The depth specific significiance, relative abundance and phylogeography of anaerobic ammonium oxidation (anammox) in marine and estuarine sediments

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THE DEPTH SPECIFIC SIGNIFICANCE, RELATIVE ABUNDANCE AND PHYLOGEOGRAPHY OF ANAEROBIC AMMONIUM OXIDATION (ANAMMOX) IN MARINE AND ESTUARINE SEDIMENTS

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ABSTRACT

The availability of fixed forms of nitrogen is critical to the regulation of primary production. Until recently, denitrification (the sequential reduction of NO_3^-, through NO_2^-, to di-nitrogen gas) was recognised as the only significant pathway facilitating N removal. The discovery of anaerobic ammonium oxidation (a process whereby NH_4^+ is anaerobically oxidised with NO_2^- to form N_2 gas), however, has redefined this concept. Environmental studies clearly indicate that anammox is a globally significant sink for N, yet the factors that govern variations in the potential for anaerobic ammonium oxidation (anammox), the abundance or the natural diversity of these organisms are poorly understood.

The purpose of this investigation was to identify the organisms responsible for anammox across a gradient of the Medway estuary, Irish Sea and North Atlantic. DNA amplification was performed using the *Planctomycete* forward primer 'S-P-Planc-0046-a-A-18' in combination with either 'S-G-Sca-1309-a-A-21' (targeting members of the genus '*Scalindua*') or 'S-*-Amx-0368-a-A-18'reverse. Analysis of 16S rRNA gene fragments indicated that the majority of sequences shared large phylogenetic distances with the 'candidate' species '*Scalindua sorokinni*' (≤93% sequence similarity). A number of the sequences extracted from both marine and estuarine sediments, however, cluster into 2 sub-groups that share common origins with the anammox lineage.

In addition, the zone of potential anammox activity was characterised using a combination of ^{15}N isotope labelling experiments, pore water oxygen profiles and depth specific rates sediment metabolism (CO_2 production). This was performed in combination with fluorescence *in situ* hybridisation (FISH), to map shifts in the abundance of anammox organisms with depth, thus potentially linking the depth integrated capacity for anammox to deviations in population size. The potential for anammox activity and positive FISH signals confirm the presence of anammox at all sites investigated. The contribution of anammox to total N_2 production (*ra%*) varied, on average, between 4-35% in estuarine and 13-49% in marine sediments relative to denitrification. This was linked to a small population of anammox organisms constituting <1-3% of total bacteria in the estuarine sediments and <1-5% in marine samples. Whilst the depth specific values of *ra* correlate with the relative abundance of anammox organisms in continental shelf (r^2=0.86, P=0.024) and slope sediments (r^2=0.84, P=0.011), no such relationship was observed in the Medway estuary. The overall capacity for therefore appears to be dependant upon the depth integrated potential for anammox and is not inherent to differences in population size.

I hereby certify that this thesis, of approximately 20,000 words in length, 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 higher degree.

The experiments described in Chapter II were conducted under the supervision of Prof. Kevin Purdy, Dr. Brian Oakley (Department of Biological Sciences, Warwick University, UK) and Dr. Markus Schmid (Radboud University, Nijmegen, The Netherlands). All work in Chapter III was performed under the supervision of Dr. Mark Trimmer (Queen Mary University of London, UK). The work presented in Chapter IV was conducted alongside Dr. Andrea Jaeschke (*NIOZ* Royal Netherlands Institute of Research, Department of Marine and Organic Biogeochemistry, The Netherlands) and resulted in a publication printed in April 2009 (Jaeschke *et al*., 2009).

I made a major contribution to all work presented in this thesis.

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CONTENTS

References

FIGURES AND TABLES

1. INTRODUCTION

1.1. Nitrogen

Nitrogen (N) is a group 5B element and exists in several oxidation states within the range of -3 (NH_4^+ and organic tissues) to +5 (NO_3^-) (Table 1.1.). In each of these oxidation states, nitrogen combines with atoms of oxygen or hydrogen to form a unique inorganic molecule. This occurs throughout aquatic environments where either the oxidation of reduced forms of N (such as organic N/ NH_4^+), or the reduction of oxidised N compounds (i.e. NO_2^-/ NO_3^-) is catalysed by a variety of autotrophic and heterotrophic bacteria (Seitzinger, 1988; Zehr and Ward, 2002).

Compound	Formula	Oxidation state
Ammonium	NH_4^+	-3
Hydrazine	N_2H_4	-2
Hydroxylamine	NH_2OH	-1
Di-nitrogen gas	N_2	0
Nitrous oxide	N_2O	+1
Nitric oxide	NO	+2
Nitrite	NO_2^-	+3
Nitric dioxide	NO_2	+4
Nitrate	NO_3^-	+5

Table 1.1. The oxidation states of N compounds in the aquatic environment. Reduced and oxidised forms of N are utilised in both autotrophic and heterotrophic metabolisms.

1.2. The turnover of nitrogen compounds in the environment

The nitrogen cycle is essentially the exchange of N between atmosphere and aquatic or terrestrial environments. The larger, abiotic pool of nitrogen constitutes 78% (vol. N_2) of the earth's atmosphere and is inaccessible to most living organisms. Di-nitrogen gas is triple bonded (N≡N) and cleavage of this molecule requires a series of complex reactions and high inputs of energy (16 ATP/mole of N_2 fixed) (Paerl and Zehr, 2000). The only organisms capable of catalysing this process (via the enzyme nitrogenase) are nitrogen fixing prokaryotes (Paerl and Zehr, 2000). It is only through this pathway that N becomes available to the biosphere. Consequently, the availability of fixed N is an important factor in regulating primary production (Ryther and Dunstan, 1971).

Until recently, denitrification was considered the only significant process returning biologically available nitrogen to the atmosphere (Devol, 1991). In recent years, an increase in anthropogenic nitrogen inputs, including the production of industrial sewage effluent and agricultural fertilizers, has caused an imbalance between nitrogen fixation and nitrogen removal processes (Herbert, 1999; Middleberg *et al*., 1996; Vitousek *et al*., 1997). In aquatic systems, the result of this imbalance is an enrichment with N, that is, hypernutrification, which can lead to multifaceted problems collectively termed eutrophication (Jickells, 1998). Classical nitrogen removal processes, however, may serve to combat the effects of nitrogen pollution and therefore have gained considerable attention in recent years (Ogilvie *et al.*, 1997; Trimmer *et al*., 1998).

1.3. Nitrogen removal pathways

The nitrogen cycle is a microbially mediated sequence of processes, consisting of two major phases; an aerobic nitrification and an anaerobic denitrification phase (Figure 1.3.1.). In aquatic environments, ammonium derived from the decomposition of organic matter is oxidised to NO_2^- and NO_3^- via nitrification. Nitrification occurs in phases and each stage is mediated by different groups of bacteria and archea (Equations 1. and 2.). To date, there are no known organisms capable of performing both reactions (Ward, 2000).

$$NH_4 + O_2 + H^+ \rightarrow NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ \quad (1)$$

Ammonium monoxygenase

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \quad (2)$$

Equations 1 and 2. The process of nitrification involves 2 phases. Equation 1 represents the first phase of nitrification where NO_2^- is produced. The second reaction involves the oxidation of NO_2^- to NO_3^- and is represented by equation 2.

Although nitrification is typically considered an aerobic process, recent evidence has suggested that some species of nitrifiers are capable of anoxic nitrfication in the presence of NO_2 (Schmidt *et al.*, 2001). Furthermore, there is growing evidence that Crenarchea are also capable of this process (Könnecke *et al*., 2005; Francis *et al*., 2007).

In the process of denitrification, facultative anaerobes sequentially reduce NO_3^-, through a series of intermediates ($NO_3^- \rightarrow NO_2^- \rightarrow N_2O$), to N_2 gas (Zumft, 1997) (Equation 3).

$$C_6H_{12}O_6 + 4NO_3^- \rightarrow 6CO_2 + 6H_2O + 2N_2 \quad (3)$$

Equation 3. During denitrification, NO_3^- is reduced through various inorganic forms to N_2. The reaction is represented by equation 3.

The nitrification and denitrification pathways are coupled across the oxic-suboxic interface, facilitated by the diffusion of NO_3^- from the oxic into the suboxic zone (Henriksen and Kemp, 1988). At low oxygen concentrations, however, water column derived NO_3^- may alternatively diffuse into the suboxic zone, where it is subsequently reduced to N_2 gas.

Until recently, denitrification was considered one the most significant sinks for fixed nitrogen in the biosphere, however, if NO_3^- is utilised in the process of 'dissimilatory nitrate reduction to ammonium' (DNRA), it will be retained in a form that is bioavailable (Patrick *et al.*, 1996). A variety of prokaryotes are capable of this process and combine the reduction of NO_3^- to the oxidation of organic carbon (or reduced Fe and S) to produce NH_4^+ (via NO_2^-) (Blackburn, 1983). The importance of DNRA in sediments is not yet fully understood (Cornwell *et al.*, 1999), although rates of DNRA equivalent to those of denitrification, have been reported in sediments with high rates of organic carbon mineralisation (Bonin *et al.*, 1998; Tobias *et al.*, 2001).

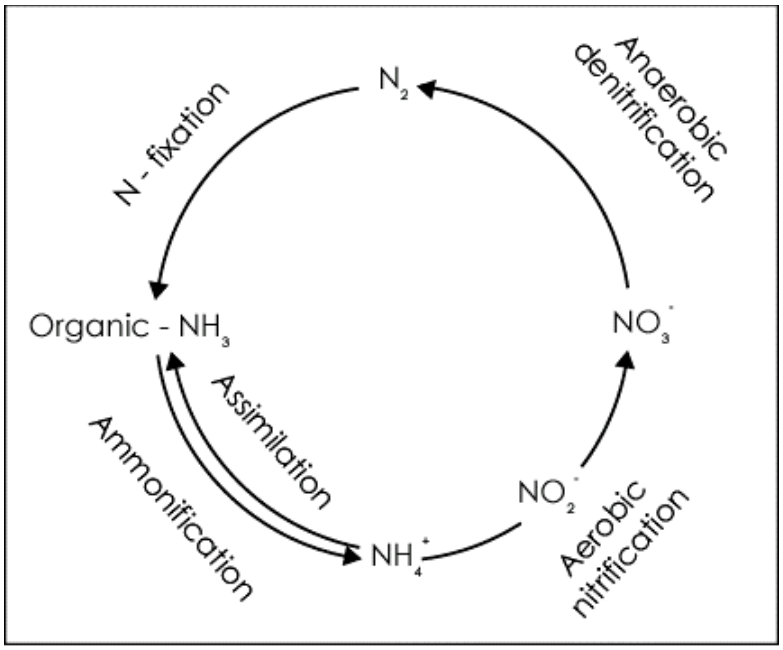


Figure 1.3.1. Schematic representation of the 'classical' nitrogen cycle illustrating critical aerobic nitrification and anaerobic denitrification phases (in the absence of anammox). (Diagram used with the permission of Dr. M. Trimmer).

1.4. A novel nitrogen removal process

Anaerobic ammonium oxidation (anammox) was first described in an anaerobic denitrifying reactor where the disappearance of NH_4^+ and NO_3^- were linked to a concomitant increase in N_2 gas (Mulder *et al.*, 1995; Van de Graaf *et al*., 1995). Further studies, using ^{15}N isotopes, revealed that NO_2^- (rather than NO_3^-) served as the oxidizing agent for anammox and that denitrification could provide the source of NO_2^- required for this process (Dalsgaard and Thamdrup, 2002; Van de Graaf *et al*., 1995) (Equation 4.). Essentially, this pathway circumvents the 'critical' aerobic phase of coupled denitrification and therefore redefines the 'classical' nitrogen cycle (Figure 1.4.1.). Further studies revealed that this process was microbially mediated by a group of

autotrophic bacteria forming a deep, monophyletic branch within the '*Planctomycetales*' (Strous *et al.*, 1999).

$$NO_2^- + NH_4^+ \rightarrow N_2 + 2H_2O \quad (4)$$

Equation 4. The anaerobic oxidation of NH_4^+ with NO_2^- (as an electron acceptor) produces N_2 gas with a stoichiometry of the ratio 1:1.

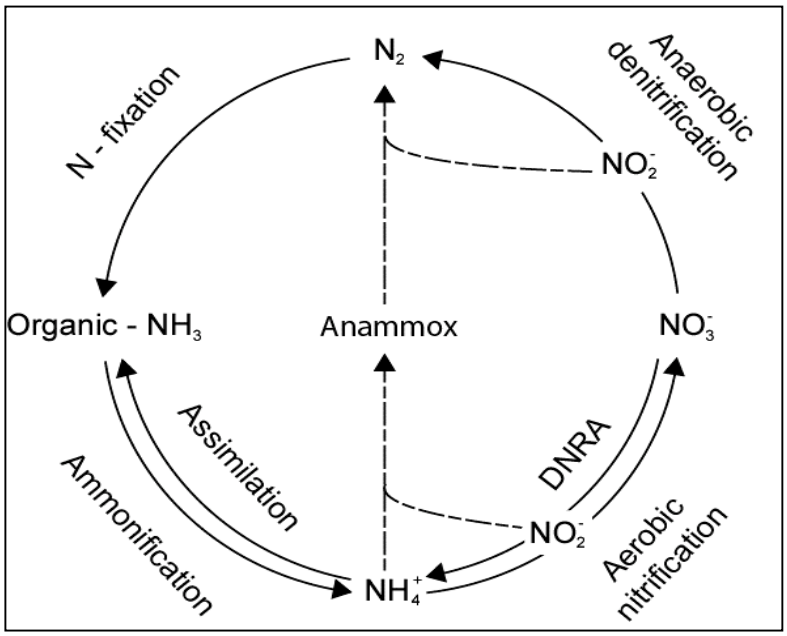


Figure 1.4.1. Links between 'classical' nitrogen removal pathways and anammox (dotted arrows) in the formation of N2 gas. (Trimmer *et al*., 2003).

1.5. The significance of anammox in sediment ecosystems

The anammox process has been detected in a variety of ecosystems including oxygen minimum zones (Kuypers *et al*., 2003; Kuypers *et al*., 2005), tropical freshwater (Schubert *et al*., 2006), Arctic sea ice (Rysgaard and Glud, 2004) and Arctic (Rysgaard *et al*., 2004), marine (Schmid *et al*., 2007; Thamdrup and Dalsgaard, 2002), and estuarine sediments (Meyer *et al*., 2005; Nicholls and Trimmer, 2009; Risgaard-Petersen *et al*., 2005; Trimmer *et al*., 2003; 2005; Tal *et al*., 2005; Rich *et al*., 2008). These studies confirmed that anammox significantly contributes to N_2 gas production; particularly in continental shelf sediments where up to 67% of total N_2 production can be attributed to this pathway (Dalsgaard and Thamdrup, 2002). Studies addressing the factors that regulate the distribution and significance of this process are, however, relatively scarce.

Dalsgaard *et al.* (2003) proposed that anammox is closely coupled to NO_3^- reduction in the environment. In a hypernutrified estuary, NO_2^- seldom accumulates in sediments and therefore the availability of this nutrient is scarce. The production of this intermediate in denitrification may, however, provide the NO_2^- required for anammox (Dalsgaard and Thamdrup, 2002). Interestingly Kuypers *et al.* (2003) reported that the highest density and activity of anammox coincided with a NO_2^- peak in the suboxic zone (of the water column) (Figure 1.5.1.). Furthermore, this NO_2^- peak arose at the base of the nitrate peak, indicating that anammox occurs just below the zone of denitrification, and in turn, provides evidence for coupling between these processes and is corroborated by Meyer *et al*. (2005).

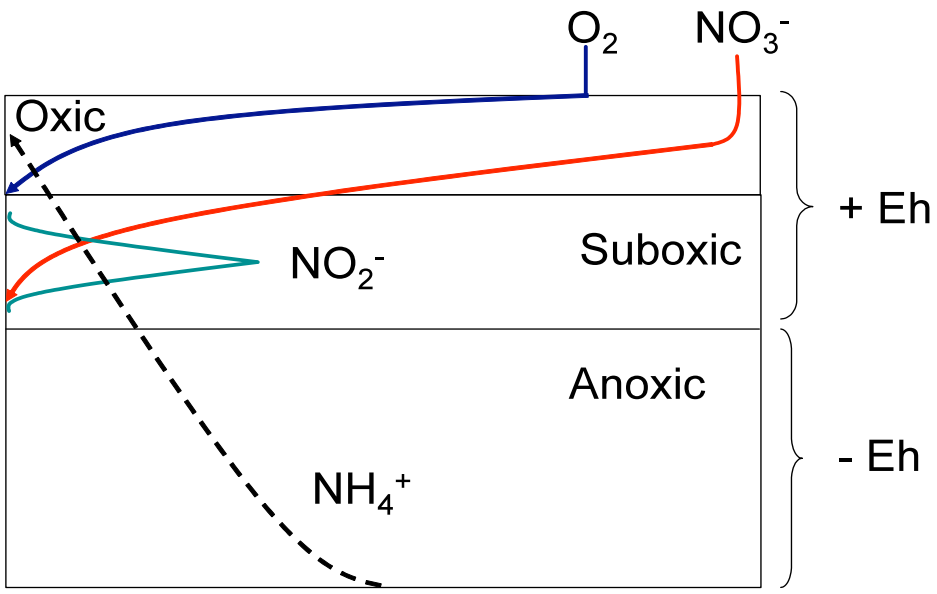


Figure 1.5.1. The theoretical distribution of O_2, NO_2^-, NO_3^- and NH_4^+ in oxic, suboxic and anoxic zones. Oxygen rapidly depletes within the first few mm of the sediment due to microbial respiration. Nitrate penetrates a little further into the sediment, but depletes as a result of denitrification and NO_3^- respiration. The zone of anammox activity is theoretically localised to suboxic zone where NO_2^- availability and the absence of O_2, provide the stable environment for the formation of an anammox community.

Interestingly, there are conflicting reports of how anammox correlates with sediment reactivity (the availability of reductants in the porewater). In the Thames estuary anammox peaks in highly reactive sediment, accounting for 8% of N_2 gas production in sediment slurries (Trimmer *et al.*, 2003) although in the less reactive sediments of the Skagerrak, 67% of N_2 gas production can be attributed to anammox (Dalsgaard and Thamdrup, 2002). Trimmer *et al.* (2005) reported rates of NO_3^- reduction in the Thames at 20 times those of the Skagerrak. It was therefore proposed that if rates of NO_3^- reduction were operating at V_{max} in the Skagerrak, 60% of all NO_3^- reduced would

accumulate as NO_2^-. However, in the context of NO_3^- reduction in the Thames, only 20% of NO_3^- reduced would be lost as NO_2^-. Consequently, an excess in NO_2^- production in the Skagerrak, could in fact, support a relatively large community of anammox organisms (Trimmer *et al.*, 2005).

In addition to a potential source of NO_2^-, anammox organisms require the absence of oxygen for stable growth. Enrichment culture studies have demonstrated that these organisms are inhibited by as little as 1 μM oxygen (Strous *et al*., 1997). The presence of bioturbating macrofauna dramatically disrupts the vertical distribution of O_2 and NO_x^- as shown in Figure 1.5.1. The overall effect of bioturbation is an increase in the surface area of the sediment-water interface. Essentially, this potentially results in an extension of the NO_3^- reducing zone, through an extension of the oxic-suboxic interface (Henriksen *et al.*1980). In terms of anaerobic ammonium oxidation, an increase in the availability of NO_2^- could enhance this process. The inhibitory effect of constant incursions of oxygen may, however, suppress the formation of a stable anammox community in such environments. The effects of bioturbation in either marine or estuarine sediments, are largely unexplored.

