2^-$ indicated that there was always capacity for both anammox and denitrification with a mean relative contribution of anammox to N_2 production of 12.5%.

Possible organammox was indicated through the production of $^{29}N_2$ when screening using slurries amended with $^{13}CO(^{15}NH_2)_2$ + $^{14}NO_2^-$ (6.9nmol ml^{-1} wet sediment of $^{29}N_2$ over the 3hr incubation), however the latter could also be attributed to urea being hydrolysed to NH_4^+ and incorporated into the pore-water NH_4^+ pool prior to oxidation via anammox.

It is noteworthy that the production of $^{29}N_2$ from $^{13}CO(^{15}NH_2)_2$ + $^{14}NO_2^-$ was considerably lower than that produced from $^{15}NH_4^+$ + $^{14}NO_2^-$, however it was comparable to previously reported rates from the classic anammox pathway in estuarine sediments (Trimmer et al., 2003). Lower rates relative to slurries amended with $^{15}NH_4^+$ + $^{14}NO_2^-$ could potentially be because the sediment had a more limited ability to oxidize urea compared to NH_4^+, or due to slow and incomplete hydrolysis of the urea lead to a low $^{15}NH_4^+$ to $^{14}NH_4^+$ ratio in the pore water.

Further investigation using time series experiments showed rapid production of $^{15}NH_4^+$ on addition of $^{13}CO(^{15}NH_2)_2$ to pore water. However, monitoring $^{29}N_2$ over time showed that, although samples amended with $^{15}NH_4^+$ + $^{14}NO_2^-$ produced $^{29}N_2$ immediately, those amended with $^{13}CO(^{15}NH_2)_2$ + $^{14}NO_2^-$ displayed a significant delay of 30 to 50 minutes before measureable quantities of $^{29}N_2$ were detected. Pore-water concentrations of $^{15}NH_4^+$ indicated that between 25% and 41% of the

$^{13}CO(^{15}NH_2)_2$ had been hydrolysed to $^{15}NH_4^+$. However, rates of $^{45}CO_2$ production indicated a higher turnover of urea than was determined from pore-water $^{15}NH_4^+$ concentration, suggesting that a portion of the $^{15}NH_4^+$ had been adsorbed onto the sediments (2004) and was unavailable to the anammox community. The reported $^{15}NH_4^+$ concentrations of between 230μM and 430μM after approximately 60 minutes were considerably lower than the initial 1000μM $^{15}NH_4^+$ concentration in the parallel $^{15}NH_4^+$ experiment. This indicates that ^{15}N labelling of the NH_4^+ pool was considerably lower than in samples amended with $^{15}NH_4^+$, which supports the scenario that limited urea hydrolysis is the cause of low $^{29}N_2$ production. Indeed, the anammox community would have had to rely almost entirely on background $^{14}NH_4^+$ during the initial part of the experiment, when only a small fraction of the urea had been hydrolysed.

There was a clear drop in the rate at which urea was hydrolysed from July and August to October, suggesting a seasonal effect. Previous seasonal studies (Therkildsen and Lomstein, 1994) have reported large variations in urea production and hydrolysis, with much greater rates in the summer months. This would suggest that the seasonal patterns observed in the previous chapter in the rates of N_2 production from anammox and denitrification are following the cycling of organic nitrogen in estuarine sediments. Furthermore NH_4^+ production from urea hydrolysis will be greatest during the time of the year when the sediment has the highest requirement for inorganic nitrogen.

Estimates of $^{29}N_2$ production rates based on the concentration of $^{15}NH_4^+$ resulting from the hydrolysis of $CO(^{15}NH_2)_2$ and taking the concentration of background $^{14}NH_4^+$ into consideration, were close to the measured rates. This supports a model in which $^{15}NH_4^+$ is produced through the hydrolysis $CO(^{15}NH_2)_2$, which is then oxidized to $^{29}N_2$ via anammox. For the presence of organammox to be confirmed, the measured rate of $^{29}N_2$ production would have had to be significantly higher than that predicted from the hydrolysis model.

The findings presented indicates that a considerable proportion of the urea is hydrolysed to NH_4^+, most of it within the first hour, and rapidly incorporated into the NH_4^+ pool where it is immediately available to the anammox community. (Berman and Bronk, 2003) demonstrated using the data compiled by Antia et al. (1991) that the composition of dissolved nitrogen in estuaries has a much higher ratio of inorganic nitrogen in the form of NH_4^+ (48%) compared to DON (13%). This study suggests that DON, in this instance urea, can be rapidly cycled into the inorganic nitrogen pool and that even if was present within the sediments of an estuary, anammox would be quantitatively more important for N_2 removal as it is able to access the much larger pool of NH_4^+.

In contrast the composition of total dissolved nitrogen pool in the ocean differs substantially from that found in estuarine systems. DON has been reported to be available in much higher quantities than NH_4^+, especially at the ocean surface (Berman and Bronk, 2003b, Antia et al., 2001). With, generally, much lower available NH_4^+ compared to DON, the microbial community could potentially have

a far higher affinity to oxidize DON rather than NH_4^+, as indicated by the oxidation of ATU via NO_2^- in the Arabian Sea (Trimmer and Purdy, 2012)

Many types of phytoplankton, particularly the more toxic species, have higher urea uptake rates than they do for NO_3^- or NH_4^+ and can therefore be stimulated by higher concentrations of urea (Berg et al., 1997, Kudela and Cochlan, 2000, Collos et al., 2004, Glibert et al., 2006). These studies have also demonstrated the ability of estuarine sediments, particularly during the summer months, to rapidly hydrolyse urea to NH_4^+, which is very quickly incorporated into the NH_4^+ pool to be removed by processes such as anammox. This could help to attenuate the problems associated with the toxic phytoplanktonic blooms and eutrophication of coastal waters.

Although not the focus of this chapter, the variation between sampling dates of anammox activity in slurries amended with a fixed $^{15}NO_2^-$ concentration suggests that the anammox community within the sediments adjusts to the *in-situ* availability of NO_2^- concentration over time. This may be regulated by the availability of bio-available organic carbon as suggested by the previous chapters and would indicate that anammox does not maintain the same capacity to oxidize NH_4^+ via NO_2^- throughout the year.