1.6. The identification of anammox organisms in enrichment culture and the environment

One of the major challenges facing ongoing research is the failure to isolate anammox organisms in pure culture. The use of 16S ribosomal RNA (rRNA) as a phylogenetic marker for anammox, however, has facilitated the identification of anammox organisms without the need for conventional cultivation techniques. rRNA is ubiquitous to all cellular life forms and consists of regions with different levels of conservation. This allows the determination of phylogenetic relationships from distantly to closely related species (Woese, 1987).

Anammox cells were purified for the first time (in suspension) by density gradient centrifugation (Strous *et al*., 1999). This enabled the extraction of DNA or RNA, amplification (with the use of a universal 16S ribosomal (rDNA) primer set) and cloning (to create a 16S rDNA gene library) (Jetten *et al.*, 2001). The dominant 16S rDNA sequence, within this library, was affiliated with the order *Planctomycetales* and the purified organism was tentatively named '*Candidatus Brocadia anammoxidans*' (Strous *et al*., 1999).

Information from the 16S rDNA sequence was used to generate oligonucleotide probes specific for *B. anammoxidans.* These probes successfully hybridised with anammox organisms (in FISH) and were used in further studies of wastewater treatment systems. Interestingly, probe binding patterns suggested that *B. anammoxidans* was not the only organism present in wastewater samples. Phylogenetic analysis of 16S rDNA gene

sequences, from a biofilm in a wastewater treatment system in Stuttgart, confirmed the presence of a distant relative of *B. anammoxidans*, capable of the anammox reaction (Schmid *et al*., 2000). This new species was also affiliated with the order *Planctomycetales* and is known as '*Candidatus Kuenenia stuttgartiensis*'. Subsequent studies have confirmed the presence of further species within two additional candidate genera '*Scalindua*' and '*Anammoxoglobus*' in wastewater treatment and natural systems (Strous *et al.*, 1999; Schmid *et al*., 2000; 2003; Kartal *et al*., 2007).

1.7. Thesis outline

This study addresses the depth distribution, significance and phylogeography of anammox organisms across a transect of estuarine, coastal and deep marine sediments.

CHAPTER II: The phylogeography of anammox organisms in marine and estuarine environments

Environmental studies clearly indicate that whilst the presence of anammox organisms has been verified in a vast range of environments, diversity is extremely limited (Penton *et al*., 2006; Nakijima *et al*., 2008; Schmid *et al*., 2007; Woebken *et al*., 2008). To date, only two 'candidate' species of anammox have been identified in natural systems. These species are constrained to the genus '*Scalindua*' and are known as '*Scalindua sorokinni*' and '*Scalindua arabica*' (Kuypers *et al*., 2003; Woebken *et al*., 2008). The objective of this investigation was to explore the diversity of the organisms responsible for anammox in marine and estuarine sediments. To investigate the composition of the anammox community, seven sites were selected representing deep sea sediments in the North

Atlantic (2000m), coastal sediments in the Irish Sea (50-100m) and shallow, organically enriched sediments in the Medway estuary. DNA was extracted from sediment samples and PCR amplified using a combination of either Pla 46F-Sca 1309R or Pla 46F-Amx 0368R. All amplificates were subsequently cloned and phylogenetically analysed.

Following a preliminary survey, a single clone sharing 96% sequence similarity with '*Scalindua sorokinni*' was isolated from sediments in the Medway estuary. This clone potentially represents a new species within the genus *Scalindua.* Further exploration was therefore required to determine whether this clone was more widely distributed in the environment. An additional survey of the anammox community was performed using fluorescent probes and primers designed according to this sequence.

CHAPTER III: The relative abundance and depth specific significance of anammox in estuarine sediments

The potential contribution of anammox to total N_2 production is highly variable in natural systems, yet the factors that regulate either the significance of the anammox process or the abundance of these organisms are largely unknown (Trimmer *et al*., 2003; 2005; Risgaard-Petersen *et al*., 2005; Meyer *et al*., 2005; Tal *et al*. 2005; Rich *et al*., 2008). The objective of this investigation was to determine whether variations in the significance of anammox were associated with changes in population size. In order to map this distribution, ^{15}N tracer experiments were employed to examine depth specific fluctuations in the potential contribution of anammox to total N_2 production (Thamdrup and Dalsgaard 2002; Dalsgaard and Thamdrup 2002; Dalsgaard *et al.*, 2003, Trimmer *et al.*,

2003; Risgaard–Petersen *et al*., 2004; Rysgaard and Glud, 2004; Risgaard–Petersen *et al*., 2005; Meyer *et al*., 2005; Trimmer *et al.*, 2005). FISH and fluorochrome 4'6-diamino-2-phenylindole dihydrochloride (DAPI) staining were used alongside this technique to map shifts in the abundance of anammox organisms with depth, thus linking potential activity to differences in population size.

Furthermore, the zone of anammox activity was characterised with the purpose of identifying the factors that may influence the distribution and activity of these organisms in an estuarine environment. This was achieved by using a combination of porewater oxygen profiles and measurements of depth specific rates CO_2 production.

Microsensor oxygen profiles were used to determine the position of the oxic-suboxic interface. As anammox is inhibited by the presence of oxygen ($\leq$1.1μM), the position of oxic-suboxic interface is potentially key to the distribution of these organisms with depth (Strous *et al.*, 1999). Moreover, the depth specific rates of CO_2 production provide an indirect measurement of sediment metabolism and are therefore an indication of sediment reactivity. In terms of the potential contribution of anammox to total N_2 production, previous estuarine studies have demonstrated that that the significance of this process is positively correlated with sediment reactivity (Trimmer *et al*., 2003; 2005). Sediment samples were retrieved from three sites forming a transect along the Medway estuary. Samples were collected both in the presence and absence of bioturbating macrofauna.

CHAPTER IV: The relative abundance and depth specific significance of anammox in marine sediments

Anammox is highly significant in deep, offshore sediments, yet the factors that maintain elevated activity are relatively unexplored (Van de Graaf *et al*., 1995; Mulder *et al*., 1995; Dalsgaard *et al*., 2003). The purpose of this study was to investigate possible links between the depth integrated potential for anammox and the overall significance of this process. In turn, fluctuations in the depth specific potential for anammox were subsequently compared with variations in the distribution of anammox organisms. The zone of potential anammox activity was characterised using porewater oxygen profiles, ^{15}N tracer experiments and FISH analysis. This investigation was performed at stations in the Irish Sea and North Atlantic.

2. INTRODUCTION

The anammox process is performed by a group of organisms that form a monophyletic branch within the phylum *Planctomycetes* (Strous *et al*., 1999; Schmid *et al*., 2005). This group consists of four genera that can be further divided into two distinct groups (as described by Woebken *et al*., 2008). The first group encompasses species that appear to be confined to wastewater treatment environments. These species have been described in enrichment culture and fall within candidate genera '*Brocadia*', '*Kuenenia*' and *'Anammoxoglobus*' (Kartal *et al*., 2007; Schmid *et al*., 2000; 2003; Strous *et al.*, 1999). The second group is represented by species that largely occur in the environment and fall within the clade '*Scalindua'* (Kuypers *et al*., 2003; Van de Vossenberg *et al*., 2008). To date, only two species of *Scalindua*, '*Candidatus Scalindua sorokinni*' and '*Candidatus Scalindua arabica*', have been identified in natural systems (Kuypers *et al*., 2003, Woebken *et al*., 2008). Both *Scalindua sorokinni* and *Scalindua arabica* are distantly related to those species that constitute the wastewater treatment group. On average they share between 85.9 - 87.9% sequence similarity with members of '*Kuenenia*' and '*Brocadia*' (Kirkpatrick *et al*., 2006; Kuypers *et al*., 2003; 2005; Risgaard–Petersen *et al*., 2004; Strous *et al*., 1997; Van de Graaf *et al*., 1996; Woebken *et al*., 2008). Whilst a wealth of knowledge regarding the implementation and diversity of anammox in wastewater treatment is available, little is known about the ecophysiology of this distantly related clade.

The recent enrichment of *Scalindua sorokinni* in mixed culture could provide some insight into the physiology and regulation of anammox, yet the resulting data cannot be directly applied to natural systems. The constant, highly controlled conditions under which anammox organisms are enriched, fail to reflect the dynamics of temperature, salinity, oxygen or nutrient concentrations within the natural environment. One of the most immediately relevant examples is the enrichment of *Scalindua sorokinni* from sediment collected in Gullmar Fjord (Van de Vossenberg *et al*., 2008). To promote the growth of this organism, the culture and reactor influent were maintained at 0.5mM NO_2^-. This is nearly two orders of magnitude above *in situ* NO_2^- concentrations, where NO_2^- rarely exceeds 5μM (Meyer *et al*., 2005). Whilst the availability of NO_2^- (derived via NO_3^- reduction) is potentially crucial to the significance, abundance and distribution of anammox in the environment, any extrapolation from enrichment culture studies should be viewed with caution (Meyer *et al*., 2005). In addition to high concentrations of NO_2^-, the enrichment vessel was continuously purged with a mixture of Ar/CO_2 in order to maintain completely anaerobic conditions. This is in line with the observation that anammox reaction is inhibited by ≤1μM oxygen in enrichment culture (Strous *et al*., 1999). *In situ* measurements of O_2, however, indicate that the concentration of O_2 in Gullmar Fjord (160μM) was highly elevated relative to the conditions maintained in the culture. This is far removed from environmental conditions where the presence of anammox has been confirmed in periphyton dominated, aerobic sediments (Penton *et al*., 2006) and from depths in the Namibian OMZ where oxygen concentrations of up to 25μM O_2 were reported (Woebken *et al*., 2007). It is therefore clear that further

ecological studies are required to resolve the factors that may regulate the distribution, diversity and activity of environmental species.

A growing number of environmental surveys suggest that whilst the distribution of anammox organisms is widespread, diversity is extremely limited (Penton *et al*., 2006; Nakajima *et al*., 2008; Schmid *et al.*, 2007; Woebken *et al*., 2008). Intriguingly, 16S rRNA gene sequences collected from a broad range of environments including oxygen minimum zones (Kuypers *et al*., 2003; 2005; Kirkpatrick *et al*., 2006; Stevens and Ulloa 2008; Woebken *et al*., 2007; 2008), freshwater (Penton *et al*., 2006; Schmid *et al*., 2007; Schubert *et al*., 2006), estuarine (Rich *et al*., 2008; Risgaard-Petersen *et al*., 2004; Tal *et al*., 2005) and marine systems (Penton *et al*., 2006; Schmid *et al*., 2007; Shu and Jiao, 2008) demonstrate a high degree of sequence similarity to each other and to *Scalindua sorokinni*. In order to classify candidate sequences as members of a given species, the candidate sequences should share at least 97% sequence identity to the organism concerned (Rosello-Mora and Amann, 2001). To date, the majority of sequences retrieved from the environment share at least 97% sequence similarity to *Scalindua sorokinni*. The only exceptions are those retrieved from Lake Tanganyika (Schubert *et al*., 2006; DQ4440) and the South China Sea sediment (EU048621). These sequences share ≤ 96% sequence identity with other *Candidatus Scalindua* sequences but can be grouped with *Scalindua arabica* (Woebken *et al*., 2008). No other species of anammox have been identified in the environment.

Given the limited data set concerning the phylogeny of anammox, in marine (Penton *et al*., 2006; Schmid *et al*., 2007; Shu and Jiao, 2008) and estuarine systems (Rich *et al*., 2008; Risgaard-Petersen *et al*., 2004; Tal *et al*. 2005) further investigation is clearly required. The purpose of this study is to explore possible shifts in the structure of the anammox community, in the transition between marine and estuarine environments. Seven sites were analysed in total, representing deep sea sediment from the North Atlantic shelf break (2000m), coastal seas (at depths ranging from 50-100m) and from estuarine sediments (along a gradient of OC,O_2 and NO_x^- availability). This study was extended to determine whether a unique clone from The Medway was distributed more widely in the environment. The MC1 clone sequence shares 96% with *Scalindua sorokinni* and could therefore represent a new species within the genus *Scalindua*. To further investigate the composition of the Anammox community in the Medway estuary, the probe 'MC1' (S-*-MC1-0043-a-20) was tested in fluorescence *in situ* hybridisation in addition to the 'MC1' primer (designed with the same target sequence).

3. METHODS

3.1. Study sites

3.1.1. Marine study

Sediment samples were collected from sites located in the Irish Sea and the Celtic Sea portion of the North Atlantic. The Irish Sea is located between Great Britain and Ireland. It connects with the North Atlantic via Saint Georges Channel (to the south) and through the North Channel (to the North). Sediment samples were retrieved from three sites in the Irish Sea (Sites 4, 5 and 6) at depths ranging from 50-100m and from 1 site (Site 1, 2000m) in the North Atlantic (Figure 3.1.1.). Details of locations and site characteristics are described in Table 3.1.

3.1.2. Estuarine study

Sediment samples were collected from three sites forming a transect along the Medway Estuary. The Medway Estuary lies to the South of the Thames Estuary in South East England, Kent. It is connected to the Thames Estuary via the Isle of Grain and drains into the North Sea through tidal channels that divide large islands of salt marsh. Samples from each site were gathered at low tide from the inter-tidal mudflats along the estuary (Figure 3.1.2). Details of the locations and site characteristics are described in Table 3.1.

Site	**Location**	**Depth**	**Latitude**	**Longitude**	**Oxygen penetration depth (mm)**	**Total organic carbon (%)**
1	North Atlantic	2006	48.03.09N	9.51.11W	12.4	0.49
4	Irish Sea	106	51.13.10N	6.05.96W	9.8	0.82
5	Irish Sea	104	53.52.95N	5.35.56W	17.0	1.36
6	Irish Sea	54	54.07.14N	5.35.05W	7.2	1.25
Gr	Grain	0	51.22.24.74N	0.27.50.43E	9.9	*NS*
Up	Upnor	0	51.24.35.85N	0.31.45.54E	3.6	*NS*
MBM	Medway Bridge Marina	0	51.27.38.90N	0.43.05.74E	5	*NS*

Table 3. 1. Site characteristics and locations. Values for total organic carbon (Sites 1-6) were reproduced from Jaeschke *et a*l, (2009). *NS = Not included in this study.*

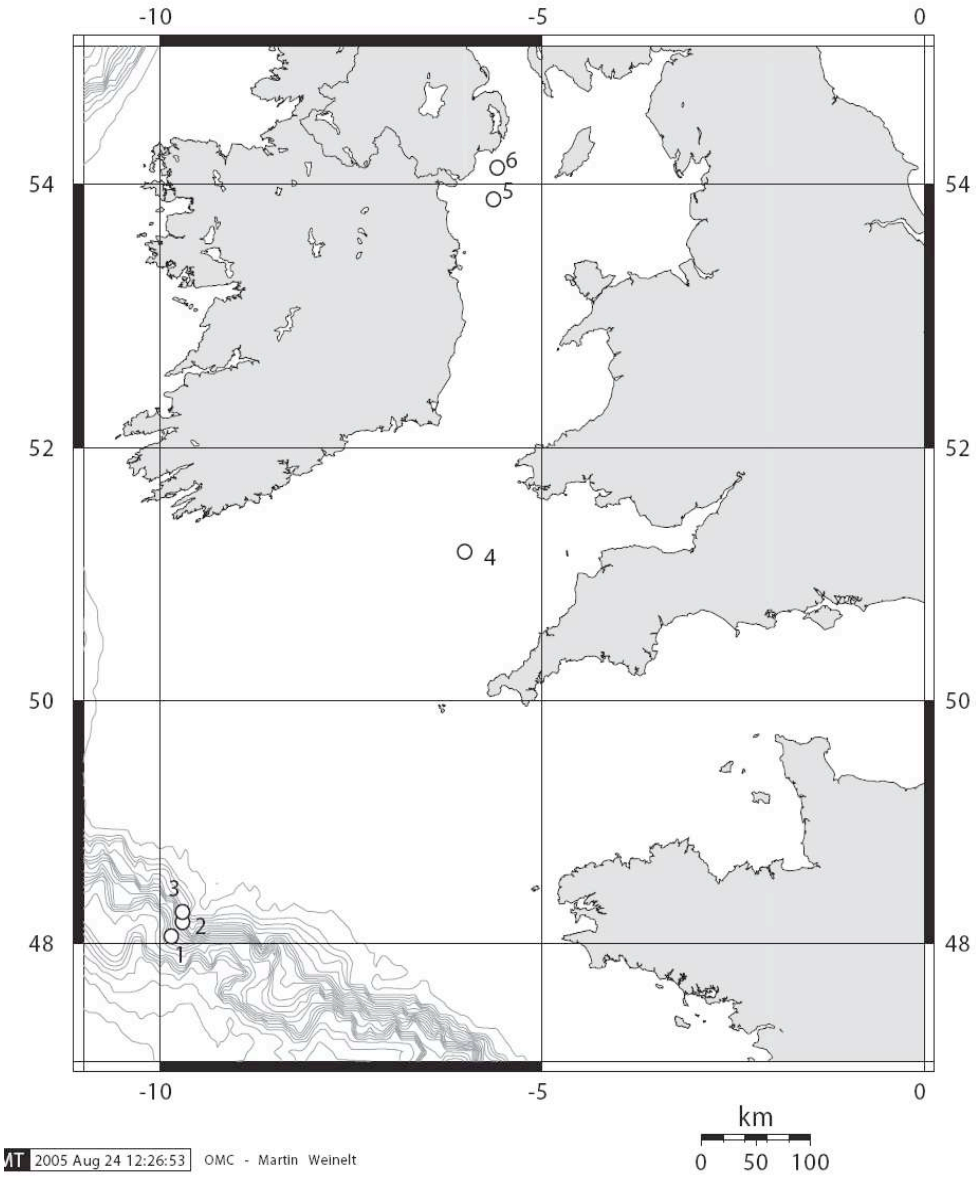


Figure 3.1.1. Map of the North Atlantic and Irish Sea showing the locations of the four sampling sites (open circles). Source: Jaeschke *et al*, (2009). Sites 2 &3 did not form part of this study.

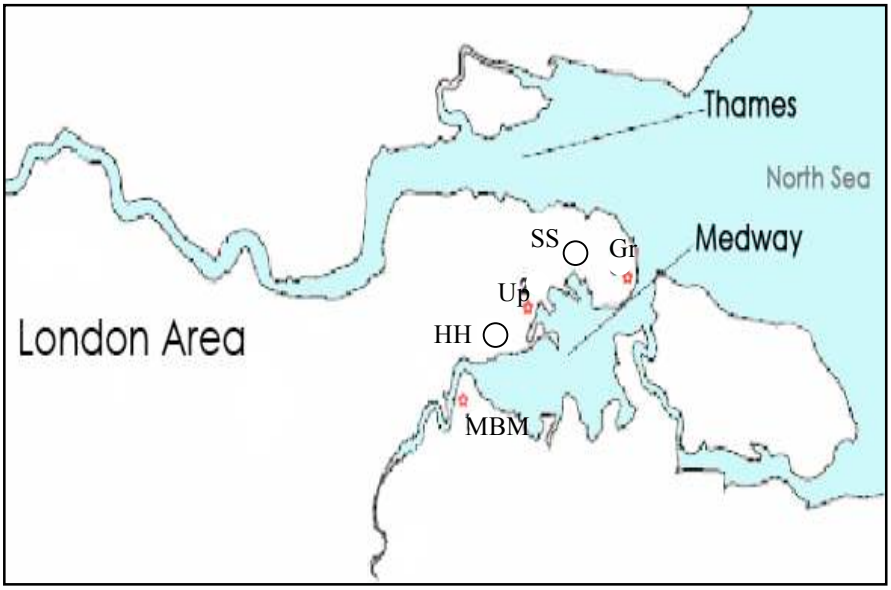


Figure 3.1.2. Map of the Medway estuary on the southeastern coast of England showing the locations of the five sampling sites used in the preliminary (April 2005, red stars and open circles) and the second survey (September 2007, red stars). Used with permission of Dr. M. Trimmer.