This study developed our understanding of nitrogen cycling within estuarine sediments. It is the first to demonstrate that NH_4^+ produced through the hydrolysis of urea is instantly available to the anammox community. It also supported previous findings that the hydrolysis of urea varies seasonally with greater rates in the

summer. Furthermore the seasonal variation in anammox activity followed a similar pattern, which indicates that the seasonal cycling of DON and DIN are intimately linked to the nitrogen cycle.

CHAPTER 5: CONCLUSIONS

Anaerobic oxidation of ammonium via nitrite is widespread in aquatic ecosystems throughout the world (Thamdrup and Dalsgaard 2002, Dalsgaard and Thamdrup 2002, Dalsgaard et al., 2003, Trimmer et al., 2003, Trimmer et al., 2009, Zhang et al., 2007, Hamersley et al., 2009, Ward et al., 2009). Importantly it negates the requirement of an aerobic step, which was once considered essential for the removal of fixed nitrogen via the coupled nitrification-denitrification pathway. Although the environments where anammox is present are well categorized, little is known about its seasonal dynamics and the drivers behind any observed seasonal variation. Previous studies have suggested that the availability of NO_3^- and bioavailable carbon could influence, not only the availability of an anammox community, but also its contribution to N_2 production (Risgaard-Petersen et al, 2004a, Risgaard-Petersen et al., 2005, Trimmer et al., 2003, Engström et al., 2005, Dalsgaard et al., 2005). The current study is the first in depth investigation into seasonal variation in an estuarine sediments' capacity for anammox using intact sediment cores, and the factors affecting it.

The seasonal study was conducted at a constant experimental temperature and NO_3^- concentration and indicated that rates of anammox activity varied seasonally with greatest rates in the spring and summer. Furthermore, denitrification, sediment metabolism and the presence of bioirrigating fauna followed a similar seasonal pattern. The seasonal pattern in sediment metabolism mirrored anammox suggesting that the capacity for anammox was indirectly driven by the availability of

bioavailable organic carbon. This stimulated heterotrophic NO_3^- reduction and denitrification and therefore the production of NO_2^-, which is essential to sustain an active anammox community (Risgaard-Petersen et al., 2004, Risgaard-Petersen et al., 2005, Meyer et al., 2005). Moreover, it is probable that the presence of bioirrigation will stimulate anammox by increasing the surface area of sediment where NO_3^- reduction and aerobic NH_4^+ oxidation can take place, both of which have the potential to generate NO_2^- (Nielsen, 2004, Mermillod-Blondin et al., 2004, Hansen and Kristensen, 1998, Rysgaard et al. 1995). *In-situ* NO_3^- concentration at the Medway Bridge Marina is relatively constant throughout the year, which would suggest that NO_3^- does not affect the capacity for anammox substantially.

The seasonal study was conducted at three different tidal elevations, which were inundated by NO_3^- rich overlying water for considerably different periods of time, ranging from an average of 90% at the lowest tidal elevation to 10% at the highest. Previous studies have suggested that the availability of NO_3^- can determine both the presence and potential for anammox activity (Meyer et al., 2005, Risgaard-Petersen 2004, Risgaard-Petersen et al., 2005). This study however indicated that the average annual rate of anammox increased as inundation decreased, suggesting that the availability of anammox was independent of the length of time the sediment was in contact with NO_3^- from the overlying water. Analysis of the rates of anammox and denitrification across the year established a highly significant relationship, suggesting that the relative contribution of anammox to denitrification is seasonally similar. Furthermore on examining the relationship between anammox and denitrification across the tidal elevations a highly significant increase in the

steepness of the slope between anammox and denitrification was observed as contact with the overlying water decreased.

As contact with the overlying water decreased, both anammox and denitrification will become increasingly reliant on nitrification to provide NO_2^- and NO_3^-. With a supply of NO_3^-, NO_3^- reduction and in particular denitrification could in turn provide NO_2^- to sustain an active anammox community. The coupling of nitrification and denitrification is well established in estuarine sediments (Jenkins and Kemp, 1984, Sebilo et al., 2006, Rysgaard et al., 1995, Dong et al., 2000), and the reported findings would suggest that anammox is coupled to denitrification but also potentially to nitrification, either directly or indirectly, which was evident as a much tighter relationship at the higher elevations.

In order to further investigate the seasonal drivers of anammox activity, its response to experimental temperature was examined in samples collected at different times of the year. This indicated that rates of anammox and denitrification generally increased with temperature within the range defined by the *in-situ* temperatures of the Medway estuary, however anammox activity peaked at 20°C whereas rates of denitrification continued to increase. This supports previous findings (Dalsgaard and Thamdrup 2002, Rysgaard et al., 2004) that the optimum temperature is lower for anammox than for denitrification. Furthermore the response of anammox and denitrification to experimental temperature varied seasonally with a steeper slope of the response in summer compared to winter, which was similar to the response of sediment metabolism and denitrification to temperature. This suggested that a lower bioavailability of organic carbon in the winter was indeed limiting the response of

both anammox and denitrification, as previously suggested (Trimmer et al., 2003, Trimmer et al., 1998).

The immediate effect of increasing NO_3^- concentration in the overlying water on rates of anammox and denitrification was examined in sediment cores with initial NO_3^- concentration ranging from 50 to 800 μM $^{15}NO_3^-$. Analysis of the $^{15}NO_3^-$ to $^{14}NO_3^-$ ratio (r_{14}) indicated an average background $^{14}NO_3^-$ concentration of 100 μM. Although rates of denitrification continued to increase with NO_3^- concentration up to 800 μM, anammox activity did not increase above 200 μM $^{15}NO_3^-$, indicating that the capacity for anammox was saturated at concentrations in the region of 300μM total NO_3^- concentration ($^{14}NO_3 + {}^{15}NO_3^-$). This suggests that as increasing layers of sediment become saturated with NO_3^- the facultative denitrifiers began to respire NO_3^- throughout the depth of NO_3^- penetration, as indicated by Meyer et al. (2008) and Neubacher et al. (2012). Anammox, however, is likely to be restricted to more specific zones within the sediment, which have a sustained supply of NO_2^-, and as the Medway estuary has a fairly constant concentration of NO_3^-, these zones are likely to be fairly narrow. Anammox cannot therefore respond throughout the entire depth of NO_x^- penetration. This would indicate that during rapid increases of NO_3^- concentration, for instance at times of increased run-off, it is the denitrifiers, through their increased NO_3^- respiration, that have the greatest potential to attenuate the NO_3^- load reaching coastal waters.

Dissolved organic nitrogen (DON) can make up a considerable proportion of the dissolved nitrogen in aquatic ecosystems (Antia et al., 1991, Berman and Bronk 2003). Urea, a very common DON, is widely available in estuarine systems (Glibert

et al., 2006). It is well-established that it can be hydrolysed by the enzyme urease to NH_4^+ before being removed from an ecosystem as N_2 via anammox or denitrification. This part of the work was designed to examine if urea can be directly oxidized, coupled to the reduction in NO_2^-, in a similar manner to the anaerobic oxidation by ATU observed by Trimmer and Purdy (2012). This was achieved by measuring the production of $^{15}NH_4^+$, $^{45}CO_2$ and $^{29}N_2$ in sediment slurries amended with $^{13}CO(^{15}NH_2)_2 + {}^{14}NO_2^-$. The measured $^{29}N_2$ values were then compared to values estimated using measured rates of anammox from concurrent experiments ($^{15}NH_4^+ + {}^{14}NO_2^-$, $^{15}NO_2^-$) and applied to the $^{15}NH_4^+$ labelling of the of the NH_4^+ pool resulting from urea hydrolysis. The predicted and measured values were in good agreement, strongly supporting the hydrolysis/anammox pathway and ruling out organammox in this case. Furthermore, it indicated that NH_4^+ produced through the hydrolysis of urea is rapidly incorporated into the NH_4^+ pool, to be rapidly utilized by the anammox community, and also supports previous studies (Therkildsen and Lomstein, 1994), which indicated that hydrolysis of urea follows a seasonal pattern.

Cycling of nitrogen within the Medway Estuary follows a distinct seasonal pattern with greater rates of anammox and denitrification being recorded in the spring and summer months indicating a far greater ability of estuarine sediments to remove fixed nitrogen as N_2 during these months. Furthermore sediment metabolism and bioirrigation was also at its greatest during the summer months, which suggests that the availability of organic carbon and delivery of substrates in to the sediment strata is stimulating N_2 removal and that both the nitrogen and carbon cycles are very closely linked within the estuarine sediments. Moreover the cycling and removal of

DON follows a similar pattern, further supporting the increased cycling of both nitrogen and carbon in the summer months.

This seasonal investigation was, however, only conducted at one location. Therefore future seasonal investigations over a wider range of aquatic ecosystems and estuaries would be useful to corroborate the findings in this thesis. The reported findings indicated a close link between the cycling of dissolved organic nitrogen (DON) to NH_4^+ and its removal by anammox. An in depth seasonal study examining the cycling of DON, its integration into the inorganic nitrogen pool and subsequent removal would extend our knowledge of the nitrogen cycle as whole. Furthermore, by examining the link between the availability of organic carbon and how it has the potential to regulate fixed nitrogen, and therefore the assimilation of CO_2, would be an interesting area of research helping to further our understanding of how the carbon and nitrogen cycle interact and how they can have regulatory effects on each other.

This thesis is the first in depth study investigating the seasonal characteristics of anammox in estuarine sediments using intact sediment cores. The results presented are important as they demonstrate how nitrogen and carbon cycling vary substantially with season. Additionally this thesis was the first to demonstrate how anammox and denitrification respond to temperature using intact sediment cores across a range of experimental temperatures. With Earth's temperatures predicted by the Inter Governmental Panel on Climate Change (IPCC, 2008) to significantly rise

due to climate change over the coming years, it is crucial to understand how the nitrogen and carbon cycles are affected by temperature as both N_2O and CO_2 are greenhouses gasses which could contribute to future global temperature rises.

REFERENCES

ANTIA, A. N., KOEVE, W., FISCHER, G., BLANZ, T., SCHULZ-BULL, D., SCHÖLTEN, J., NEUER, S., KREMLING, K., KUSS, J. & PEINERT, R. (2001) Basin-wide particulate carbon flux in the Atlantic Ocean: Regional export patterns and potential for atmospheric CO2 sequestration. *Global Biogeochemical Cycles,* 15**,** 845-862.

ANTIA, N. J., HARRISON, P. & OLIVEIRA, L. (1991) The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology and ecology. *Phycologia,* 30**,** 1-89.

BANTA, G. T., HOLMER, M., JENSEN, M. H. & KRISTENSEN, E. (1999) Effects of two polychaete worms, Nereis diversicolor and Arenicola marina, on aerobic and anaerobic decomposition in a sandy marine sediment. *Aquatic microbial ecology,* 19**,** 189-204.

BEMAN, J. M., ARRIGO, K. R. & MATSON, P. A. (2005) Agricultural runoff fuels large phytoplankton blooms in vulnerable areas of the ocean. *Nature,* 434**,** 211-214.

BERG, G. M., GLIBERT, P. M., LOMAS, M. W. & BURFORD, M. A. (1997) Organic nitrogen uptake and growth by the chrysophyte Aureococcus anophagefferens during a brown tide event. *Marine Biology,* 129**,** 377-387.

BERG, P., RISGAARD-PETERSEN, N. & RYSGAARD, S. (1998) Interpretation of measured concentration profiles in sediment pore water. *Limnol. Oceanogr,* 43**,** 1500-1510.

BERMAN, T. & BRONK, D. A. (2003) Dissolved organic nitrogen: a dynamic participant in aquatic ecosystems. *Aquatic microbial ecology,* 31**,** 279-305.

BERNHARD, A. (2010) The Nitrogen Cycle: Processes, Players, and Human Impact. *Nature Education Knowledge,* 2**,** 12.

BINNERUP, S. J. R., JENSEN, K., REVSBECH, N. P., JENSEN, M. H. & SÃ¸RENSEN, J. (1992) Denitrification, dissimilatory reduction of nitrate to ammonium, and nitrification in a bioturbated estuarine sediment as measured with 15N and microsensor techniques. *Applied and Environmental Microbiology,* 58**,** 303-313.

BRODA, E. (1977) Two kinds of lithotrophs missing in nature. *Zeitschrift für allgemeine Mikrobiologie,* 17**,** 491-493.