3.2. Sediment collection and storage

The marine sediment samples were collected with a box-corer aboard the R/V *Pelagia* during March 2006. Only intact undisturbed cores containing clear overlying water were sub sampled using Perspex tubes (height = 6cm, inner diameter = 9cm). 5 sediment cores were retrieved from the box corer and transferred to a water bath containing aerated site water. The water bath was maintained at constant temperature (8ºC) using a Grant thermo circulator (Grant instruments, Cambridgeshire, UK). The sediment cores were re-equilibrated over night and subsequently sliced at 3mm intervals to a depth of 36mm. Approximately 1g of sediment from the suboxic zone was collected from each core and transferred to a 1.5ml Eppendorf tube. All samples were stored at -20ºC until required for further processing.

Samples were gathered at low tide from the Medway Estuary (September 2007) using 5 identical Perspex tubes (dimensions as above). The cores were then returned to the lab (within two hours) and transferred to a water bath containing aerated low nutrient sea water (diluted to site salinity). The cores were re-equilibrated over night and maintained at 12ºC using a Grant thermo circulator (Grant instruments, Cambridgeshire, UK). Sediment was sliced at 0.5cm and intervals to a depth of 2.5cm for FISH analysis. Approximately 1g of sediment from the suboxic zone was transferred to a 1.5ml Eppendorf tube. All samples were subsequently stored at -20 ºC until required.

3.3. DNA isolation

DNA was extracted using the hydroxytapatite spin column method (Purdy *et al*., 1996). In brief this method lyses cells using bead-beating in an alkaline phosphate solution, in the presence of phenol and sodium dodecyl sulphate (SDS). Nucleic acids were separated from cellular components and sediment contaminants using a hydroxyapatite spin column. Nucleic acids were purified by ethanol and then PEG precipitation. PEG precipitation was performed using an amended method, with 10% (w/v) PEG as described by Arbeli and Fuentes (2007). Nucleic acid extracts were examined ethidium bromide-stained agarose gel electrophoresis (1.5% (w/v) gel in 1x TAE) (Figure 3.3.1.).

3.4. Optimisation of anammox directed amplification using a sequential PCR approach

Early attempts to directly amplify the 16S rRNA gene, from DNA extracted from sediment samples, failed to produce PCR products. Consequently, a general bacterial PCR was used as the first step in a nested PCR approach.

Universal bacterial primers are widely used in the amplification of entire 16S rRNA genes (Bruce *et al*., 1992; Purdy *et al*., 2003). Nested within the 16S rRNA gene is a signature region unique to anammox organisms that can be amplified in an anammox directed PCR, using template DNA derived from the preceding general bacterial PCR.

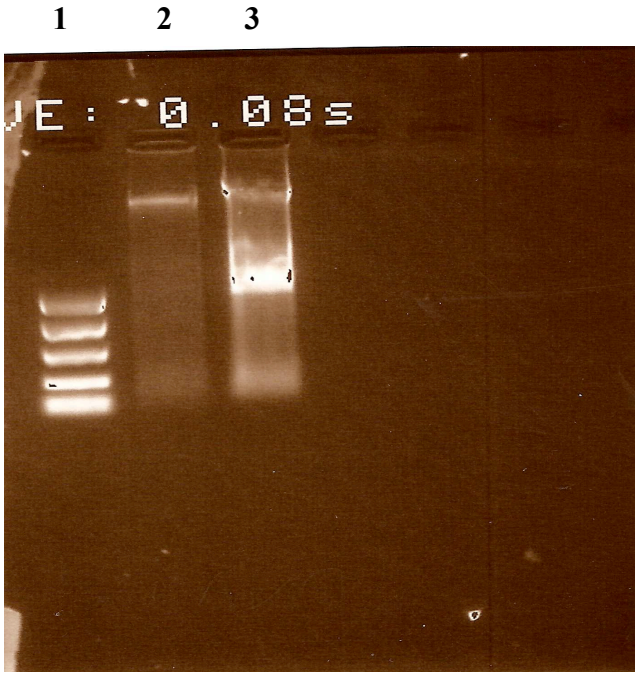


Figure 3.3.1. Verification of DNA isolation using 1.5% (wt/vol) agarose gel electrophoresis. Lanes 1, 1kb DNA ladder; lane 2, Site 5 (Irish Sea sediment); lane 3, positive control (pond sediment).

The universal primers 'BF' and '1541R' (see Table 3.4.1) were used in the amplification of 16S rDNA gene sequences. PCR reactions (50 µl) were performed with Go-Taq from Promega (Southampton, UK) using their 5X PCR buffer, 1.75 mM $MgCl_2$, 1mM dNTPs, 20 pmols of each primer, 200 ng BSA and 1U of Go-Taq.

Thermal cycling (EP Mastercycle, Eppendorf, Germany) was performed with an initial hotstart at 96°C for 2 minutes, followed by 10 cycles of a denaturation step of 1 minute at 96ºC, an annealing step at 50ºC for 30 seconds and elongation at 72ºC for 2 minutes, then 20 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 2.5 minutes and finally an elongation step at 72°C for 5 minutes. Positive (DNA extracted from pond sediment) and negative controls (no DNA) were included in all reactions. PCR reactions were analysed by ethidium bromide-stained agarose gel electrophoresis (1.5% (w/v) gel in 1x TAE) to determine the presence and size of amplicons.

The general bacterial PCR product was used as a template for the following PCR assays: 'Pla 46F' and 'Amx 0368R', targeting all anammox organisms; 'Pla 46F' and 'Sca 1309R', targeting species of the genera *Scalindua*; and 'Pla 46F' and 'MC1R', designed using the probe design tool in ARB (see section 3.6.). Details of the primers are provided in Table 3.4.1.

The PCR mixture (50µl) was as above, except they contained 1.5mM $MgCl_2$ and 10pmol of each primer. Thermal cycling was carried out with a 5 minute hotstart at 94ºC, followed by

PCR primers	**Specificity**	**Sequence 5'-3'**	**Reference**
Bf	*Bacteria*	TCAGAWYGAACGCTGGCGG	KJ Purdy pers comms.
1541R	*Bacteria*	AAGGAGGTGATCCAGCC	Embley, 1991.
Pla 46F	*Planctomycetales*	GACTTGCATGCCTAATCC	Neef *et al*., 1998.
Sca 1309R	*Scalindua*	TGGAGGCGAATTTCAGCCTCC	Schmid *et al*., 2003.
Amx 0368R	All anammox organisms	CCTTTCGGGCATTGCGAA	Schmid *et al*., 2000.
MC1R	MC1 clone	TGTTAAGAAATGTAGGTCTG	This study

Table 3.4. PCR primers used in this study. All oligonucleotide primers were primers were purchased from MWG-Biotech (London, UK).

an annealing step of for 1 minute and elongation at 72ºC for 1.5 minutes. This was repeated between the second and last step 14 times. The program continued with a denaturation step of 94ºC for 1 minute, an annealing phase of 56 ºC for 1 minute and elongation at 72ºC for 1.5 minutes. This was repeated 14 times. In addition, samples were held at 72ºC at the end of the cycle for 10 minutes. Positive (DNA extracted from enrichment culture) and negative controls (no DNA template) were used in each PCR amplification and analysed by agarose gel electrophoresis (5% (w/v) gel in 1x TAE) to determine the presence and size of amplicons. In order to reduce the probability of preferential amplification bias (Polz *et al*., 1998), 5 replicate PCR amplifications were performed per primer pair for each sample. PCR products were subsequently checked by agarose gel electrophoresis (Figure 3.4.1.) and bulked prior to cloning.

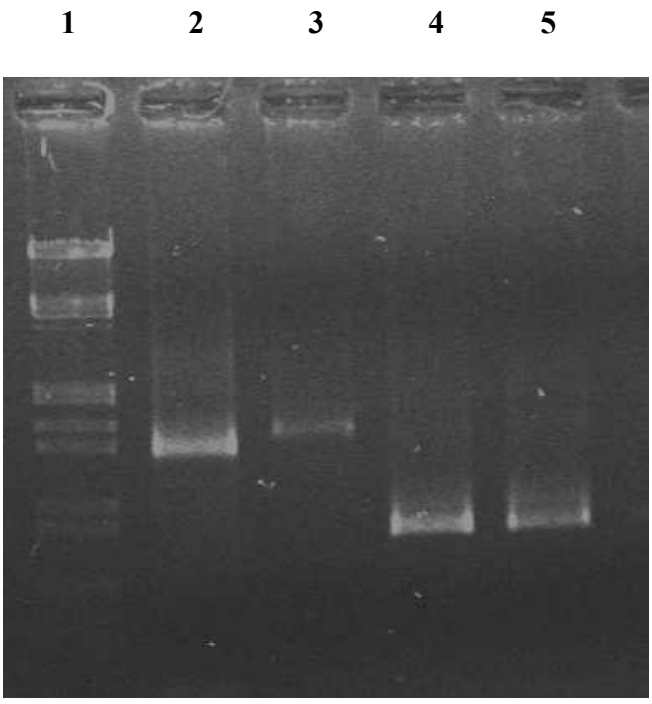


Figure 3.4.1. Agarose gel electrophoresis verifying the size and presence of amplicons. Lane 1, 1kb ladder; Lanes 2-3, DNA amplified with Pla 46F Sca 1309R (1500bp); Lanes 4 and 5, DNA amplified with Pla 46F and Amx 0368R (650bp).

3.5. Cloning and comparative sequence analysis

3.5.1. Clone libraries

PCR amplified 16S rDNA fragments were transformed into *Escherichia coli* using a TOPO TA cloning kit (Invitrogen, UK) by following the manufacturer's instructions. The presence and size of cloned inserts was verified by colony PCR amplification using vector based primers (M13F and M13R) and subsequent agarose gel electrophoresis. The clone libraries consisted of twenty clones (per primer set) for each of the marine and estuarine sites. In total, 280 clones were sequenced on an ABI Prism 3700 DNA analyser, at the Natural History Museum London, using the vector based primers M13F and M13R.

3.5.2. Phylogenetic analysis

The sequences were initially aligned using the alignment function in 'green genes' and corrected by visual inspection in ARB (http://greengenes.lbl.gov/cgi-bin/nph-NAST_align.cgi; De Santis *et al*., 2006; http://www.arb-home.de; Ludwig *et al.*, 2004). All partial and redundant sequences removed. Phylogenetic trees were calculated using the maximum likelihood algorithm with a 50% variability filter in ARB. The topology of each resulting tree was verified by bootstrapping in PAUP (n=300). Similarity matrices were calculated by using the 'neighbour-joining' tool using the similarity option in ARB. The Sim Rank algorithm was used (as a component of the green genes classification function) to initially classify all aligned sequences (De Santis *et al*., 2006).

3.6. Evaluating the specificity of MC1

Approximately 1g of sediment from the suboxic zone from each site was fixed with 4% (v/v) paraformaldehyde in PBS solution and allowed to stand at room temperature for 2 hours. Each sample was then washed twice with PBS and re-suspended in a 50% (v/v) PBS-ethanol solution. All samples were subsequently stored at -20ºC until required.

FISH experiments were conducted based on the methods described by Schmid *et al*., (2000; 2003). Optimal hybridisation conditions for MC1 were determined by a series of FISH experiments. This involved sequentially increasing the concentration of formamide in the hybridisation buffer whilst decreasing the corresponding concentration of sodium chloride in the washing buffer. The hybridisation buffers were prepared between concentrations of 0 and 70% formamide, at 5% intervals. These indicated that in order to maximise probe binding, 25% formamide was required in the hybridisation buffer. Following hybridisation, sediment samples were counterstained with DAPI (fluorochrome 4'6-diamino-2-phenylindole dihydrochloride) mounted in Vectashield (Vector Laboratories, Peterborough, UK) and viewed using a Leica DM RA2 epiflouresecence microscope (Leica microsystems, UK). Images were captured from two visual fields using Openlab (Improvision, Coventry, UK and processed with Adobe Photoshop (Adobe Systems, Edinburgh, UK). Adjustments to colour contrast and brightness were uniform across all images. Five replicate samples were analysed using FISH at each depth interval. Following the confirmation of positive FISH signals, the probe MC1 was tested in conjunction with the probes Pla 46F and Amx 0368R. All labelled oligonucleotide probes were purchased as Cy3, Cy5 and 5(6)-

carboxyfluorescein-N-hydroxysuccinimide ester (Fluos) labelled derivatives from MWG-Biotech (London, UK) and Thermo Electron (Ulm, Germany).

4. RESULTS

4.1. Phylogenetic analysis based on 16S rRNA gene sequences retrieved from the North Atlantic, Irish Sea and the Medway Estuary

Early attempts to directly amplify 16S rDNA fragments with anammox specific primers, failed to produce any PCR products. Consequently, a nested PCR using general bacterial primers BF and 1541R was performed followed by an anammox-specific PCR. This produced full length 16S rDNA fragments which then served as templates in a further, anammox directed PCR. By adopting this sequential approach and several purification steps, the efficiency with which full and partial 16S rDNA fragments were amplified increased substantially (see Figure 3.4.1).

In total, two marine clone libraries were constructed using the primers Pla 46F - Amx 0368R, (forming the first clone library) and Pla 46F - Sca 1309R (forming the second). Within each clone library, clones derived from Sites 1, 4, 5 and 6 were screened for overall diversity and the presence of sequences affiliated with the anammox group. This was performed with the Sim Rank algorithm using the classification function in 'green genes' (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi, De Santis *et al*., 2006). The results suggested the presence of sequences closely related to *Verrucomicrobia*, amongst classified and unclassified *Planctomycetes*. Of the classified *Planctomycetes*, the majority of sequences were closely affiliated with *Pirellula*, *Rhodopirellula* and *Plantomyces* but no candidate anammox sequences were identified.

An additional three clone libraries were created from PCR products using the primer pairs Pla 46F-Amx 0368R, Pla 46F - Sca 1309R and Pla 46F – MC1R (targeting a unique sequence obtained from a previous phylogenetic survey, see 4.2.). Clones derived from three sites along the Medway estuary were initially classified (as above) using the Sim Rank algorithm. The results again suggested the presence of *Verrucomicrobia* amongst classified and unclassified *Planctomycetes*. The majority of '*Planctomycete*-like' sequences were closely affiliated to *Gemmata*, *Isosphaera*, *Pirellula* and *Rhodopirellula.* Of the 180 estuarine clones screened, only 3 candidates were identified as potentially harbouring '*Scalindua*–like' sequences. These sequences were generated using the Pla 46F capture primer in combination with Amx 0368R (Sites Gr and Up) and MC1R (Sites MBM and Up). A total of 280 clones were further analysed from both the marine and estuarine sites.

4.2. Evaluation of the efficiency of the primer MC1 in PCR amplification

To further investigate the composition of the anammox community in the Medway estuary, the primer 'MC1' was designed on the basis of a sequence retrieved (in an earlier survey) from Medway Bridge Marina (in April 2005, Figure 4.2.1.). This sequence shares 96% identity with *Scalindua sorokinni* suggesting that the MBM sequence is a novel member of the genus *Scalindua* (Rosello-Mora and Amann, 2001). The primer 'MC1' was designed to specifically amplify these sequences as it has no mismatches with the MC1 clone sequence and three mismatches with closely related members of the genus *Scalindua*. This primer pair Pla 46F - MC1R successfully amplified partial 16S rDNA fragments from Sites MBM and Up but not from samples retrieved at Site Gr.

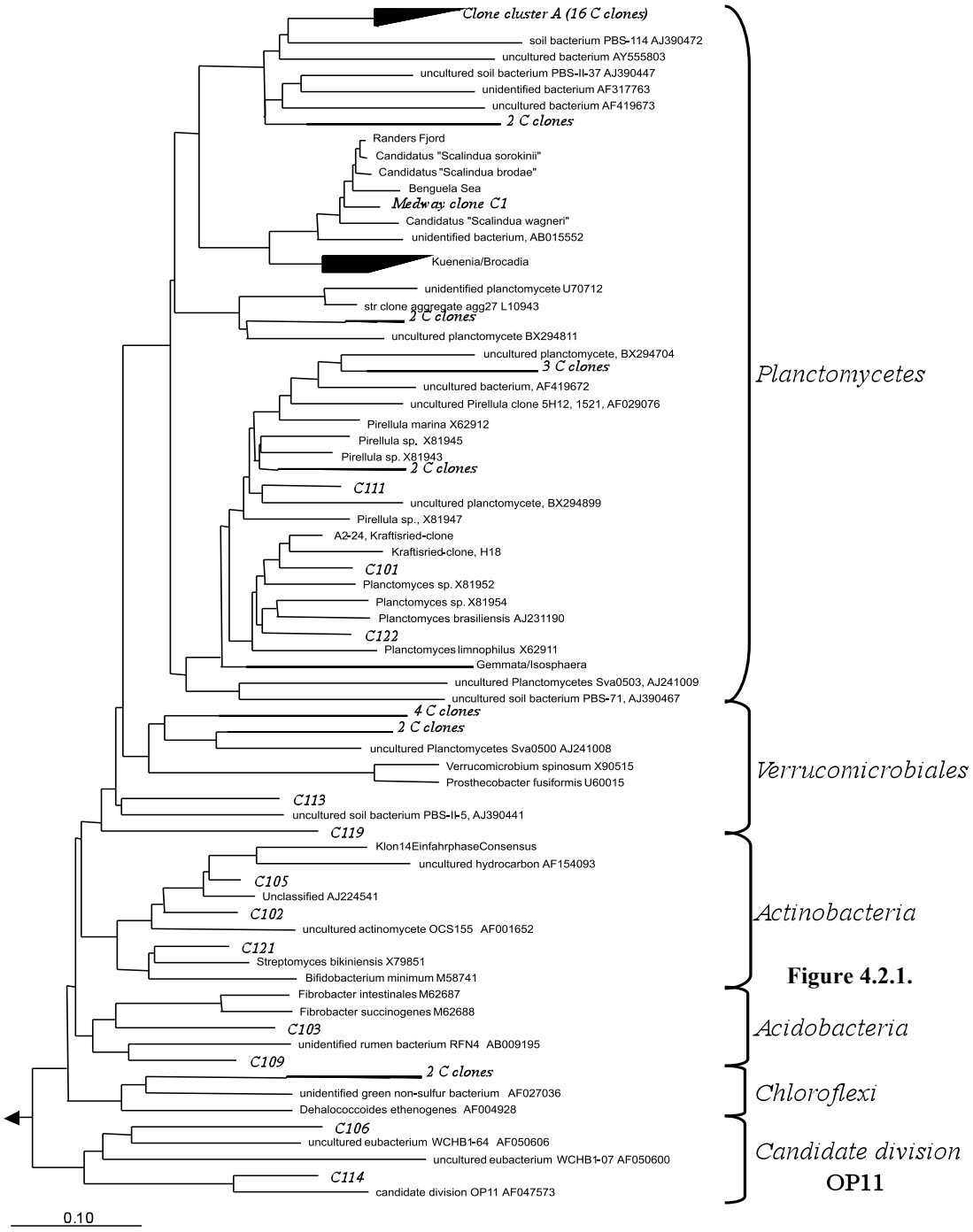


Figure 4.2.1.

Figure 4.2.1. Phylogenetic tree of 16S rRNA gene sequences amplified with Pla 46F – Sca 1309R in April 2005. The MC1 (Medway Clone C1) sequence shares 96% sequences similarity with *Scalindua sorokinni*.