BRONK, D. A. & WARD, B. B. (1999) Gross and net nitrogen uptake and DON release in the euphotic zone of Monterey Bay, California. *Limnology and Oceanography,* 44**,** 573-585.

CABRITA, M. T. & BROTAS, V. (2000) Seasonal variation in denitrification and dissolved nitrogen fluxes in intertidal sediments of the Tagus estuary, Portugal. *Marine Ecology-Progress Series,* 202**,** 51-65.

CANFIELD, D. E., GLAZER, A. N. & FALKOWSKI, P. G. (2010) The evolution and future of Earth's nitrogen cycle. *Science,* 330**,** 192-196.

CAPONE, D. G., FERRIER, M. D. & CARPENTER, E. J. (1994) AMINO-ACID CYCLING IN COLONIES OF THE PLANKTONIC MARINE CYANOBACTERIUM TRICHODESMIUM-THIEBAUTII. *Applied and Environmental Microbiology,* 60**,** 3989-3995.

COLLOS, Y., GAGNE, C. L., LAABIR, M., VAQUER, A., CECCHI, P. & SOUCHU, P. (2004) NITROGENOUS NUTRITION OF ALEXANDRIUM CATENELLA (DINOPHYCEAE) IN CULTURES AND IN THAU LAGOON, SOUTHERN FRANCE1. *Journal of Phycology,* 40**,** 96-103.

DALSGAARD, T., CANFIELD, D. E., PETERSEN, J., THAMDRUP, B. & ACUÃA-GONZÃLEZ, J. (2003) N2 production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica. *Nature,* 422**,** 606-608.

DALSGAARD, T. & THAMDRUP, B. (2002) Factors controlling anaerobic ammonium oxidation with nitrite in marine sediments. *Applied and Environmental Microbiology,* 68**,** 3802-3808.

DALSGAARD, T., THAMDRUP, B. & CANFIELD, D. E. (2005) Anaerobic ammonium oxidation (anammox) in the marine environment. *Research in Microbiology,* 156**,** 457-464.

DIAZ, F. & RAIMBAULT, P. (2000) Nitrogen regeneration and dissolved organic nitrogen release during spring in a NW Mediterranean coastal zone (Gulf of Lions): implications for the estimation of new production. *Marine Ecology Progress Series,* 197**,** 51-65.

DIAZ, R. J. & ROSENBERG, R. (2008) Spreading dead zones and consequences for marine ecosystems. *Science,* 321**,** 926-929.

DOLLAR, S. J., SMITH, S. V., VINK, S. M., OBREBSKI, S. & HOLLIBAUGH, J. T. (1991) Annual cycle of benthic nutrient fluxes in Tomales Bay, California, and contribution of the benthos to total ecosystem metabolism. *Marine ecology progress series. Oldendorf,* 79**,** 115-125.

DONG, L. F., SMITH, C. J., PAPASPYROU, S., STOTT, A., OSBORN, A. M. & NEDWELL, D. B. (2009) Changes in Benthic Denitrification, Nitrate Ammonification, and Anammox Process Rates and Nitrate and Nitrite Reductase Gene Abundances along an Estuarine Nutrient Gradient (the Colne Estuary, United Kingdom). *Applied and Environmental Microbiology,* 75**,** 3171-3179.

DONG, L. F., THORNTON, D. C. O., NEDWELL, D. B. & UNDERWOOD, G. J. C. (2000) Denitrification in sediments of the River Colne estuary, England. *Marine Ecology-Progress Series,* 203**,** 109-122.

ENGSTRÖM, P., DALSGAARD, T., HULTH, S. & ALLER, R. C. (2005) Anaerobic ammonium oxidation by nitrite (anammox): Implications for N_2 production in coastal marine sediments. *Geochimica et Cosmochimica Acta,* 69**,** 2057-2065.

ERISMAN, J. W., SUTTON, M. A., GALLOWAY, J., KLIMONT, Z. & WINIWARTER, W. (2008) How a century of ammonia synthesis changed the world. *Nature Geoscience,* 1**,** 636-639.

FUHRMAN, J. A. (1999) Marine viruses and their biogeochemical and ecological effects. *Nature,* 399**,** 541-548.

GALAN, A., MOLINA, V., THAMDRUP, B., WOEBKEN, D., LAVIK, G., KUYPERS, M. M. M. & ULLOA, O. (2009) Anammox bacteria and the anaerobic oxidation of ammonium in the oxygen minimum zone off northern Chile. *Deep-Sea Research Part Ii-Topical Studies in Oceanography,* 56**,** 1125-1135.

GALLOWAY, J. N. & COWLING, E. B. (2002) Reactive Nitrogen and The World: 200 Years of Change. *AMBIO: A Journal of the Human Environment,* 31**,** 64-71.

GALLOWAY, J. N., SCHLESINGER, W. H., LEVY, H., MICHAELS, A. & SCHNOOR, J. L. (1995) Nitrogen fixation: Anthropogenic enhancement environmental response. *Global Biogeochemical Cycles,* 9**,** 235-252.

GALLOWAY, J. N., TOWNSEND, A. R., ERISMAN, J. W., BEKUNDA, M., CAI, Z., FRENEY, J. R., MARTINELLI, L. A., SEITZINGER, S. P. & SUTTON, M. A. (2008) Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. *Science,* 320**,** 889-892.

GLIBERT, P. M., GARSIDE, C., FUHRMAN, J. A. & ROMAN, M. R. (1991) Time-dependent coupling of inorganic and organic nitrogen uptake and regeneration in the plume of the Chesapeake Bay estuary and its regulation by large heterotrophs. *Limnology and Oceanography,* 36**,** 895-909.

GLIBERT, P. M., HARRISON, J., HEIL, C. & SEITZINGER, S. (2006) Escalating worldwide use of urea a global change contributing to coastal eutrophication. *Biogeochemistry,* 77**,** 441-463.

GLUD, R. N. (2008) Oxygen dynamics of marine sediments. *Marine Biology Research,* 4**,** 243-289.

GRIFFITH, D. P., MUSHER, D. M. & ITIN, C. (1976) Urease. The primary cause of infection-induced urinary stones. *Investigative urology,* 13**,** 346.

HAMERSLEY, R. M., WOEBKEN, D., BOEHRER, B., SCHULTZE, M., LAVIK, G. & KUYPERS, M. M. M. (2009) Water column anammox and denitrification in a temperate permanently stratified lake (Lake Rassnitzer, Germany). *Systematic and applied microbiology,* 32**,** 571-582.

HÃGSLUND, S., REVSBECH, N. P., CEDHAGEN, T., NIELSEN, L. P. & GALLARDO, V. A. (2008) Denitrification, nitrate turnover, and aerobic respiration by benthic foraminiferans in the oxygen minimum zone off Chile. *Journal of Experimental Marine Biology and Ecology,* 359**,** 85-91.

HANSEN, K. & KRISTENSEN, E. (1998) The impact of the polychaete Nereis diversicolor and enrichment with macroalgal (Chaetomorpha linum) detritus on benthic metabolism and nutrient dynamics in organic-poor and organic-rich sediment. *Journal of Experimental Marine Biology and Ecology,* 231**,** 201-223.

HENRIKSEN, K., HANSEN J. I., BLACKBURN, T.H., (1980) The influence of benthic infauna on exchange rates of inorganic nitrogen between sediment and water *Ophelia (Suppl)* 1:249-256.

HENRIKSEN, K. & KEMP W. M., (1988) Nitrification in estuarine and coastal marine sediments In *Nitrogen cycling in coastal marine environments*.Proc. *SCOPE Symp. 33. Wiley.* 207-249.

HENRIKSEN, K., RASMUSSEN, M. B., JENSEN, **A.** (1983). Effect of bioturbation and microbial nitrogen transformations in the sediment and fluxes of ammonium and nitrate to the overlaying water. In: Hallberg, R. (ed.) *Ecol. Bull. Vol. 35,Environmental biogeochemistry*. Publishing House/FRN, Stockholm, p. 193-205

HIETANEN, S. & KUPARINEN, J. (2008) Seasonal and short-term variation in denitrification and anammox at a coastal station on the Gulf of Finland, Baltic Sea. *Hydrobiologia,* 596**,** 67-77.

HOWARTH, R. W. (2008) Coastal nitrogen pollution: a review of sources and trends globally and regionally. *Harmful Algae,* 8**,** 14-20.

HOWARTH, R. W., JAWORSKI, N., SWANEY, D., TOWNSEND, A. & BILLEN, G. (2000) Some Approaches for Assessing Human Influences on Fluxes of Nitrogen and Organic Carbon to Estuaries. *Estuarine science: a synthetic approach to research and practice***,** 17.

HOWARTH, R. W. & MARINO, R. (2006) Nitrogen as the limiting nutrient for eutrophication in coastal marine ecosystems: Evolving views over three decades. *Limnology and Oceanography,* 51**,** 364-376.

JAESCHKE, A., ROOKS, C., TRIMMER, M., NICHOLLS, J. C., HOPMANS, E. C., SCHOUTEN, S. & DAMSTE, J. S. S. (2009) Comparison of ladderene phospholipid and core lipids as indicators for anaerobic ammonium oxidation (anammox) in marine sediments. *Geochimica Et Cosmochimica Acta,* 73**,** 2077-2088.

JENKINS, M. C. & KEMP, W. M. (1984) THE COUPLING OF NITRIFICATION AND DENITRIFICATION IN 2 ESTUARINE SEDIMENTS. *Limnology and Oceanography,* 29**,** 609-619.

JENSEN, M. M., KUYPERS, M. M. M., LAVIK, G. & THAMDRUP, B. (2008) Rates and regulation of anaerobic ammonium oxidation and denitrification in the Black Sea. *Limnology and Oceanography,* 53**,** 23.

JETTEN, M. OPDEN CAMP, HJM, KUENEN, JG, AND STROUS, M. (2010) Description of the order Brocadiales. *Bergey's Manual of Systematic Bacteriology,* 4**,** 596-603.

JETTEN, M. S. M., STROUS, M., VAN DE PAS-SCHOONEN, K. T., SCHALK, J., VAN DONGEN, U., VAN DE GRAAF, A. A., LOGEMANN, S., MUYZER, G., VAN LOOSDRECHT, M. C. M. & KUENEN, J. G. (1998) The anaerobic oxidation of ammonium. *Fems Microbiology Reviews,* 22**,** 421-437.

JETTEN, M.S.M., L. VAN NIFTRIK, M. STROUS, B. KARTAL, J.T. KELTJENS, AND H.J.M. OP DEN CAMP. (2009) Biochemistry and molecular biology of anammox bacteria. *Crit. Rev. Biochem. Mol. Biol*. 44:65–84.

JØRGENSEN, B. B. & SØRENSEN, J. (1985) SEASONAL CYCLES OF O-2, NO-3-3 AND SO-4(2-) REDUCTION IN ESTUARINE SEDIMENTS - THE SIGNIFICANCE OF AN NO-3-REDUCTION MAXIMUM IN SPRING. *Marine Ecology-Progress Series,* 24**,** 65-74.

JØRGENSEN, K. S. (1989) ANNUAL PATTERN OF DENITRIFICATION AND NITRATE AMMONIFICATION IN ESTUARINE SEDIMENT. *Applied and Environmental Microbiology,* 55**,** 1841-1847.

JØRGENSEN, K. S. & SORENSEN, J. (1988a) 2 ANNUAL MAXIMA OF NITRATE REDUCTION AND DENITRIFICATION IN ESTUARINE SEDIMENT (NORSMINDE-FJORD, DENMARK). *Marine Ecology-Progress Series,* 48**,** 147-154.

KARTAL, B., KUENEN, J. G. & VAN LOOSDRECHT, M. C. M. (2010) Sewage treatment with anammox. *Science,* 328**,** 702-703.

KRISTENSEN, E. (1984) Effect of natural concentrations on nutrient exchange between a polychaete burrow in estuarine sediment and the overlying water. *Journal of Experimental Marine Biology and Ecology,* 75**,** 171-190.

KRISTENSEN, E., & HANSEN, K., (1999) Transport of carbon dioxide and ammonium in bioturbated (Neresis divericolor) coastal, marine sediments. *Biogeochemistry* 45**:** 147–168.