4.3. Evaluating the specificity of probe MC1 in FISH at a range of formamide and Na Cl concentrations

Several FISH experiments were performed to test the specificity of the new 'MC1' probe against target organisms potentially carrying the MC1 sequence. Subsequent epifluorescence microscopy revealed bright and clear FISH signal intensities originating from candidate anammox cells. Optimal hybridisation conditions for MC1 were determined by a series of FISH experiments. This involved sequentially increasing the concentration of formamide (in the hybridisation buffer) whilst decreasing the corresponding concentration of sodium chloride (Na Cl) (in the washing buffer). The hybridisation buffers were prepared between concentrations of 0 and 70% formamide, at 5% intervals. The FISH protocol was followed as previously described (Schmid *et al*., 2000; 2003), with subsequent observation of cells by epifluorescence microscopy. The results indicated that the optimum concentration of formamide in the hybridisation buffer was 25%. At this concentration the probes hybridised with Anammox-like cells producing a clear and bright signal (4.3.1).

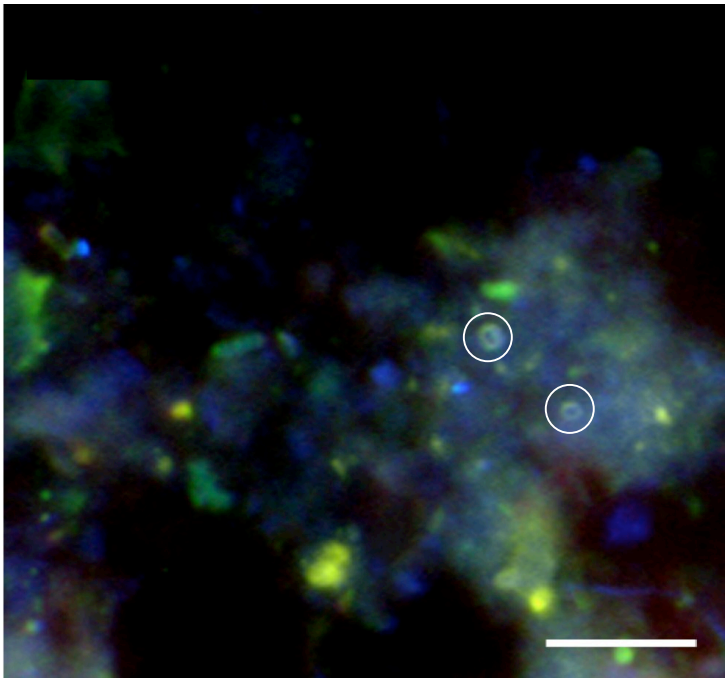


Figure 4.3.1. The detection of anammox cells with the Cy3 labelled probe MC1. 'doughnut - like' anammox cells are clearly visible at a concentration of 25% formamide in the hybridisation buffer (white circles). The blue DAPI stained cells indicate the presence of DNA-containing bacterial cells. The diameter of the anammox cells is within the expected size range of 0.7 µm. The scale bar represents 5µm.

4.4. Phylogenetic analysis of sequences affiliated with the anammox group

All phylogenetic trees were calculated using the maximum likelihood algorithm in combination with a 50% variability filter. This method was performed using the Arb program package (http://www.arb-home.de; Ludwig *et al.*, 1998). The topology of the resulting trees was further tested by bootstrapping (n=300 re-samplings) (PAUP). Surprisingly, of the 280 clones screened, only three sequences were retrieved that fell within the anammox group. All phylogenetic trees were constructed with reference *Planctomycetes* and anammox sequences.

4.4.1 Sequences amplified using the primer MC1

The sequence 'Up 92' (Site Up) clearly groups with *Scalindua sorokinni* (Figure 4.4.1). This sequence shares just 93% sequence similarity to this species and 96% identity with the MC1 clone. Although this value is relatively low, all remaining sequences shared <93% sequence similarity with *Scalindua sorokinni*. (This is applicable to all sequences analysed, throughout all clone libraries).

The remaining sequences formed 3 sub-groups near to the known anammox group (Groups A, B and C). Despite the position of these clusters there is little evidence that these sub-groups represent new environmental clusters of anammox (Figure 4.4.1). On average, these sequences share just 72% sequence similarity to *Scalindua sorokinni*, 90% sequence similarity to each other and 77-100% sequence similarity with the MC1 clone. It is therefore

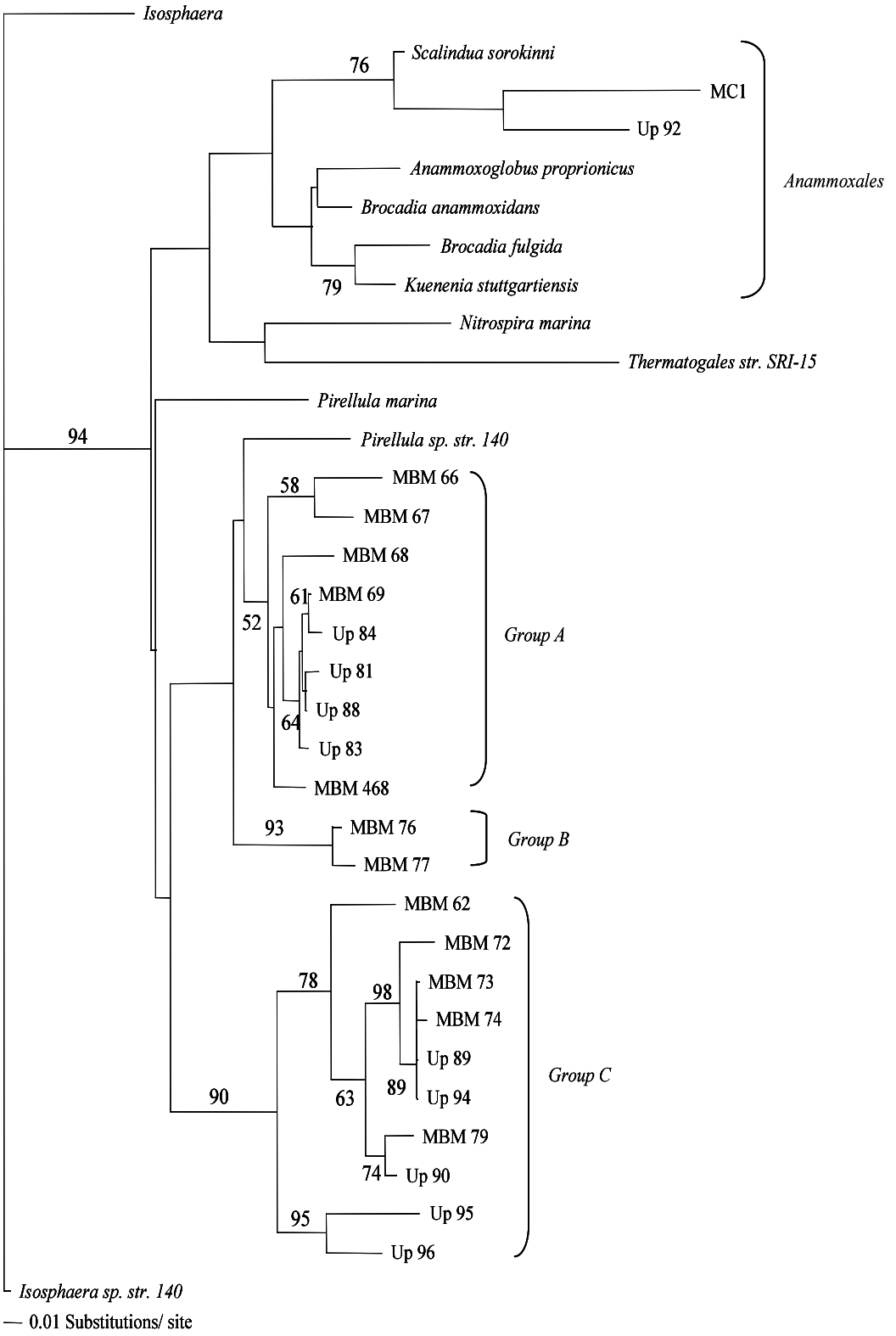


Figure 4.4.1. Phylogenetic tree of 16S rRNA gene sequences amplified with Pla 46F – MC1R. These sequences were retrieved from the Medway Estuary at Sites MBM and Up. The scale bar represents 0.01 substitutions/ site. Associated bootstrap values are displayed on supported nodes.

clear that the primer MC1 was non specific and consequently failed to retrieve the target sequence. This perhaps indicates that probe design cannot be directly applied to the synthesis of corresponding primers. All other known anammox primers, however, are based on probe sequences.

4.4.2 Sequences affiliated with the Anammox group in the Medway estuary

Samples amplified with the primer pair Pla 46F - Amx 0368R yielded 2 sequences that were placed near or within the anammox group. These sequences were retrieved from samples collected at Sites Gr (G 51) and Up (Up 36). 'G 51' appears to be affiliated with *Scalindua sorokinni* where as Up 36 forms an independent branch stemming from the Anammox clade (Figure 4.4.2.). These sequences, however, share as little as 86 and 77% sequence similarity with *Scalindua sorokinni*. Moreover, few conclusions can be drawn from the relationships of these and the remaining clones, given the low bootstrap values associated with the corresponding phylogenetic tree.

4.5. Phylogenetic analysis of sequences falling outside the known anammox group

Sequences from Sites 1 and 5 formed two phylogenetic clusters close to the Anammox group (Groups D and E) (Figure 4.5.1). The clustering of these sub-groups is well supported by the bootstrap values. Group D consists of 6 clone sequences (amplified with primers Pla 46F – Sca 1309R) derived from Site 5. These sequences share between 74-98% sequence similarity to each other and 75% sequence similarity to *Scalindua sorokinni*. Similarly, the 6 sequences that

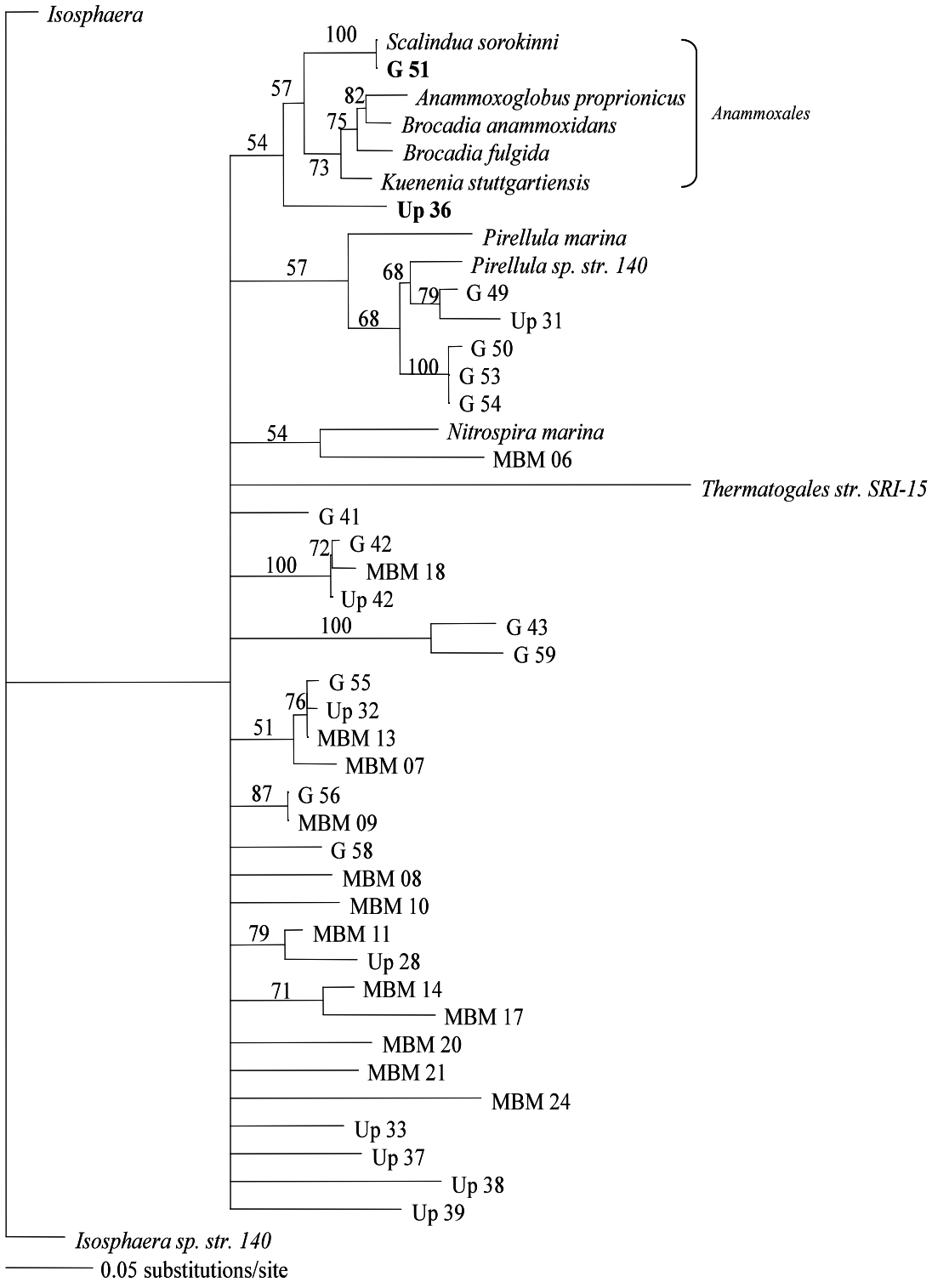


Figure 4.4.2. Phylogenetic tree of 16S rRNA gene sequences amplified with primer pairs Pla 46F – Amx 0368R. These sequences were retrieved from Sites MBM, Up and Gr (G). The scale bar represents 0.05 substitutions/ site. Associated bootstrap values are displayed at supported nodes.

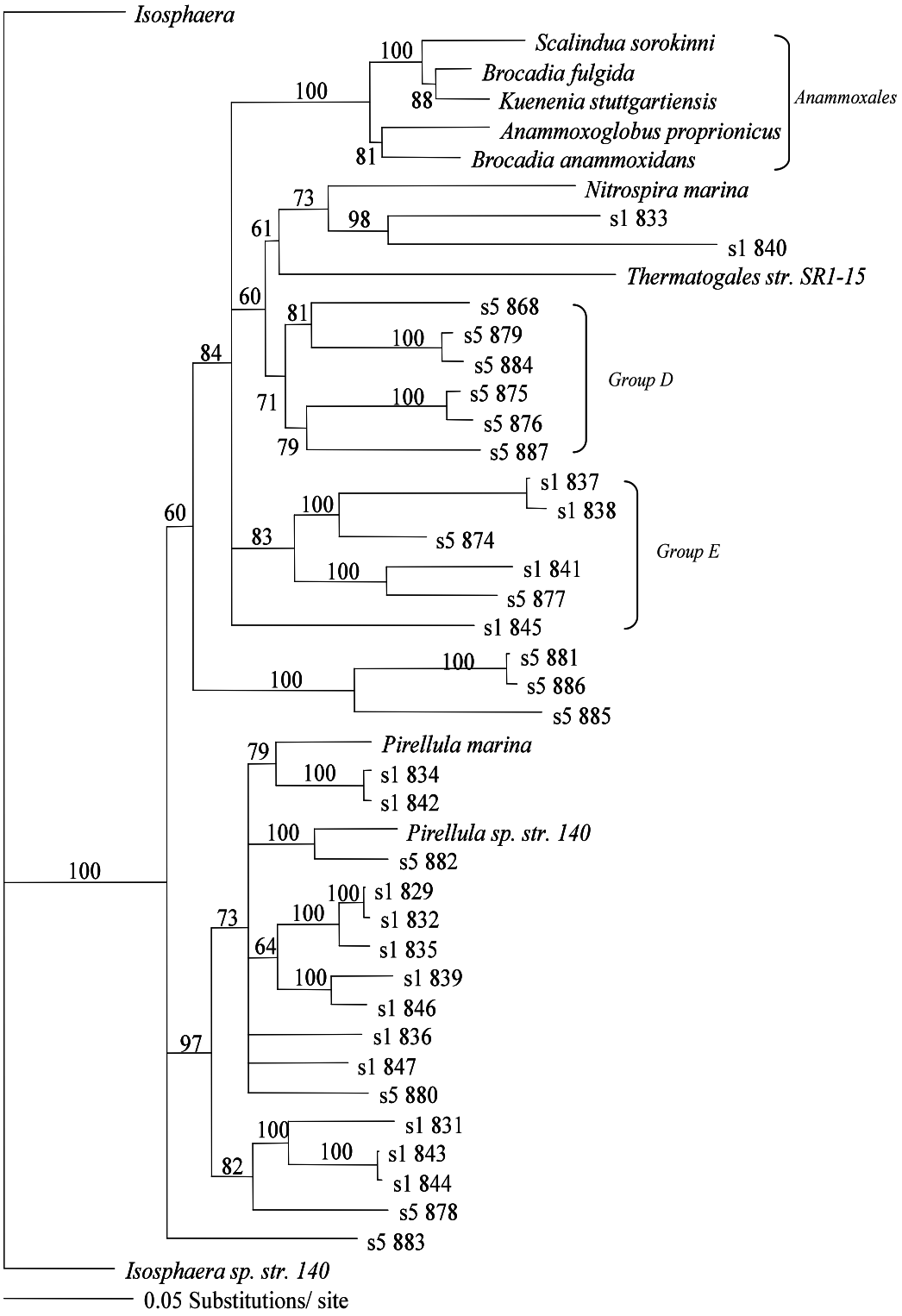


Figure 4.5.1. Phylogenetic tree of 16S rRNA gene sequences amplified with Pla 46F- Sca 1309R. These sequences were retrieved from Sites 1 (S1) and 5 (S5). The scale bar reprents 0.05 substitutions/ site. Associated bootstrap values are displayed at supported nodes.

comprise Group E (Site1 and 5), share between 73-97% sequence similarity to one another and 75% sequence similarity to *Scalindua sorokinni*. Despite the large phylogenetic distances between members of Groups D and E, to *Scalindua sorokinni*, these sequences share common origins with the anammox group and potentially represent environmental clusters of anammox.

Three sequences (collected from Sites Gr and Up) also branched separately from the anammox group. The sequence identities of Up 66, Up 69 and G 88, to *Scalindua sorokinni*, were 77%, 75% and 74% respectively. Again, it is clear that large phylogenetic distances exist between these sequences and candidate members of the anammox group (4.5.3.).

Bootstrap values could not be applied to the sequences retrieved with primers Pla 46F and Amx 0368R. It was therefore difficult to comprehensively analyse sequences retrieved from Sites 4 and 6. The above observations are not in line with previous environmental studies. In the majority phylogenetic surveys, the sequences identified were closely affiliated with the members of *Scalindua*. The efficiency, with which these sequences are retrieved, however, is highly variable (Penton *et al*., 2006; Schmid *et al*., 2007; Tal *et al*., 2005).

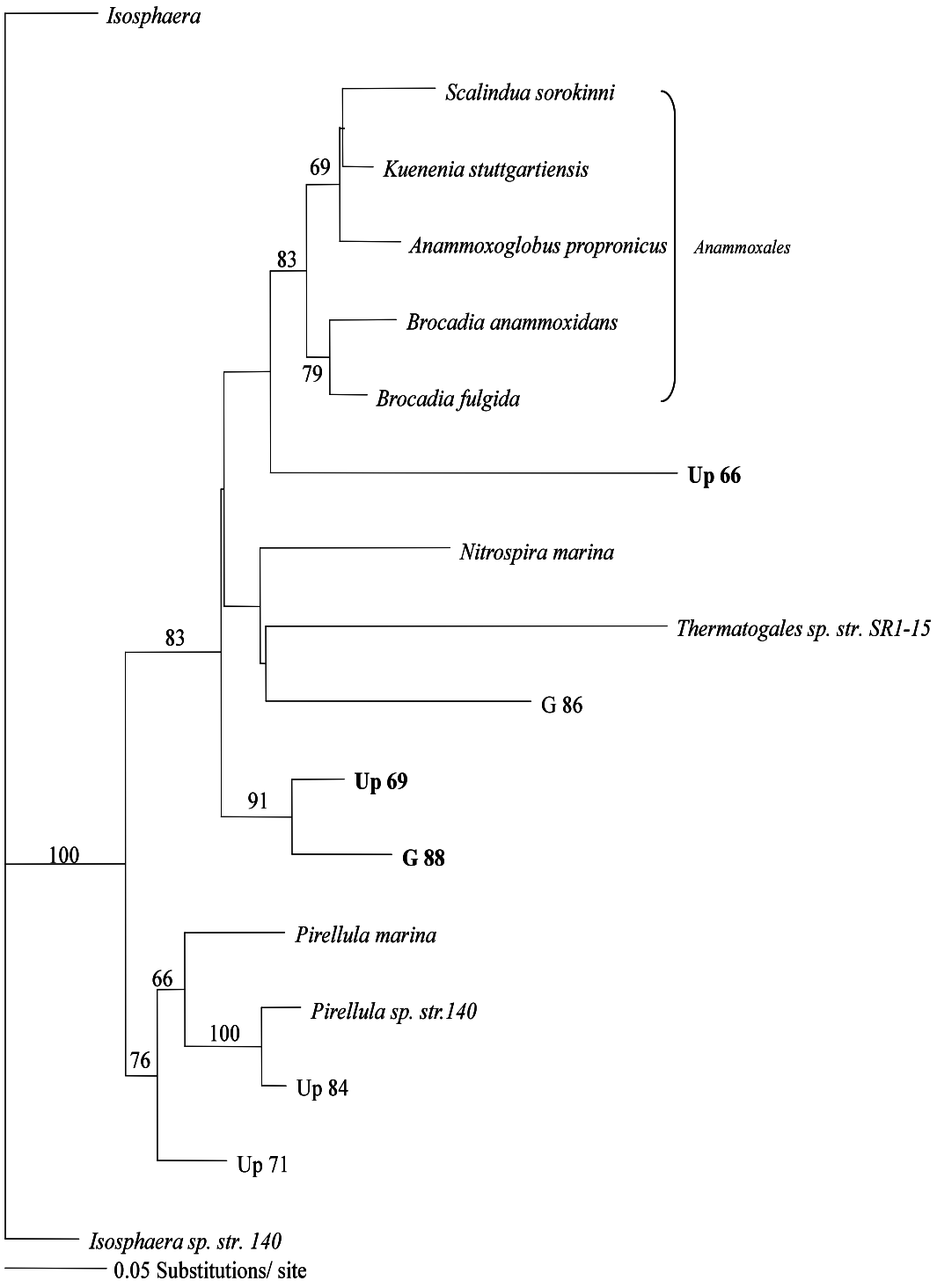


Figure 4.5.2. Phylogenetic tree of the 16S rRNA gene sequences amplified with primer pairs Pla 46F-Sca 1309R. These sequences were retrieved from Site MBM, Up and Gr (G). The scale bar represents 0.05 substitutions/ site. Associated bootstrap values are displayed at supported nodes.

5. DISCUSSION

To date, only two candidate species of anammox have been identified in natural systems. These species are affiliated with the candidate genus *'Scalindua*', and are known as '*Scalindua sorokinni*' and '*Scalindua arabica*' (Kuypers *et al*., 2003, Woebken *et al*., 2008). It is generally accepted that whilst anammox organisms are distributed across a vast range of environments, their diversity appears to be extremely limited. In fact, only members of the genus '*Scalindua*' have been reported in natural systems (Penton *et al*., 2006; Schmid *et al*., 2007, Woebken *et al*. 2008; Nakajima *et al*. 2008).

The sequences retrieved from the North Atlantic, Irish Sea and the Medway estuary do not share the reported similarities. The highest sequence similarity, to known species of anammox, is limited to a single clone retrieved from the Medway estuary (Up 92). This clone shares just 93% sequence similarity with '*Scalindua sorokinni*'. This is evidently not in line with previous observations where the majority of environmental sequences, share at least 97% sequence identity with either '*Scalindua sorokinni*' or '*Scalindua arabica*' (Schubert *et al*., 2006; Woebken *et al*., 2008). It is important to note, however, that whilst '*Scalindua arabica*' sequences cluster within the '*Scalindua*' clade, their presence has not, as yet, been confirmed with probes/primers specifically targeting the signature region of this species in the environment (Woebken *et al*., 2008). Essentially, this further constrains current knowledge of anammox organisms to '*Scalindua sorokinni*' in natural systems.

The majority of sequences in this study are phylogenetically distant from '*Scalindua sorokinni*' and with other members of the anammox group. One possible argument for this is PCR bias (Polz and Cavanaugh, 1998). The isolation of microorganisms in pure culture represents <1% of the total bacterial diversity in the environment, yet PCR amplification facilitates the retrieval of sequences from uncultured bacteria (Amann *et al*., 1995). Although this method is free from the bias associated with traditional isolation techniques, template-product ratios can be skewed by the preferential amplification of specific sequences (Polz and Cavanaugh, 1998). The probability of this occurring, however, was reduced by performing 5 PCR amplifications in parallel/ sample. Further steps were also taken to reduce the effect of sediment contaminants in PCR amplification. This prevented the co-extraction of sediment contaminants alongside DNA. It is probable that the absence of '*Scalindua*-like' sequences, in this study, cannot be directly attributed to either the interference of sediment contaminants in amplification or to inherent PCR bias.

Intriguingly, the efficiency with which '*Scalindua*-like' sequences are retrieved from the environment is highly variable. This occurs whether sequences are recovered using general bacterial primers, anammox specific primers or a nested PCR approach. The collection of 'anammox-like' sequences, in a study surveying several marine oxygen minimum zones, is in good agreement with this type of variation. Of the total sequences amplified with Pla 46F and the (general bacterial) primer 1037R, just 1 of 480 sequences from the Black Sea could be affiliated with *Scalindua sorokinni* and 8/672 from the Namibian OMZ. This is in contrast to 31/136 collected from the Arabian Sea (Woebken

et al., 2008). Given that the frequency of anammox organisms in OMZ systems is approximately 1% of the total bacterial population, there is perhaps discrepancy between the number of anammox organisms postulated and the reported target retrieval efficiency of the primers (Kuypers *et al*., 2003; 2005; Hamersley *et al*. 2007). As a result, this could point towards a greater *in situ* diversity than previously acknowledged. Using an anammox directed PCR approach, however, Schmid *et al*. (2007) successfully retrieved a high proportion of '*Scalindua*-like' sequences from marine sediments in Gullmar Fjord and the Golfo Dulce (6/12, 3/12 respectively). Interestingly, these sequences were of such high purity that the PCR products were directly sequenced without the need for prior cloning. Nevertheless, Tal *et al*. 2005, failed to report more than 2 clones (of several hundred retrieved) affiliated with '*Scalindua sorokinni*' (in estuarine sediments). Moreover, this was only achieved following a pre-amplification step (using general bacterial primers) preceding an anammox directed PCR. Given that anammox organisms constitute 2-8% of the total microbial population in sediments, this indicates that current primer sets are not suited to the consistent retrieval of all 'anammox-like' sequences (Schmid *et al*., 2007). Furthermore, it has been demonstrated that the available primer sets are at best semi-specific for anammox organisms and therefore underrepresent these organisms (by approximately 50%) *in situ* (Penton *et al*., 2006). In the context of this study, this may explain the limited retrieval of '*Scalindua*-like' sequences in the North Atlantic, Irish Sea and Medway estuary.

In order to extend this survey, beyond the limitations of the available primer sets, an additional primer was specifically designed to retrieve a sequence sharing <97%

sequence similarity to '*Scalindua sorokinni*'. According to current understanding, candidate sequences sharing ≥ 97% sequence identity to a particular species can be classified as members of the same species (Rosello-Mora and Amann, 2001). As the MC1 clone shares 96% sequence similarity to '*Scalindua sorokinni*', this sequence serves as a good candidate for a new species within the genus '*Scalindua*'. The primer MC1, however, proved non specific for the MC1 clone and therefore failed to retrieve target sequence. This is in contrast to the efficiency with which the probe hybridised with candidate organisms. This further demonstrates the difficulty with which anammox-like sequences can be retrieved from the environment and the adaptation of fluorescent probes as oligonucleotide primers.

Interestingly, a number of unidentified *Planctomycetes* from Sites 1 and 5 form two phylogenetic clusters close to the anammox group. On average, these sequences share just 75% sequence similarity to '*Scalindua sorokinni*' and yet exhibit common origins with the anammox clade (Figure 4.5.1.). This is strongly supported by the associated bootstrap values and suggests that these sequences perhaps represent organisms capable of the anammox process. In a previous phylogenetic survey, 16 clones were retrieved from Thames estuary (UK) that branched deep within the anammox lineage. Similarly, these sequences shared just 75% similarity with '*Scalindua sorokinni*' and >96% sequence similarity to one another. Although anammox activity has been identified in sediments of the Thames estuary, amongst the sites investigated in this study, the ecophysiology of these organisms is unresolved (see Trimmer *et al*., 2003; 2005; Jaeschke *et al*., 2009 and Chapter III). Such sequences are often reported environmental

studies but are largely disregarded (Tal *et al*., 2005; Schubert *et al*., 2006; Stevens and Ulloa, 2008; Shu and Jiao, 2008; Woebken *et al*., 2008).

In the context of this study, no sequences were retrieved that could be directly affiliated with known species of anammox. This does not however suggest the complete absence of organisms capable of anammox in the Medway estuary, North Atlantic or Irish Sea. Anammox activity, ladderane lipids (biomarkers unique to anammox organisms) and positive FISH signals confirm the presence of these organisms at all sites explored (Jaeschke *et al*., 2009, Chapters III and IV). It is therefore clear that in the absence of sequences affiliated with anammox, and in the presence of both anammox activity and ladderane lipids, that the available molecular tools were incapable of detecting the full diversity of the anammox organisms present.

The biodiversity of the microorganisms responsible for anammox may expand well beyond the known anammox group and potentially, outside the phylum *Planctomycetes*. This is evident amongst the bacteria that account for aerobic ammonium oxidation in marine environments. In contrast to the described diversity of anammox, the betaproteobacteria responsible for aerobic ammonium oxidation in marine systems, are relatively diverse. This diversity extends to members of the genera *Nitrospira*, *Nitrosoccous*, *Nitrosomonas* and even amongst marine crenarcheota (Freitag and Prosser, 2003; Wuchter *et al*., 2006). In view of this, it is not unreasonable to suggest that the organisms capable of anaerobic ammonium oxidation are not inherently confined to the candidate genera '*Brocadia*', '*Kuenenia*', '*Anammoxoglobus*' or to '*Scalindua*'. Further

studies are clearly required to explore the diversity of anammox in natural systems. This includes the development of efficient primers and detailed investigations of sequences, that are distantly related, but share common origins with anammox organisms.

6. INTRODUCTION

The availability of fixed forms of nitrogen is an important factor in regulating primary production (Vitousek and Howarth, 1991). In aquatic environments, NH_4^+ derived from the decomposition of organic matter is oxidised to NO_3^- (via NO_2^-) in nitrification. The NO_3^- produced in this process subsequently diffuses into the suboxic zone, where it is denitrified to N_2 gas in coupled denitrification. Until recently, the removal of fixed nitrogen was almost entirely attributed to coupled denitrification (Devol, 1991). The discovery of anaerobic ammonium oxidation (a process whereby NH_4^+ is anaerobically oxidized with NO_2^- to form N_2 gas), however, redefined this concept. Consequently, anammox represents an alternative N removal pathway that circumvents the critical aerobic phase typically associated with coupled denitrification.

Environmental studies have confirmed the presence of anammox in a diverse range of geographically and biochemically distinct environments such as oxygen minimum zones (Kuypers *et al*., 2003; 2005), tropical freshwater (Schubert *et al*., 2006), Arctic sea ice (Rysgaard and Glud, 2004) and Arctic (Rysgaard *et al*., 2005), marine (Schmid *et al*., 2007; Thamdrup and Dalsgaard, 2002), and estuarine sediments (Meyer *et al*., 2005; Rich *et al*., 2008; Risgaard –Petersen *et al*., 2005; Tal *et al*., 2005; Trimmer *et al*., 2003; 2005). Although the occurrence of anammox is relatively widespread, the significance of this process is highly variable. The potential contribution of anammox to total N_2 production, in marine and estuarine sediments, varies between 35-67% (Rysgaard *et al*., 2004; Thamdrup and Dalsgaard, 2002) and <1-26% (Nicholls and Trimmer, 2009; Risgaard-

Petersen *et al*., 2004), yet the specific factors that govern this variation, are largely unknown.

As NH_4^+ is rarely limiting in sediments, the physical and microbiological mechanisms by which NO_2^- is transported to anammox organisms are crucial. In particular, denitrification may play a key role in the regulation and abundance of anammox organisms via the delivery of NO_2^-. The potential contribution of anammox to total N_2 production in the Thames estuary does not exceed 8% yet the significance of this process in deep, offshore sediments is 67-79% of the total N_2 production (Skagerrak, Denmark) (Nicholls and Trimmer et al., 2009; Trimmer *et al*., 2003; Thamdrup and Dalsgaard, 2002). Trimmer *et al*. (2005) proposed that this could be attributed to shifts in the microbial population as a direct consequence of environmental conditions. Essentially, highly reactive sediments, enriched with NO_3^- and organic carbon, favour the growth of heterotrophic denitrifiers over anammox organisms. Whilst the denitrifying community facilitates the delivery of NO_2^- to anammox organisms, NO_2^- production balances NO_2^- consumption in the Thames. Consequently, the availability of free NO_2^- (to anammox organisms) is limited in estuarine environments, and in turn, may reduce the overall importance of anammox to total N_2 production (Trimmer *et al*., 2005). In deep, offshore sediments, however, the availability of NO_3^- and organic carbon are limited. This promotes a chemolithoautotrophic lifestyle which potentially favours autotrophs such as anammox organisms. In the Skagerrak, NO_2^- production outweighs NO_2^- consumption and therefore could increase the availability of this free intermediate and the overall importance of anammox as a sink for nitrogen. Consequently, this may maintain a

comparatively larger anammox population in deeper offshore waters, relative to shallow estuarine sediments.

Whilst NO_2^- is a prerequisite for anammox activity, the position of the oxic and suboxic interface could also serve as a governing factor in the spatial distribution of anammox organisms throughout marine and estuarine sediments. Enrichment culture studies have shown that anammox organisms are reversibly inhibited by as little as 1μM oxygen (Strous *et al*., 1999). This would suggest that the formation of a stable anammox community can only occur in the suboxic zone of the sediment substrata. The disruption of the sediment substrata by bioturbating infauna, however, strongly influences sediment processes by altering the supply of nutrients and oxygen to microorganisms. The overall effect of this is an increase in the surface area of the sediment-water interface. This causes an extension of oxic and suboxic zones into otherwise highly reduced, anoxic sediment (Henriksen *et al.*1980). Consequently, this has the potential to enlarge the size of the nitrifying and denitrifying communities and thus, their potential contribution to N_2 production (Blondin *et al*., 2004). Whilst bioturbation could sustain a larger community of NO_2^- producing organisms, the relatively slow growth (maximum doubling time 11 days) and sensitivity of anammox organisms to oxygen, could suppress anammox activity (Strous *et al*., 1999).

Nevertheless there is a growing body of evidence for sustainable anammox activity under unfavourable conditions. Woebken *et al*. (2007) put forward an argument for the existence of anammox in oxygen depleted microniches (in planktonic snow of the

Namibian shelf waters). Essentially, in such environments, a protective layer of microorganisms (consuming oxygen) shields anammox bacteria from ambient O_2 up to 25μM. In view of this, the distribution of anammox organisms is not necessarily constrained by environmental conditions.

The purpose of this study was to determine whether variations in the significance of anammox are related to the changes in the size of the anammox community. The zone of anammox activity was characterised by ^{15}N isotope labelling experiments, oxygen profiles and depth specific rate measurements of CO_2 production (sediment metabolism). Fluorescence *in situ* hybridisation and 4'6-diamino-2-phenylindole dihydrochloride (DAPI) were used alongside these techniques to determine shifts in the abundance of anammox organisms with depth, thus potentially linking anammox activity to deviations in population size. This was explored both at sites in the presence and absence of bioturbationg invertebrates, along a transect of the Medway estuary.

7. METHODS

7.1. Study sites

The sediment characteristics and site locations are described in section 3.1.2., Table 3.1. and Figure 3.1.2. (Chapter II). This is with the exception of two sites (Sites 2 and 4) that were included in a preliminary survey of the Medway Estuary. Site 2 (Stoke Saltings) and Site 4 (Horrid Hill) are located between Sites Gr and Up, and Sites Up and MBM respectively. The locations of these sites are included in Figure 3.1.2. (Chapter II).

7.2. Sediment collection, storage and sample preparation

Samples were retrieved from the Medway Estuary during October 2007 using the methods described in section 3.1.2 (Chapter II).

The position of the oxic-suboxic interface was determined by measuring porewater oxygen profiles in each core (as described in 7.3.). The sediment was then sub-sampled at 1cm intervals to a total depth of 5cm in all cores (-1cm, -2cm, -3cm, -4cm and -5cm). This was for the purpose of ^{15}N isotope labelling experiments and measurements of sediment metabolism. The second subset of samples was represented by depth intervals of 0.5cm. These samples were used in FISH labelling experiments and were retrieved over a depth of 2.5cm.

For the purpose of measuring the potential contribution of anammox to total N_2 production, 1g of sediment (from each depth interval) was transferred to a 3ml gas tight,

glass vial (Exetainer, Labco Ltd., High Wycombe, UK). In total, 1 reference and 2 'end point' incubation samples were collected from each depth in 5 replica cores. The wet weight of each sediment sample was measured and recorded prior to further processing. (For details of end point incubation experiments, see 7.4). Aliquots of 2.5g of sediment were distributed into gas tight, 12ml glass vials (Exetainer, Labco Ltd., High Wycombe, UK). This was for the purpose of measuring changes in the rate of CO_2 production with depth. One sample was collected from each depth interval from 5 replica cores. The wet weight of each sediment sample was then measured and recorded prior to further processing. (For details of 'CO_2 production' experiments, see 7.5.). To map shifts in the abundance of anammox organisms with depth, approximately 1g of sediment from each interval was transferred to a 1.5ml Eppendorf tube (see 7.6). This was repeated 4 times in each core providing 4 separate sub-samples. These samples were fixed, and then stored at -20 ºC for further FISH analysis.

7.3. Porewater oxygen profiles

Porewater oxygen profiles were measured using a Clark-type oxygen microsensor with an outer tip diameter of 40 - 60µm (OX50, Unisense, Aarhus, Denmark) (Revsbech, 1989). Prior to each measurement, a two point linear calibration was conducted between 0% and 100% oxygen with sodium ascorbate-NaOH solution and air saturated seawater respectively. Individual sediment cores were then transferred from the water bath and submerged in a Perspex container filled with 1.9L of aerated low nutrient seawater (diluted to site salinity). Following transfer, oxygen microelectrodes were positioned perpendicular to the sediment surface and driven into the sediment (using an automated

system) at 100µm depth intervals. Readings from the microelectrode were displayed on a picoammeter (PA 2000, Unisense, Aarhus, Denmark) and recorded every 4 s, (during which time the signal stabilised).During profiling the cores were maintained at 12ºC.