KUDELA, R. M. & COCHLAN, W. P. (2000) Nitrogen and carbon uptake kinetics and the influence of irradiance for a red tide bloom off southern California. *Aquatic microbial ecology,* 21**,** 31-47.

KUENEN, J. G. & JETTEN M. S. M. (2001) Extra ordinary anaerobic ammonium oxidizing bacteria *ASM News,* 67: 456-463.

KUYPERS, M. M., SLIEKERS, A. O., LAVIK, G., SCHMID, M., JØRGENSEN, B. B., KUENEN, J. G., DAMSTÉ, J. S. S., STROUS, M. & JETTEN, M. S. (2003) Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature,* 422**,** 608-611.

LEE, C. (1988) Amino acid and amine biogeochemistry in marine particulate material and sediments. *Nitrogen cycling in coastal marine environments***,** 125-141.

LUTHER, G. W., SUNBY, B., LEWIS, B. L., BRENDEL, P. J., & SILVERBERG, N. (1997).Interactions of manganese with the nitrogen cycle: alternative pathways to dinitrogen. *Geochim. Cosmochim. Acta* 61, 4043–4052.

MCCARTHY, J. J. & WHITLEDGE, T. E. (1972) Nitrogen excretion by anchovy (Engraulis mordax and E. ringens) and jack mackerel (Trachurus symmetricus). *Fish. Bull,* 70**,** 395-401.

MERMILLOD-BLONDIN, F., ROSENBERG, R., FRANCOIS-CARCAILLET, F., NORLING, K. & MAUCLAIRE, L. (2004) Influence of bioturbation by three benthic infaunal species on microbial communities and biogeochemical processes in marine sediment. *Aquatic Microbial Ecology,* 36**,** 271-284.

MEYER, R. L., ALLEN, D. E. & SCHMIDT, S. (2008) Nitrification and denitrification as sources of sediment nitrous oxide production: A microsensor approach. *Marine chemistry,* 110**,** 68-76.

MEYER, R. L., RISGAARD-PETERSEN, N. & ALLEN, D. E. (2005) Correlation between anammox activity and microscale distribution of nitrite in a subtropical mangrove sediment. *Applied and Environmental Microbiology,* 71**,** 6142-6149.

MULDER, A., GRAAF, A., ROBERTSON, L. & KUENEN, J. (1995) Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. *FEMS Microbiology Ecology,* 16**,** 177-184.

NEDWELL, D. B., DONG, L. F., SAGE, A. & UNDERWOOD, G. J. C. (2002) Variations of the nutrients loads to the mainland UK estuaries: Correlation with catchment areas, urbanization and coastal Eutrophication. *Estuarine Coastal and Shelf Science,* 54**,** 951-970.

NEDWELL, D. B., JICKELLS, T. D., TRIMMER, M. & SANDERS, R. (1999) Nutrients in estuaries. *Advances in Ecological Research,* 29**,** 43-92.

NEUBACHER, E. C., PARKER, R. E. & TRIMMER, M. (2012) The potential effect of sustained hypoxia on nitrogen cycling in sediment from the southern North Sea: a mesocosm experiment. *Biogeochemistry***,** 1-16.

NICHOLLS, J. C. & TRIMMER, M. (2009) Widespread occurrence of the anammox reaction in estuarine sediments. *Aquatic Microbial Ecology,* 55**,** 105-113.

NIELSEN, K., RISGAARD-PETERSEN, N., SOMOD, B., RYSGAARD, S. & BERGO, T. (2001) Nitrogen and phosphorus retention estimated independently by flux measurements and dynamic modelling in the estuary, Randers Fjord, Denmark. *Marine Ecology-Progress Series,* 219**,** 25-40.

NIELSEN, L. P. (1992) DENITRIFICATION IN SEDIMENT DETERMINED FROM NITROGEN ISOTOPE PAIRING. *Fems Microbiology Ecology,* 86**,** 357-362.

NIELSEN, M., GIESEKE, A., DE BEER, D. & REVSBECH, N. P. (2009) Nitrate, nitrite, and nitrous oxide transformations in sediments along a salinity gradient in the Weser Estuary. *Aquatic Microbial Ecology,* 55**,** 39-52.

NIELSEN, O. I. E., GRIBSHOLT, B., KRISTENSEN, E. & REVSBECH, N. P. (2004) Microscale distribution of oxygen and nitrate in sediment inhabited by Nereis diversicolor: spatial patterns and estimated reaction rates. *Aquatic microbial ecology,* 34**,** 23-32.

OGILVIE, B., NEDWELL, D. B., HARRISON, R. M., ROBINSON, A. & SAGE, A. (1997) High nitrate, muddy estuaries as nitrogen sinks: the nitrogen budget of the River Colne estuary (United Kingdom). *Marine ecology progress series,* 150**,** 217-228.

PAERL, H. W. & ZEHR, J. P. (2000) Marine nitrogen fixation. *Wiley Series in Ecological and Applied Microbiology*.

PEIERLS, B. L., CARACO, N. F., PACE, M. L. & COLE, J. J. (1991) Human influence on river nitrogen. *Nature,* 350**,** 386-387.

PINHEIRO, J., BATES, D., DEBROY, S. & SARKAR, D. (2009) the R Core team (2009) nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-96. *R Foundation for Statistical Computing, Vienna*.

R SATISTICAL SOFTWARE - R DEVELOPMENT CORE (2006)

RABALAIS, N. N., TURNER, R. E. & WISEMAN JR, W. J. (2002) Gulf of Mexico hypoxia, AKA" The dead zone". *Annual Review of ecology and Systematics***,** 235-263.

REVSBECH, N. P., RISGAARD-PETERSEN, N., SCHRAMM, A. & NIELSEN, L. P. (2006) Nitrogen transformations in stratified aquatic microbial ecosystems. *Antonie van Leeuwenhoek,* 90**,** 361-375.

RICH, J. J., DALE, O. R., SONG, B. & WARD, B. B. (2008) Anaerobic ammonium oxidation (Anammox) in Chesapeake Bay sediments. *Microbial Ecology,* 55**,** 311-320.

RICHARDS, F. A. (1965a) Anoxic basins and fjords In RILEY J.P. and SKIRROW, g. [eds.] *Chemical Oceanography*, 611-645. Academic Press

RICHARDS, F. A. (1965b) Chemical observations in some anoxic, sulfide-bearing basins and fjords, In PEARSON, E. A. [ed.] *Advances in water pollution Research, 215-232.* Pergamon Press.

RISGAARD-PETERSEN, N., LANGEZAAL, A. M., INGVARDSEN, S., SCHMID, M. C., JETTEN, M. S. M., OP DEN CAMP, H. J. M., DERKSEN, J. W. M., PINA-OCHOA, E., ERIKSSON, S. P., NIELSEN, L. P., REVSBECH, N. P., CEDHAGEN, T. & VAN DER ZWAAN, G. J. (2006) Evidence for complete denitrification in a benthic foraminifer. *Nature,* 443**,** 93-96.

RISGAARD-PETERSEN, N., MEYER, R. L. & REVSBECH, N. P. (2005) Denitrification and anaerobic ammonium oxidation in sediments: effects of microphytobenthos and NO3. *Aquatic Microbial Ecology,* 40**,** 67-76.

RISGAARD-PETERSEN, N., MEYER, R. L., SCHMID, M. C., JETTEN, M. S. M., ENRICH-PRAST, A., RYSGAARD, S. R. & REVSBECH, N. P. (2004) Anaerobic ammonium oxidation in an estuarine sediment.

RISGAARD-PETERSEN, N., NIELSEN, L. P., RYSGAARD, S., DALSGAARD, T. & MEYER, R. L. (2003) Application of the isotope pairing technique in sediments where anammox and denitrification coexist. *Limnology and Oceanography-Methods,* 1**,** 63-73.

RISGAARD-PETERSEN, N., NIELSEN, L. P., RYSGAARD, S., DALSGAARD, T. & MEYER, R. L. (2004b) Application of the isotope pairing technique in sediments where anammox and denitrification co-exist (vol 1, pg 63, 2003). *Limnology and Oceanography-Methods,* 2**,** 315-315.

ROOKS, C., SCHMID, M. C., MEHSANA, W. & TRIMMER, M. (2012a) The depth-specific significance and relative abundance of anaerobic ammonium-oxidizing bacteria in estuarine sediments (Medway Estuary, UK). *Fems Microbiology Ecology,* 80**,** 19-29.

RYSGAARD, S., CHRISTENSEN, P. B. & NIELSEN, L. P. (1995b) Seasonal variation in nitrification and denitrification in estuarine sediment colonized by benthic microalgae and bioturbating infauna. *Marine ecology progress series,* 126**,** 111-121.

RYSGAARD, S., GLUD, R. N., RISGAARD-PETERSEN, N. & DALSGAARD, T. (2004) Denitrification and anammox activity in Arctic marine sediments. *Limnology and Oceanography,* 49**,** 1493-1502.

RYSGAARD, S. R., RISGAARD-PETERSEN, N., NIELSEN, L. P. & REVSBECH, N. P. (1993) Nitrification and denitrification in lake and estuarine sediments measured by the 15N dilution technique and isotope pairing. *Applied and Environmental Microbiology,* 59**,** 2093-2098.

RYTHER, J. H. & DUNSTAN, W. M. (1971) Nitrogen, phosphorus, and eutrophication in the coastal marine environment. *Science,* 171**,** 1008-1013.

SCHUBERT, C. J., DURISCHÂ€ • KAISER, E., WEHRLI, B., THAMDRUP, B., LAM, P. & KUYPERS, M. M. M. (2006) Anaerobic ammonium oxidation in a tropical freshwater system (Lake Tanganyika). *Environmental microbiology,* 8**,** 1857-1863.

SEBILO, M., BILLEN, G., MAYER, B., BILLIOU, D., GRABLY, M., GARNIER, J. & MARIOTTI, A. (2006) Assessing nitrification and denitrification in the seine river and estuary using chemical and isotopic techniques. *Ecosystems,* 9**,** 564-577.

SEITZINGER, S. P. & GIBLIN, A. E. (1996) Estimating denitrification in North Atlantic continental shelf sediments. *Biogeochemistry,* 35**,** 235-260.

SEITZINGER, S. P. & SANDERS, R. W. (1999) Atmospheric inputs of dissolved organic nitrogen stimulate estuarine bacteria and phytoplankton. *Limnology and Oceanography,* 44**,** 721-730.

SHAND, C. A., WILLIAMS, B. L., DAWSON, L. A., SMITH, S. & YOUNG, M. E. (2002) Sheep urine affects soil solution nutrient composition and roots: differences between field and sward box soils and the effects of synthetic and natural sheep urine. *Soil Biology & Biochemistry,* 34**,** 163-171.

SHARP, J. (1983) The distributions of inorganic nitrogen and dissolved and particulate organic nitrogen in the sea. *Nitrogen in the Marine Environment. Academic Press, New York,* 1-35.

SMIL, V. (2002) Nitrogen and food production: proteins for human diets. *AMBIO: A Journal of the Human Environment,* 31**,** 126-131.

STIEF, P. & DE BEER, D. (2002) Bioturbation effects of Chironomus riparius on the benthic N-cycle as measured using microsensors and microbiological assays. *Aquatic Microbial Ecology,* 27**,** 175-185.

STROUS, M., FUERST, J. A., KRAMER, E. H. M., LOGEMANN, S., MUYZER, G., VAN DE PAS-SCHOONEN, K. T., WEBB, R., KUENEN, J. G. & JETTEN, M. S. M. (1999) Missing lithotroph identified as new planctomycete. *Nature,* 400**,** 446-449.

TEIXEIRA, C., MAGALHÃES, C., JOYE, S. B. & BORDALO, A. A. (2012) Potential rates and environmental controls of anaerobic ammonium oxidation in estuarine sediments. *Aquatic Microbial Ecology,* 66**,** 23-32.

THAMDRUP, B. (2012) New Pathways and processes in the global nitrogen cycle. *Annual Review of Ecology, Evolution, and Systematics,* 43**,** 407-428.

THAMDRUP, B. & DALSGAARD, T. (2002) Production of N2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. *Applied and Environmental Microbiology,* 68**,** 1312-1318.

THERKILDSEN, M. S. & LOMSTEIN, B. A. (1994) Seasonal variation in sediment urea turn-over in a shallow estuary. *Marine Ecology-Progress Series,* 109**,** 77-77.