7.4. End point incubation experiments

To determine the relative contribution of anammox to total N_2 production, 1g of sediment (from each depth interval) was distributed into 3ml, gastight glass vials (Exetainer, Labco Ltd., High Wycombe, UK). The glass vials were then transferred into an anaerobic glove box (Belle Technology, Dorset, UK) where they were filled with 1ml of LNSW (diluted to site salinity and degassed with oxygen free nitrogen), sealed and shaken vigorously (to create an anaerobic slurry). This was followed by a pre-incubation period of 24 hours in order to remove all background NO_x^- and O_2. During this preincubation period, the vials were placed on rotating rollers and maintained at constant temperature (12ºC) in the dark. The samples were then injected with a solution containing an isotopic mixture of $Na^{15}NO_3^-$ (99.2 ^{15}Natm %) and $^{14}NH_4^+Cl$ using a Hamilton syringe (Sigma-Aldrich, Poole, United Kingdom). The final concentrations of $^{15}NO_3^-$ and $^{14}NH_4^+$, in each slurry, were 200µM and 500µM respectively. To maintain completely anaerobic conditions the working solutions were degassed with (oxygen free nitrogen) prior to injection. The vials were then placed on rollers for a further 24 hours and incubated at 12ºC in a constant temperature room (as above). At the end of the incubation period, the samples were injected with 50% $ZnCl_2$ (w/v) to inhibit further microbial activity.

The abundance of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ were measured directly from the headspace (of each vial) using a continuous flow isotope ratio mass spectrometer (Delta Matt plus, Thermo-Finnigan, Bremen, Germany). The potential contribution of anammox to total N_2 production (*ra%*) was calculated according to the equations described by Thamdrup and Dalsgaard (2002).

$$D_{30} = P_{30} \times F_N^{-2} \quad (1)$$

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

Total denitrification was calculated from equation 1 where D_{30} is equal to the production of $^{30}N_2$. The denitrification of $^{15}NO_3^-$ and unlabelled $^{14}NO_3^-$ produces $^{29}N_2$ ($^{14}N^{15}N$) and $^{30}N_2$ ($^{15}N^{15}N$) labeled N through random isotope pairing. Given that anammox only produces $^{29}N_2$ (from 1 atom of $^{15}NO_3^-$ and $^{14}NH_4^+$), $^{30}N_2$ is exclusive to denitrification (Thamdrup and Dalsgaard, 2002). The total production of $^{30}N_2$ is therefore equal to denitrification (D_{tot}) and is denoted by the term P_{30} (equation 3). The fraction of ^{15}N labelled NO_3^- in the combined $^{14/15}$ NO_3^- pool corresponds to F_N. In this case F_N is equal to 0.992 (from a stock solution of 99.2 ^{15}N atm %).

$$D_{tot} = P_{30} \times F_N^{-2} \quad (3)$$

Total anammox can be calculated from equation 2 where A_{tot} represents the total production of N_2 via anammox and P_{29} is equal to total $^{29}N_2$ production. The relative

contribution of anammox to total N_2 production can be calculated from equation 4 where ra is simply:

$$ra\ (\%) = [100 \times (A_{tot} / D_{30})]. \quad (4)$$

7.5. Rates of CO_2 production with sediment depth

The accumulation of CO_2 was measured directly from the headspace of each sealed vial by gas chromatography-flame ionisation detection (GC-FID) (Sanders *et al*., 2007; Nicholls and Trimmer, 2009). Samples were injected into the gas chromatograph at intervals of 84 minutes. The rate of CO_2 production was calculated by the linear regression of CO_2 production, plotted against time (Nicholls and Trimmer, 2009).

7.6. Fluorescence *in situ* hybridisation (FISH)

Approximately 1g of sediment from each section was fixed with 4% (v/v) paraformaldehyde in PBS solution and allowed to stand at room temperature for 2 hours. Each sample was then washed twice with PBS and re-suspended in a 50% (v/v) PBS-ethanol solution. All samples were subsequently stored at -20ºC until required.

FISH experiments were conducted on the basis of the methods described by Schmid *et al*. (2000; 2003). In the preliminary phylogenetic survey of the Medway Estuary (April 2005, Chapter II), the '*Scalindua*' specific probes Sca 1114 and BS 820 were used alongside Pla 46F, to detect anammox organisms at 5 sites along the Medway Estuary

(top 2cm of sediment). This investigation was performed in combination with ^{15}N labelling experiments (top 2cm) as described by Trimmer *et al*. (2003). For probe details refer to Schmid *et al*. (2005).

Subsequently, the abundance of anammox organisms was examined with depth using the anammox specific probes Amx 0368 and Sca1309 in combination with Pla 46 (for target specificity refer to Table 3.4., Chapter II). In order to maximise probe binding, the hybridisation buffer was set a concentration of 25% formamide. All hybridisations were conducted at 56ºC. Following hybridisation, the sediment samples were counterstained with DAPI (fluorochrome 4'6-diamino-2-phenylindole dihydrochloride) and viewed using an epiflouresecence microscope (Leica microsystems, UK). Images were captured from 20 visual fields with a black and white digital camera (Leica microsystems, UK). A total of five FISH experiments were performed at each depth interval per core. All labelled oligonucleotide probes were purchased as Cy3, Cy5 and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (Fluos) labelled derivatives from MWG-Biotech (London, UK) and Thermo Electron (Ulm, Germany).

Only anammox cells hybridising with all three probes and sharing typical 'doughnut-shaped' morphology were considered during manual counts. All anammox organisms and bacterial cells were manually counted in the first survey. In the second survey, however, the total number of bacteria per micrograph was calculated using the software Image Pro Plus version 5.0 (Media Cybernetics, Inc.). Manual counts of total bacteria were

compared with automated counts to establish the degree of accuracy within the automated dataset.

8. RESULTS

8.1. The *in situ* detection and potential significance of anammox

Anammox organisms were detected at all sites in the first survey of the Medway Estuary (April 2005). The fluorescently labelled probes Scabr 1114 (Cy3) and BS 820 (Cy5) were used in combination with Pla 46 (Fluos). This resulted in the triple hybridisation of probes with target organisms, pointing towards the presence of '*Scalindua*-like' cells (Figure 8.1.1). The cells depicted, were typically 'doughnut-shaped' and fell within the reported size range for anammox organisms. Only cells that positively hybridised with these probes were included in anammox specific counts. All samples were counterstained with DAPI, which was used to identify the total number of bacteria per micrograph.

The relative abundance of anammox organisms decreased in a seaward direction from 8% at Site 4 (landward) to 1% at Site1 (seaward). This is in good agreement with values of *ra*, where the potential contribution of anammox to total N_2 production decreased in a seaward direction from 19 to 1% (Figure 8.1.2., Table 8.1.). Interestingly, there is a discrepancy between the relative abundance of anammox organisms and values of *ra* (30%) at Site 5. Here, anammox organisms formed just 2% of the total prokaryotic population. Consequently, this lead to the detailed re-examination of three sites (Sites MBM, Up and Gr) from the Medway estuary. The results of this investigation are described below.

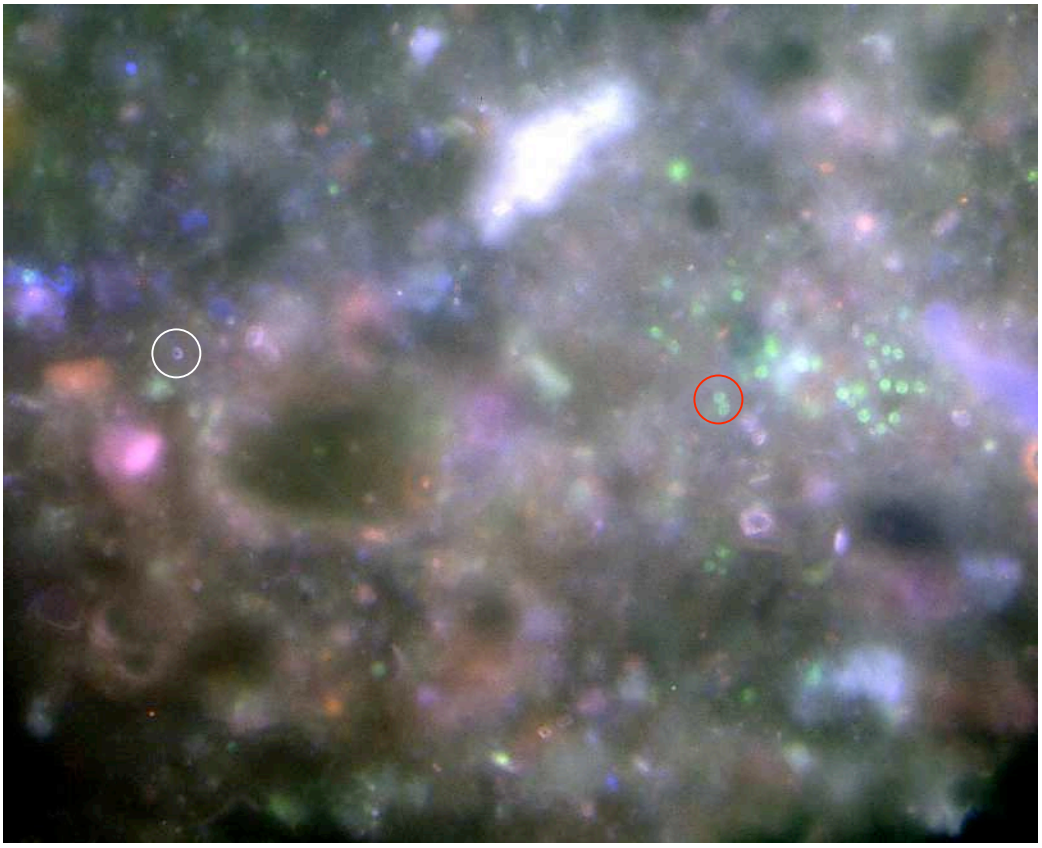


Figure 8.1.1. Combined epifluorescence micrograph from a sample retrieved from Site 5. The triple hybridisation of the anammox specific probes Scabr 1114 (Cy3), BS 820 (Cy5) and Pla 46 (Fluos) produces a white signal. In combination with a 'doughnut-like' morphology, this points towards the presence of anammox cells (white circle). Cells hybridising with Pla 46 (Fluos) only, represent members of the *Planctomycetales* (red circles). DAPI stained signals indicate the presence of DNA-containing bacterial cells. Autofluorescence is emitted by sediment particles and takes the form of large fragments of 'white debris'.

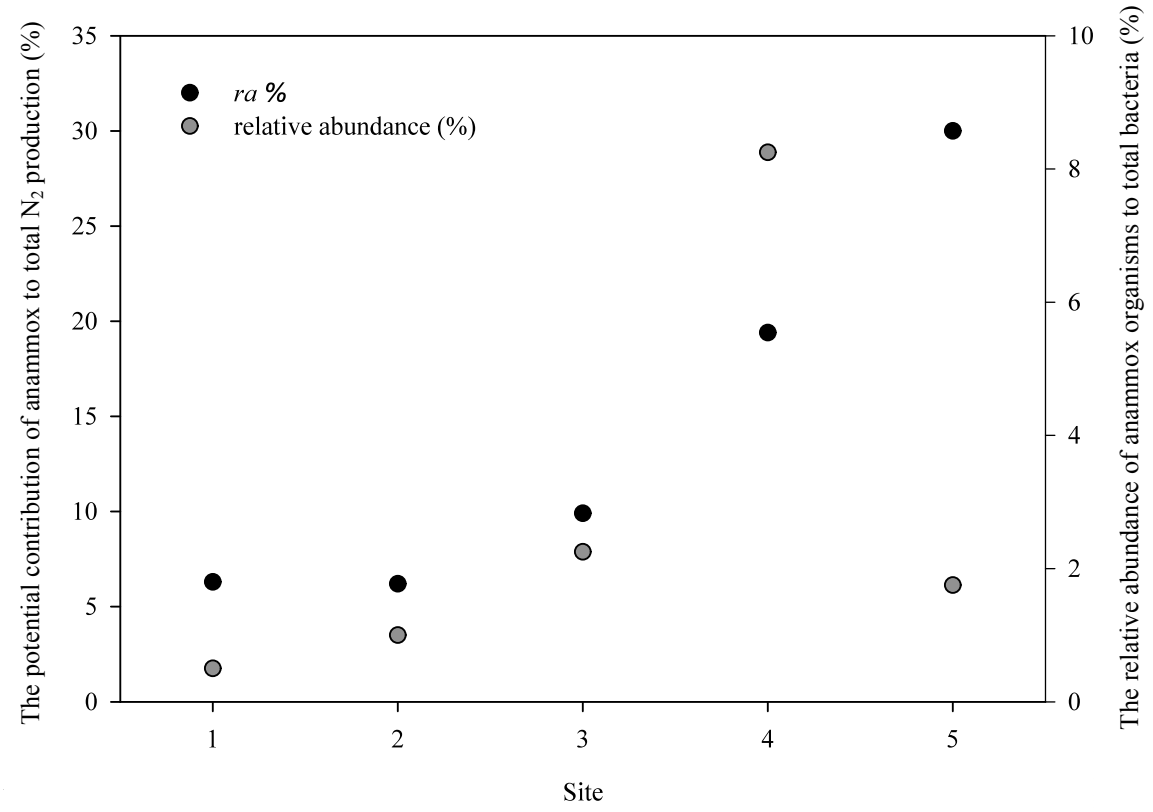


Figure 8.1.2. The potential contribution of anammox to total N_2 production (black dots) at 5 sites along the Medway Estuary (April 2005). The proportion of *ra* decreased in a seaward direction coinciding with a reduction in the relative abundance of anammox cells (grey dots). The only discrepancy in the overall correlation is at Site 5, where *ra*= 30% and the abundance of anammox organisms constitutes just 2% of the microbial population. Each data point represents the mean of 5 (*ra*) and 20 replicates (relative abundance of anammox organisms).

Site	*ra* (%)	SEM	Relative abundance of anammox organisms to total bacteria (%)	SEM
1	6	0.2	0.4	0.1
2	6	0.4	1.1	0.4
3	11	0.3	2.2	0.2
4	20	0.3	8.3	0.4
5	30	1.2	1.8	0.5

Table 8.1. The potential contribution (*ra*%) and relative abundance of anammox organisms (%) at 5 sites along the Medway estuary (April 2005). Each value represents the mean of 5 (*ra*) and 20 replicates (relative abundance of anammox organisms). All values are 1 +/- SEM.

8.2. Porewater oxygen profiles

Dissolved oxygen profiles were measured in each intact sediment core (Figure 8.2.1). The position of the oxic-suboxic interface was on average 9.9mm, 3.6mm and 3.5mm at Sites MBM, Up and Gr respectively. Interestingly, the dissolved oxygen profiles measured at Medway Bridge Marina indicate major disruption of the sediment substrata by bioturbating macrofauna (Figure 8.2.1 A). Here, multiple peaks in the oxygen profiles can be attributed to an extensive network of invertebrate burrows (personal observation). Further examination of the sediment revealed the presence of *Nereis diversicolor* and *Corophium volutator* at mean densities of 6 (*SE* = +/-1.4) and 18 (*SE* = +/-5.6) per core respectively.

In contrast, the dissolved oxygen profiles measured at Site Up and Gr demonstrate the separation of the sediment substrata into clear oxic and sub-oxic zones (Figure 8.2.1 B & C). In the absence of bioturbating fauna, the oxic zone is constrained to the upper 3.5mm of the sediment. (This is one third of the oxygen penetration depth described at Medway Bridge Marina). The position of the oxic-suboxic interface and the oxygen profiles at these sites were almost identical.

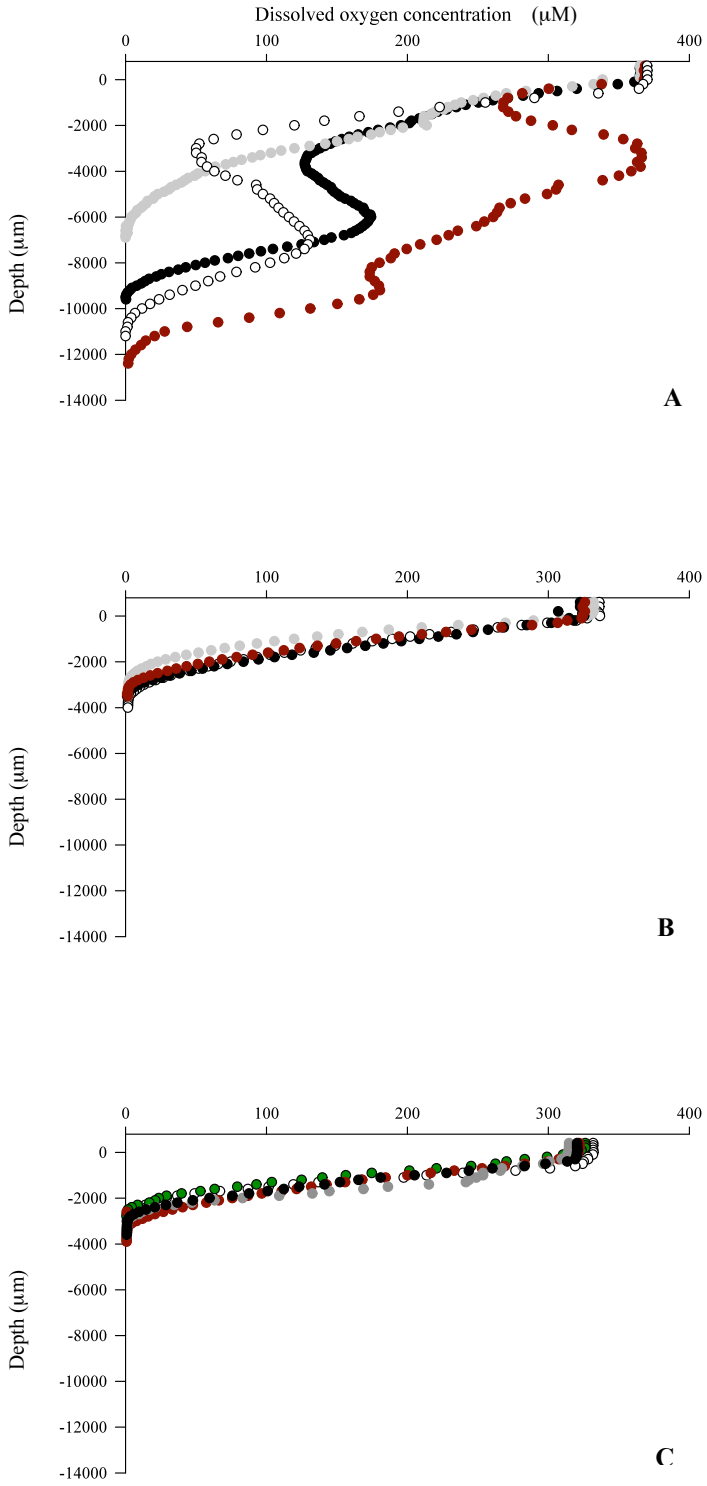


Figure 8.2.1. Measurements of multiple dissolved oxygen profiles in the presence and absence of bioturbating macrofauna. Panel A represents Site MBM, B corresponds to Site Up and C is representative of Gr. Each profile corresponds to 1 core.

8.3. Depth specific rates of CO_2 production

The production of CO_2 was generally linear with time at all sites and the majority of depth intervals. The average rates of CO_2 production were 0.0014µmol CO_2 g^{-1} wet sediment h^{-1}, 0.0002µmol CO_2 g^{-1} wet sediment h^{-1} and 0.0003µmol g^{-1} wet sediment h^{-1} at Sites MBM, Up and Gr respectively. The depth specific rates of CO_2 production varied between sites and at depth intervals (Table 8.3.1.). The greatest depth specific rates of CO_2 production were measured at Site MBM. Here, the rates of sediment metabolism peaked in the first cm of the sediment (0.0016 µmol CO_2 g^{-1} wet sediment h^{-1}) and decreased almost linearly to a depth of -4.5cm (0.0012 µmol CO_2 g^{-1} wet sediment h^{-1}) (Figure 8.3.1.). At Site Up, the rate of CO_2 production decreased within the first 2cm of the sediment from 0.00025 to 0.00015 µmol CO_2 g^{-1} wet sediment h^{-1}. This was followed by an increase in sediment metabolism to 0.00027µmol CO_2 g^{-1} wet sediment h^{-1} (Figure 8.3.2.). In general, there was no depth specific trend in CO_2 production at Site Up. Similarly, there was little variation in the depth specific rates of CO_2 production at Site Gr. Here, the rate of sediment metabolism peaked within the first 2 cm of the sediment (0.00044µmol CO_2 g^{-1} wet sediment h^{-1}) and generally exceeded the rates of CO_2 production measured at Site Up (Figure 8.3.2).

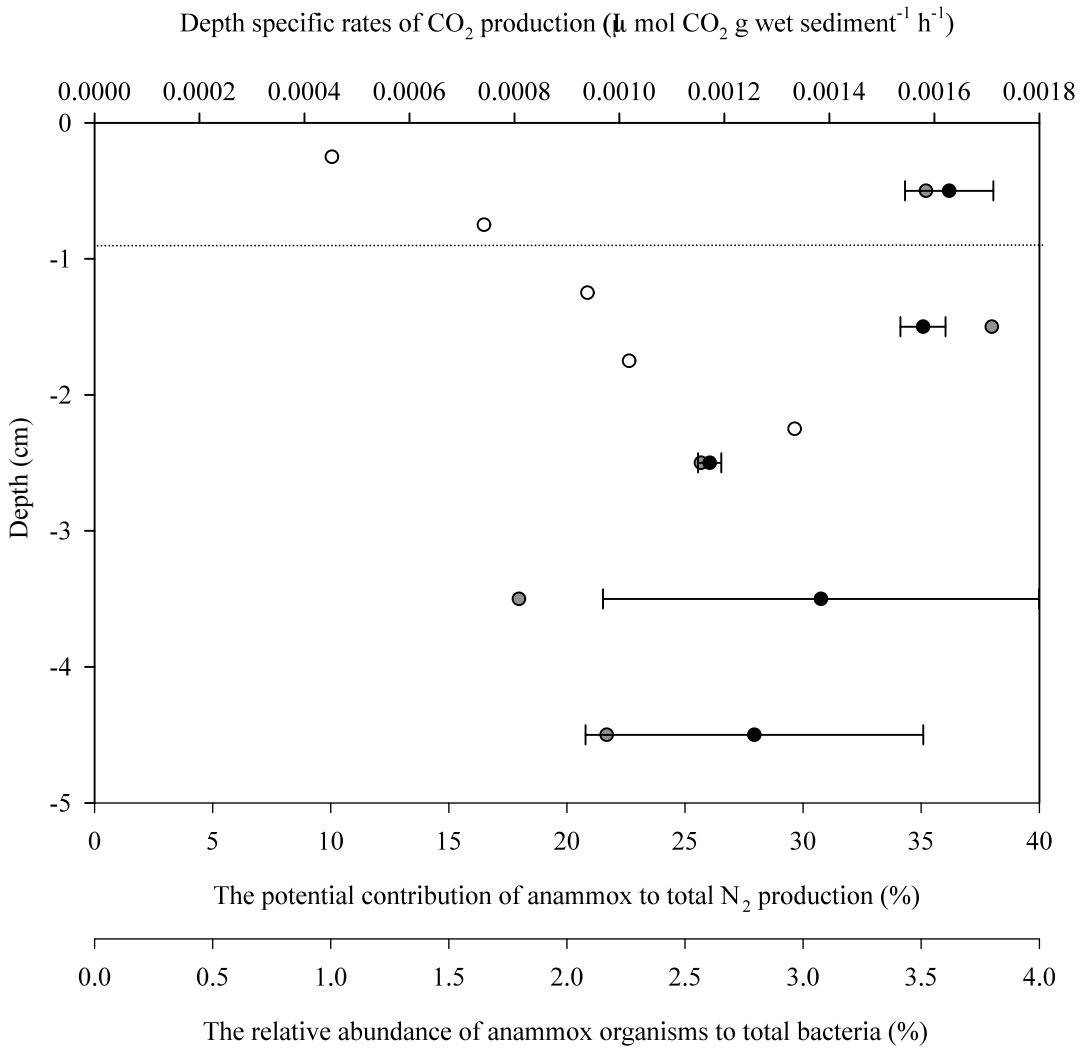


Figure 8.3.1. The depth specific distribution of CO_2 production (black dots), *ra* (grey dots) and the relative abundance of anammox organisms to total bacteria (white dots) at Site MBM. The dotted line represents the position of the oxic-suboxic interface (-9.9mm). Values of CO_2 production are 1+/- SEM (n=5). Each data point represents the mean of 5 (*ra*) and 20 replicates (relative abundance of anammox organisms).

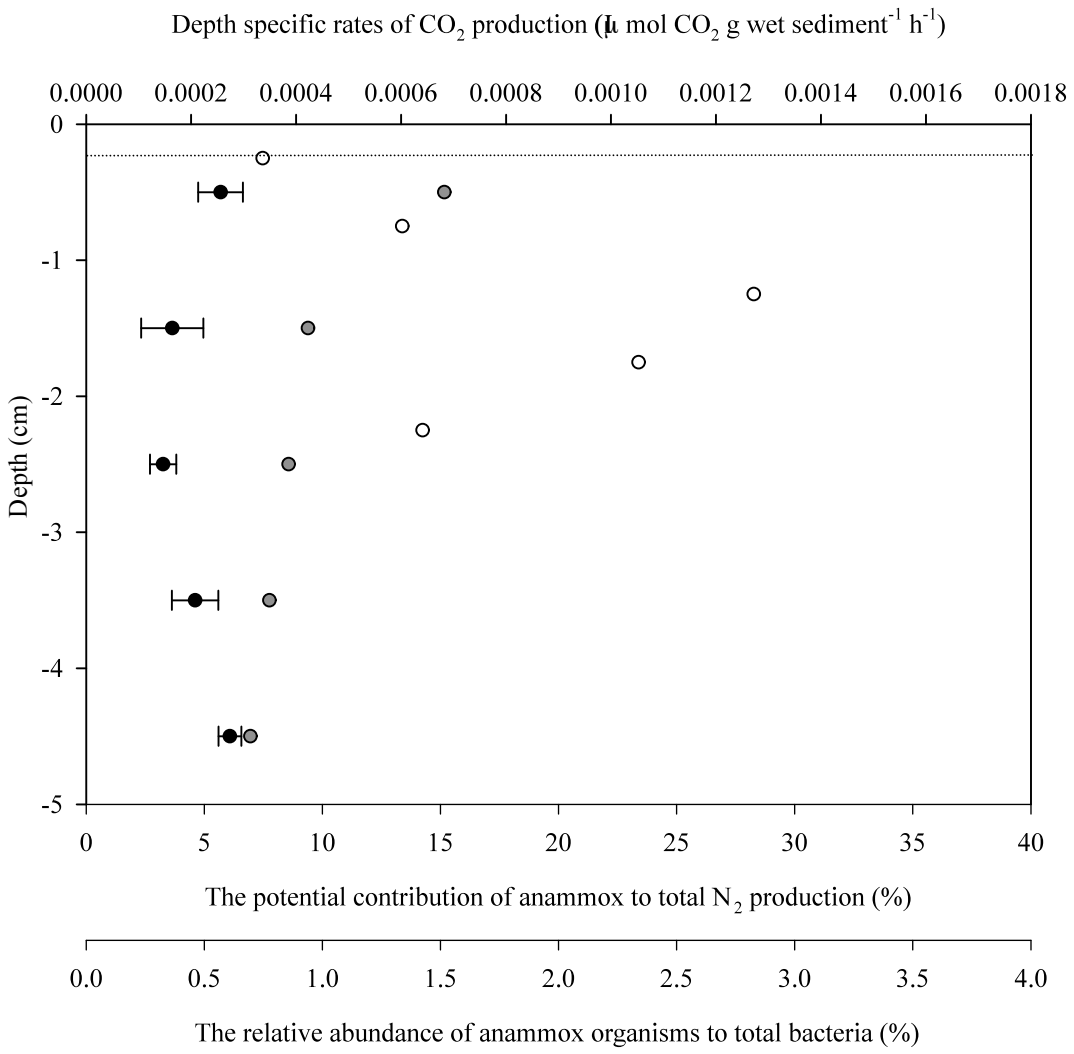


Figure 8.3.2. The depth specific distribution of CO_2 production (black dots), *ra* (grey dots) and the relative abundance of anammox organisms to total bacteria (white dots) at Site Up. The dotted line represents the position of the oxic-suboxic interface (-3.6mm). Values of CO_2 production are 1 +/- SEM (n=5). Each data point represents the mean of 5 (*ra*) and 20 replicates (relative abundance of anammox organisms).

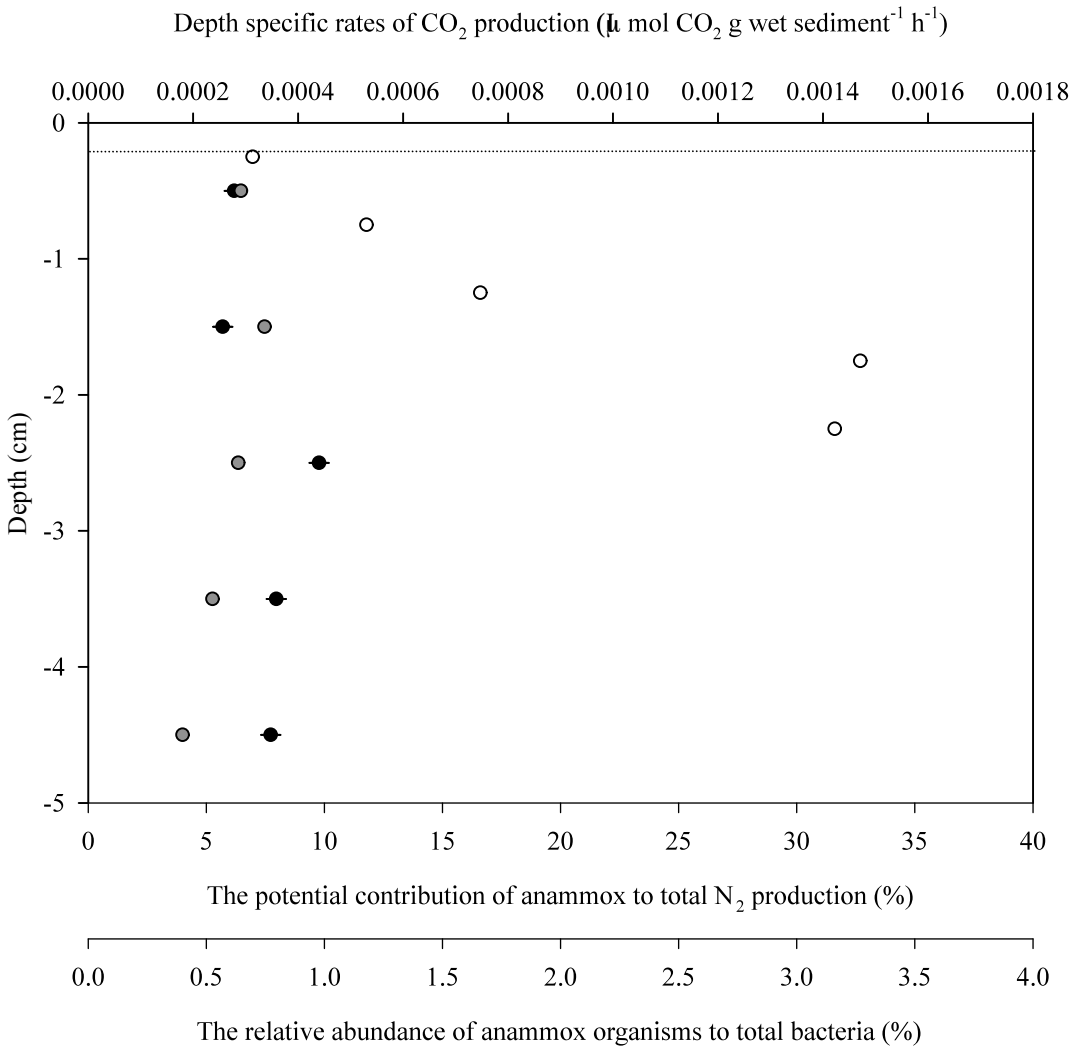


Figure 8.3.3. The depth specific distribution of CO_2 production (black dots), *ra* (grey dots) and the relative abundance of anammox organisms to total bacteria (white dots) at Site Gr. The dotted line represents the position of the oxic-suboxic interface (-3.5mm). Values of CO_2 production are 1 +/- SEM (n=5). Each data point represents the mean of 5 (*ra*) and 20 replicates (relative abundance of anammox organisms).

Site	Depth (cm)	Depth specific rates of CO_2 production (µ mol CO_2 g wet sediment^{-1} h^{-1})	SEM	*ra* (%)	SEM
MBM	0-1	0.0016283	0.0000880	35.2	5.8
	1-2	0.0015784	0.0000431	38.0	4.8
	2-3	0.0011720	0.0000225	25.7	4.2
	3-4	0.0013841	0.0004154	18.0	3.9
	4-5	0.0012571	0.0003218	21.7	7.0
Up	0-1	0.0002562	0.0000256	15.2	5.6
	1-2	0.0001640	0.0000164	9.4	0.8
	2-3	0.0001466	0.0000146	8.6	0.6
	3-4	0.0002076	0.0000208	7.8	0.7
	4-5	0.0002740	0.0000274	7.0	0.6
Gr	0-1	0.0002781	0.0000503	6.5	1.3
	1-2	0.0002562	0.0000604	7.5	0.6
	2-3	0.0004399	0.0000861	6.3	0.7
	3-4	0.0003580	0.0000758	5.3	1.0
	4-5	0.0003784	0.0000001	4.0	0.7

Table 8.3.1. Depth specific values of CO_2 production and *ra* (%) across all sites in the Medway estuary. All values are 1 +/- SEM (n=5).

Site	Depth (cm)	Relative abundance of anammox organisms to total bacteria (%)	SEM
MBM	0-0.5	1.0	0.4
	0.5-1.0	1.6	0.5
	1.0-1.5	2.1	0.6
	1.5-2.0	2.3	0.4
	2.0-2.5	3.0	0.9
Up	0-0.5	0.7	0.3
	0.5-1.0	1.4	0.4
	1.0-1.5	2.9	0.7
	1.5-2.0	2.3	0.8
	2.0-2.5	1.4	0.4
Gr	0-0.5	0.7	0.4
	0.5-1.0	1.1	0.4
	1.0-1.5	1.7	0.3
	1.5-2.0	3.3	0.6
	2.0-2.5	3.2	0.9

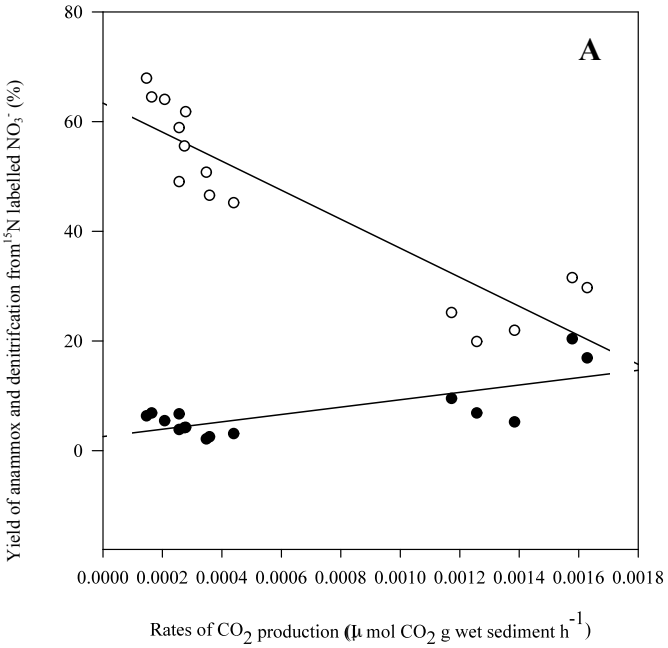
Table 8.3.2. The relative abundance of anammox organisms to total bacteria (%) across all sites in the Medway estuary. All values are 1 +/- SEM (n=20).

8.4. The significance of anammox relative to denitrification with sediment depth

The relative contribution of anammox to total N_2 production was on average 28%, 10% and 6% at Sites MBM, Up and Gr respectively. This is in line with the preliminary survey where values of *ra* decreased in a seaward direction (see Figure 8.1.2, Table 8.3.1.). Across all sites the greatest potential for anammox was localised to the upper 2cm of the sediment. The greatest depth specific values of *ra* were observed at Site MBM. Here, *ra* peaked at 37% and subsequently decreased downcore to 17% at -3.5cm (Figure 8.3.1). Similarly, a peak in the potential anammox was evident in the subsurface of the sediment at Site Up (Figure 8.2.1.). Here, *ra* peaked at 15% followed by a decrease to 7% (-4.5cm). The depth specific values of *ra* at Site Gr were the lowest observed. At this site, the potential for anammox decreased from 7.5 to 4% (Figure 8.3.3).

8.5. Depth specific rates of CO_2 production relative to the potential for anammox

Depth specific rates of CO_2 production are an indirect measurement of sediment metabolism. Interestingly, depth specific values of *ra* increased as a function of CO_2 production. This is clear at Site MBM where the maximum rate of sediment metabolism (0.0016µmol CO_2 g $^{-1}$ wet sediment h $^{-1}$) corresponds to a peak in *ra* (35%) (Figure 8.5.1. B). Contrastingly, the depth specific potential for denitrification decreased in response to sediment metabolism (Figure 8.5.1 B). This was evident at Site Up, where the lowest rate of CO_2 production (0.00015µmol CO_2 g $^{-1}$ wet sediment h $^{-1}$) was associated with a peak in denitrification (91%). When the yield of anammox and denitrification (from ^{15}N



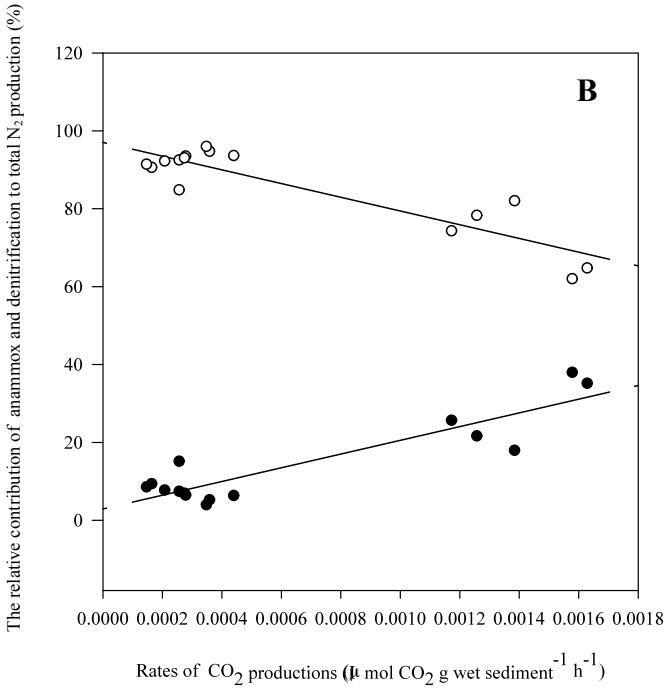


Figure 8.5.1. (A) The yield of ^{15}N labelled gas from anammox (black dots, y=67.13x+32.577, r^2=0.541) and denitrification (white dots, y=26.459x+53.35, r^2=0.816). (B) The relative contribution of anammox (black dots, y=17612x+2.947, r^2=0.816) and denitrification (white dots, y=-17612x+97.05, r^2=0.816) to total N_2 production as a function of CO_2 production. Values are a scatter of data points across all sites.

labelled NO_3^-) were plotted as a function of sediment metabolism, a similar relationship was apparent. The slope, however, indicates that proportionately less $^{15}NO_3^-$, is recovered as labelled gas, in denitrification (Figure 8.5.1 A). At maximum values of CO_2 production, the yield of ^{15}N labelled gas from anammox is 17%, relative to just 30% in denitrification (Site MBM).