TRIMMER, M. & ENGSTRÖM, P. (2011) Distribution, activity, and ecology of anammox bacteria in aquatic environments. *Nitrification, edited by: Ward, BB, Arp, DJ, and Klotz, MG, ASM Press, Washington DC.*

TRIMMER, M., NEDWELL, D. B., SIVYER, D. B. & MALCOLM, S. J. (1998) Nitrogen fluxes through the lower estuary of the river Great Ouse, England: the role of the bottom sediments. *Marine ecology progress series,* 163**,** 109-124.

TRIMMER, M., NEDWELL, D. B., SIVYER, D. B. & MALCOLM, S. J. (2000) Seasonal benthic organic matter mineralisation measured by oxygen uptake and denitrification along a transect of the inner and outer River Thames estuary, UK. *Marine ecology progress series,* 197**,** 103-119.

TRIMMER, M., NEDWELL, D. B., SIVYER, D. B. & MALCOLM, S. J. (2000) Seasonal organic mineralisation and denitrification in intertidal sediments and their relationship to the abundance of Enteromorpha sp. and Ulva sp. *Marine ecology. Progress series,* 203**,** 67-80.

TRIMMER, M., NICHOLLS, J. C. & DEFLANDRE, B. (2003) Anaerobic ammonium oxidation measured in sediments along the Thames estuary, United Kingdom. *Applied and Environmental Microbiology,* 69**,** 6447-6454.

TRIMMER, M., NICHOLLS, J. C., MORLEY, N., DAVIES, C. A. & ALDRIDGE, J. (2005) Biphasic behaviour of anammox regulated by nitrite and nitrate in an estuarine sediment. *Applied and Environmental Microbiology,* 71**,** 1923-1930.

TRIMMER, M. & PURDY, K. J. (2012) Evidence for the direct oxidation of organic nitrogen to N2 gas in the Arabian Sea. *ISME J,* 6**,** 1798-1800.

TRIMMER, M., RISGAARD-PETERSEN, N., NICHOLLS, J. C. & ENGSTRÖM, P. (2006) Direct measurement of anaerobic ammonium oxidation (anammox) and denitrification in intact sediment cores. *Marine Ecology-Progress Series,* 326**,** 37-47.

TYRRELL, T. (1999) The relative influences of nitrogen and phosphorus on oceanic primary production. *Nature,* 400**,** 525-531.

VAN DE GRAAF, A.A., A. MULDER, P. DE BRUIJN, M.S.M. JETTEN, L.A. ROBERTSON, AND J.G. KUENEN. (1995) Anaerobic oxidation of ammonium is a biologically mediated process. *Appl. Environ. Microbiol.*61:1246–1251.

VAN DE GRAAF, A. A., DE BRUIJN, P., ROBERTSON, L. A., JETTEN, M. S. & KUENEN, J. G. (1997) Metabolic pathway of anaerobic ammonium oxidation on the basis of 15N studies in a fluidized bed reactor. *Microbiology,* 143**,** 2415-2421.

VAN DE VOSSENBERG, J., WOEBKEN, D., MAALCKE, W. J., WESSELS, H. J., DUTILH, B. E., KARTAL, B., JANSSEN-MEGENS, E. M., ROESELERS, G., YAN, J. & SPETH, D. (2012) The metagenome of the marine anammox bacterium 'Candidatus Scalindua profunda illustrates the versatility of this globally important nitrogen cycle bacterium. *Environmental microbiology*.

VIDAL, M., DUARTE, C. M. & AGUSTI, S. (1999) Dissolved organic nitrogen and phosphorus pools and fluxes in the central Atlantic Ocean. *Limnology and Oceanography,* 44**,** 106-115.

VISEK, W. J. (1972) Effects of urea hydrolysis on cell life-span and metabolism. *Federation proceedings.*

VITOUSEK, P. M., CASSMAN, K., CLEVELAND, C., CREWS, T., FIELD, C. B., GRIMM, N. B., HOWARTH, R. W., MARINO, R., MARTINELLI, L. & RASTETTER, E. B. (2002) Towards an ecological understanding of biological nitrogen fixation. *Biogeochemistry,* 57**,** 1-45.

VITOUSEK, P. M. & HOWARTH, R. W. (1991) Nitrogen limitation on land and in the sea: how can it occur? *Biogeochemistry,* 13**,** 87-115.

WARD, B. B. (2000) Nitrification and the marine nitrogen cycle. *Wiley Series in Ecological and Applied Microbiology*.

WARD, B. B., DEVOL, A. H., RICH, J. J., CHANG, B. X., BULOW, S. E., NAIK, H., PRATIHARY, A. & JAYAKUMAR, A. (2009) Denitrification as the dominant nitrogen loss process in the Arabian Sea. *Nature,* 461**,** 78-81.

YVON-DUROCHER, G., CAFFREY, J.M., CESCATTI, A., DOSSENA, M., DEL GIORGIO, P., GASOL, J.M. et al. (2012). Reconciling the temperature dependence of respiration across timescales and ecosystem types. *Nature,* 487, 472–476.

ZEHR, J. P. & WARD, B. B. (2002) Nitrogen cycling in the ocean: new perspectives on processes and paradigms. *Applied and Environmental Microbiology,* 68**,** 1015-1024.

ZHANG, Y., RUAN, X. H., OP DEN CAMP, H. J. M., SMITS, T. J. M., JETTEN, M. S. M. & SCHMID, M. C. (2007) Diversity and abundance of aerobic and anaerobic ammonium oxidizing bacteria in freshwater sediments of the Xinyi River (China). *Environmental microbiology,* 9**,** 2375-2382.

ZHAO, Y., XIA, Y., KANA, T. M., WU, Y., LI, X. & YAN, X. (2013) Seasonal variation and controlling factors of anaerobic ammonium oxidation in freshwater river sediments in the Taihu Lake region of China. *Chemosphere*.

ZUMFT, W. G. (1997) Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews,* 61**,** 533.

ZUUR, A. F. (2009) *Mixed effects models and extensions in ecology with R*, Springer.

ZUUR, A. F., IENO, E. N. & ELPHICK, C. S. (2010) A protocol for data exploration to avoid common statistical problems. *Methods in Ecology and Evolution,* 1**,** 3-14.