8.6. *In situ* detection and the depth distribution of anammox organisms

The presence of anammox organisms was verified by FISH at all sites. The fluorescently labelled probes Amx 0368 (CY3), Sca 1309 (fluos) and Pla 46 (CY5) clearly hybridised with anammox cells (8.6.1). The probe binding patterns therefore pointed towards the presence of '*Scalindua*-like' bacteria. The cells depicted were typically 'doughnut-shaped' and fell within the reported size range for anammox organisms (Figure 8.6.2.). Only cells that positively hybridised with these probes were included in anammox specific counts. All samples were counterstained with DAPI, which was used to identify the total number of bacteria per micrograph.

Initially, 'manual' counts were used to determine the total number of cells in each of the captured images. These counts were then compared with automated counts as shown in Figure 8.6.3. The total number of bacteria counted manually clearly correlated with the number of cells counted using the automated procedure. It is important to note, however, that whilst the automated method proved more efficient, the linear relationship indicated an error of 16% (r^2 = 0.843, P=0.04) (Figure 8.6.3.).

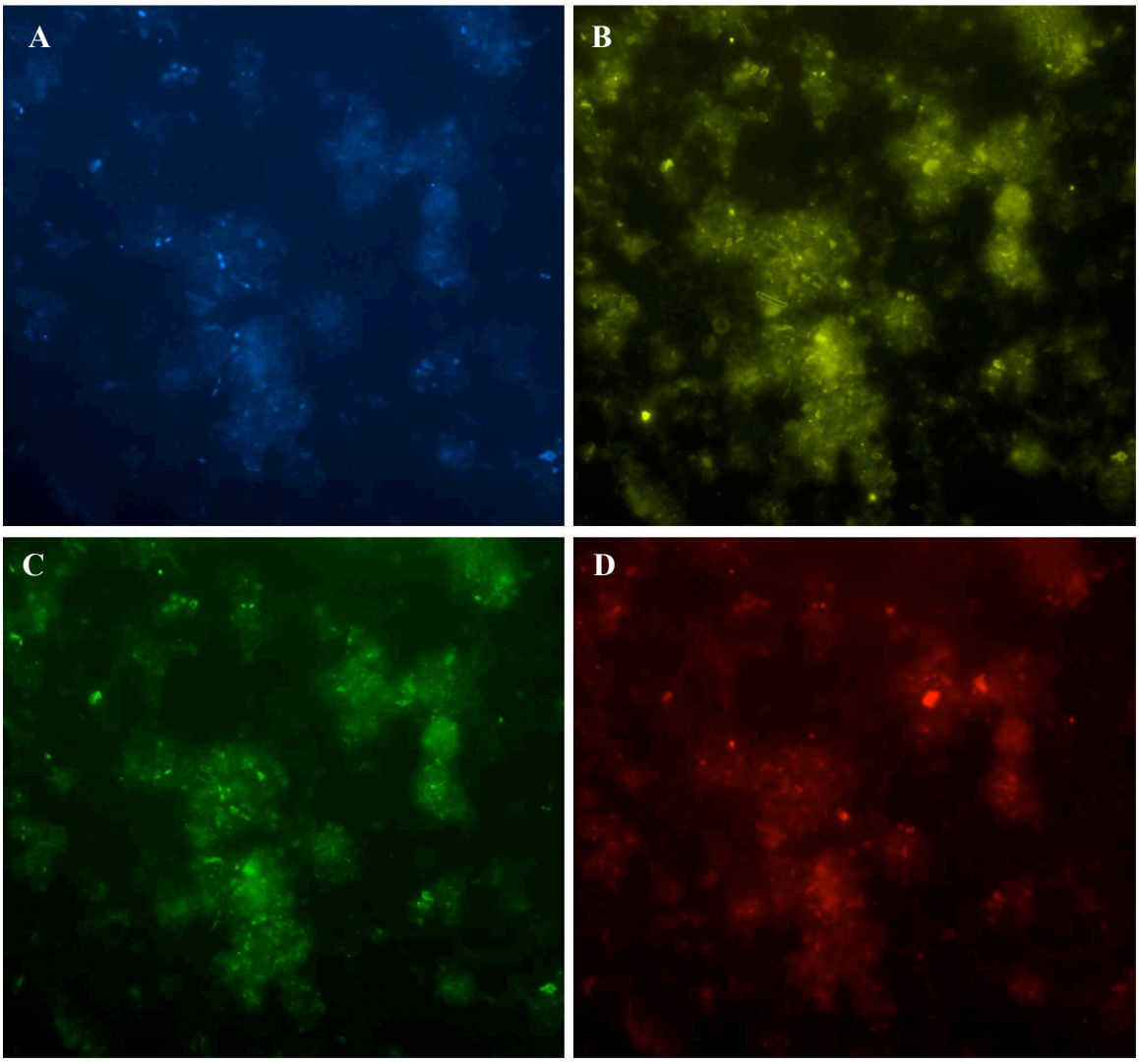


Figure 8.6.1. Confirmation of probe hybridisation in different filter sets in a sample from Site MBM. (A) DAPI stained DNA-containing cells, (B) cells hybridising with the probe Amx 0368 (cy3), (C) Sca 1309 (Fluos) and (D) Pla 46 (Cy5). The combined micrograph is shown below.

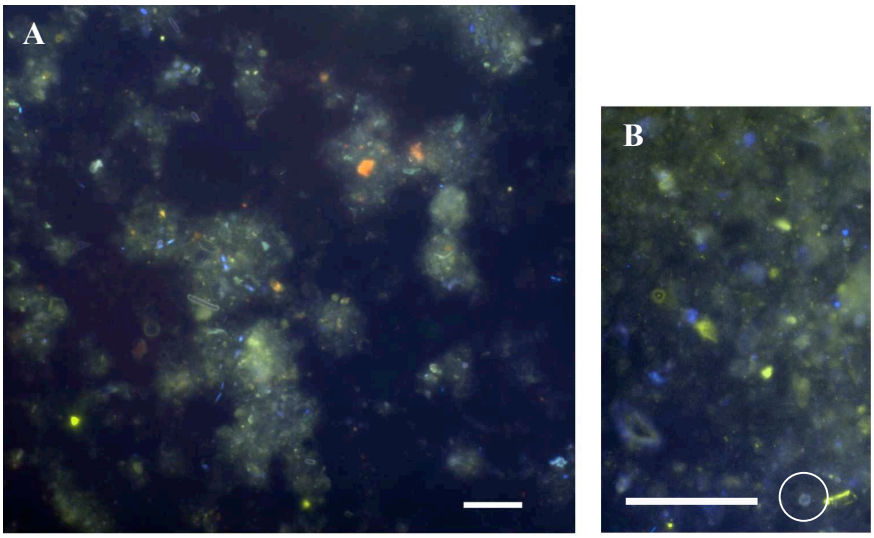


Figure 8.6.2. (A) Combined epifluorescence micrograph of a sample from Site MBM. The scale bar represents 20µm. (B) An enlargement of panel A depicting a white, 'doughnut-like' anammox cell (white circle). The scale bar represents 7µm.

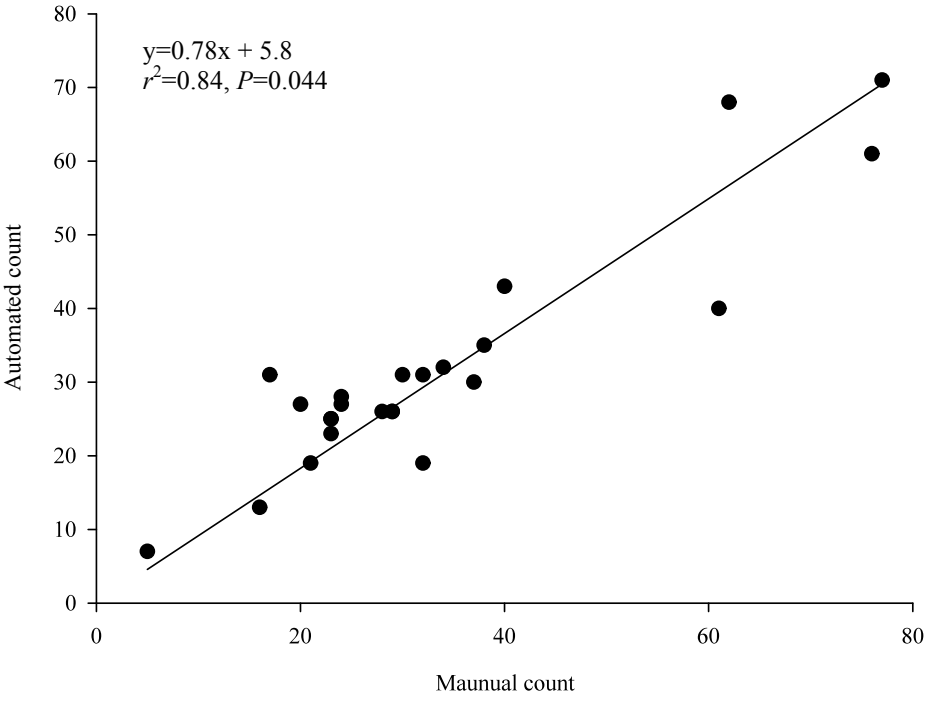


Figure 8.6.3. Linear regression of the total number of bacteria included in the manual and automated counts (n=21). Values are a scatter of data points across sites selected at random.

There was no significant variation in the total number of bacteria with depth (as previously reported, Schmid *et al*., 2007). The total number of anammox organisms detected varied between 0 and 3% at all sites and at all depth intervals (Table 8.3.2.) which is in line with previous observations (Schmid *et al*., 2007). The only site where the number of anammox organisms, relative to total bacteria, varied significantly was at Grain (1 way ANOVA, F=7.15, P=0.01). The overall trend with depth at this site was an increase from 0.7% at -5mm to 3.3% at -20mm (Figure 8.3.3.). Here, the maximum depth specific value of *ra* (5%) coincides with a peak in the abundance of anammox organisms (3.3%). This is not the case, however, at Site Up where the greatest number of anammox organisms accumulates within the first 1.5cm (2.9%). Either side of this peak, the total N_2 production does not vary significantly, although there is a definite increase in the relative number of anammox organisms from 0.7% at -0.75cm and a decrease to 1.4% at -2.25cm (Figure 8.3.2.). Interestingly, anammox organisms were present within the oxic zone and at the oxic-suboxic zone at Medway Bridge Marina. This is not consistent with the production of N_2 at these specific depths in the sediment (Figure 8.3.1.). The general trend at this site is a linear increase in the relative number of anammox organisms from 1% (at -0.25cm) to 3% (at -2.25m).

9. DISCUSSION

The presence of NO_2^- is a prerequisite for anaerobic ammonium oxidation (Van de Graaf *et al*., 1995; Dalsgaard *et al*., 2003). It is therefore likely that the capacity for anammox, in any given environment, is dependant on the physical and microbiological mechanisms by which NO_2^- is delivered to anammox organisms. In temperate estuarine sediments, the availability of NO_2^- is potentially governed by denitrification (whereby NO_2^- is released into the surrounding porewater as an intermediate of NO_3^- reduction) (Meyer *et al*., 2005). Sediments enriched with NO_3^- and organic carbon, stimulate the growth of heterotrophic denitrifying organisms which, in turn, could increase the overall availability of NO_2^- to the anammox community (Trimmer *et al*., 2005; Stief *et al*., 2002). Studies addressing the factors that regulate anammox activity and the microbial community structure, however, are limited (Trimmer *et al*., 2003; 2005; Risgaard –Petersen *et al*., 2005; Meyer *et al*., 2005, Tal *et al*., 2005; Rich *et al*., 2008). Nevertheless, it was suggested that the thickness of the NO_3^- reducing zone and the total number of the anammox organisms, could be directly linked to the significance of anammox in N removal (Trimmer *et al*. 2005; Dalsgaard *et al*., 2005). The purpose of this study was to characterise the zone of anammox activity and to subsequently connect this with the abundance of anammox organisms and the significance of anammox in N removal. Given the slow growth rate (max doubling time 9 days) and anaerobic physiology of anammox organisms (reversible inhibition <1μM oxygen) in bioreactors, the formation of a stable anammox community is theoretically limited to the suboxic zone in sediments (Strous *et al*., 1999).

The relative contribution of anammox to total N_2 production, in the Medway Estuary, is in line with previous findings where *ra* is positively correlated with sediment reactivity and organic loading (Trimmer *et al*., 2003; 2005; Rich *et al*., 2008). The importance of anammox in N removal, however, at Site MBM is substantially elevated in relation to comparative systems (Trimmer *et al*., 2003; Nicholls and Trimmer, 2009). In the Thames estuary, *ra* does not exceed 8% (in sediment slurries) yet the contribution of anammox to total N_2 production was, on average, 28% at Site MBM. Moreover, this data set is at best an estimate, given that measurements in sediment slurries under-represent *ra* by 10-15% (where *ra* >5%). (Trimmer *et al*., 2006). In the context of this error, the potential for anammox could exceed the values of *ra* measured at this site. According to current knowledge, this would therefore place the results amongst the highest values of *ra* reported in estuarine systems (maximum *ra*=24%) (Risgaard–Petersen *et al*., 2004).

In terms of the depth specific distribution of *ra*, the results fall within the expected environmental conditions that serve as a prerequisite for anammox activity (Strous *et al*., 1999; Dalsgaard *et al*., 2003). This is evident across all sites, where the greatest potential for anammox was constrained to subsurface sediment layers (Figures 8.3.1., 8.3.2.and 8.3.3.). Moreover, in sediments where there is a sufficient supply of organic matter, NO_2^- concentrations peak within the upper cm of the sediment (Stief *et al*., 2002). Consequently, this could provide an adequate supply of NO_2^- for the formation of a stable anammox community (in an oxygen deficient environment). Below the first 0.5-1cm of the sediment, the capacity for anammox decreases, as indicated by a reduction in the

significance of *ra*, to 4% (Site Gr), 7% (Site Up) and 17% (Site MBM). This could be linked to a decline in the availability of porewater NO_2^- down-core, thus providing less favourable conditions for the formation of N_2 via anammox (Figures 8.3.3 and 8.3.2.).

Local variations in NO_2^- alone, however, do not explain the elevated values of *ra* observed at Site MBM. Here, the overall contribution of anammox to total N_2 production is at least 3 x greater than the values of *ra* reported at the other sites. Interestingly, depth specific rates of CO_2 production are positively correlated with the formation of N_2 via anammox (Figure 8.5.1. A). This is clear at Site MBM, where the maximum rate of CO_2 production (0.0016μmol CO_2 g^{-1} wet sediment h^{-1}) coincides with a peak in *ra* (35%). High rates of CO_2 production are an indirect indication of elevated sediment metabolism. In environments where there is an abundant supply of organic matter, this increases sediment metabolism by stimulating the growth of heterotrophic, denitrifying bacteria (Sloth *et al*., 1995). Consequently, this may enhance the supply of freely diffusible NO_2^- to the anammox community, and therefore, promote close coupling between these processes (Kuypers et al., 2003; Thamdrup and Dalsgaard, 2002). This is further corroborated by the positive correlation between *ra* and the availability of organic carbon (see Nicholls and Trimmer, 2009), but does not explain the significance of the overall yield in ^{15}N labelled gas.

The recovery of ^{15}N labelled gas, from the addition of $^{15}NO_3^-$, indicates that denitrification is perhaps not the only significant process enabling the delivery of NO_2^- to anammox organisms. In terms of the total yield of ^{15}N labelled gas, it was evident that

whilst anammox increased as a function of CO_2 production, the corresponding yield from denitrification decreased by a factor of 4 (Figure 8.5.1. B). This deficit could perhaps be attributed to dissimilatory nitrate reduction to ammonium (DNRA), whereby NO_3^- is anaerobically reduced through NO_2^- to NH_4^+ (Patrick *et al*., 1996). If DNRA is active in these sediments, this could reduce the pool of $^{15}NO_3^-$ available for denitrification. This implies that whilst anammox perhaps benefits from the release of NO_2^- (derived as an intermediate of DNRA), denitrifying organisms may compete for the NO_3^- reduced in this process. The potential relationship between anammox in the presence of DNRA, however, is largely unexplored and may add to the complexity of N transformation in estuarine systems.

Overall, the relative abundance of anammox organisms increased with sediment depth. With the exception of Site Gr (Figure 8.3.3.), this conflicted with a decrease in the depth specific values of *ra*. This can perhaps be attributed to the limitations of FISH analysis in sediments. The stability of prokaryotic rRNA is determined by several factors in the environment (Binder *et al*., 1998) and therefore, positive signals do not necessarily represent active organisms. Moreover, Schmid *et al*. (2007) reported that mapping the abundance of marine anammox bacteria using FISH is potentially misleading. This may help to explain the depth distribution at Site Up (Figure 8.3.2.), but cannot account for the disproportionately large values of *ra* measured at Site MBM.

In first cm of the sediment the depth specific values of *ra* varied between 35-37%, yet the anammox organisms constitute just 1-1.7% of the microbial population. This is further

corroborated by the preliminary survey of the Medway Estuary. At the same site, just 2% of the microbial population coincided with an average value of *ra* equal to 30% (Figure 8.1.2.). Consequently, this could point organisms that are phylogenetically unrelated to anammox, yet capable of the anammox process. This was explored using a novel sequence (retrieved from Site MBM) sharing 96% similarity with *Candidatus* '*Scalindua sorokinni*' (see Chapter II). A fluorescent probe was designed according to this unique sequence, and positively hybridised with anammox cells (see Figure 4.3.1., Chapter II). The preliminary counts, however, did not resolve the discrepancy between *ra* and the overall abundance of anammox organisms.

In addition to the potential role of DNRA in N transformations, the presence of bioturbating macrofauna may enhance the significance of anammox by extending the zone of NO_3^- reduction (Dalsgaard *et al*., 2005; Henriksen *et al.* 1980). Total counts of invertebrates in the sediment, at Site MBM, point towards the presence of *Nereis diversicolor* and *Corophium volutator* at mean densities of 6 (≤ 3cm in length) and 18 per core respectively. The effect of this is demonstrated in Figure 8.2.1.A, where the multiple peaks in the dissolved oxygen profiles suggest ventilation of the sediment substrata with surface water. This can be attributed to a dense network of *Nereis* burrows (personal observation).

The linings of invertebrate burrows are generally considered microbial 'hot spots' for heterotrophic bacteria. Consequently, bioturbation significantly contributes to total N_2 production. Nielsen *et al.* (2004) reported that as much as 82% of bulk NO_3^- reduction in

sediments was the result of microbial activity in the lining of burrow structures. Invertebrate burrows have been associated with large numbers of nitrifying bacteria (Kristensen *et al.,* 1995) and coupled denitrification (Blondin *et al.,* 2004). As a result, bioturbation may enhance the supply of NO_2^-, by effectively extending the overall area of the NO_3^- reducing zone. In combination with high rates of sediment metabolisms and the potential for DNRA, bioturbation is perhaps amongst several governing factors that enhance the significance of anammox. This combined effect may explain why values of *ra* at Site MBM exceed those reported at Site Up and Gr. The effect of bioutrbation, in the role of anammox, however is largely unexplored.

Intriguingly, it is also evident that values of *ra* unexpectedly peak above the oxic-suboxic interface (-9.9mm) at this site in the presence of highly elevated and variable concentrations of oxygen (Figure 8.3.1.). This is perhaps the result of experimental design, whereby samples were incubated under completely anaerobic conditions. However, the physical presence of these organisms within the oxic zone, as demonstrated by FISH, implies that these bacteria are perhaps more metabolically flexible than previously understood. Penton *et al*. (2006) suggest that anammox organisms are not necessarily restricted by unfavourable environmental conditions. Following a phylogenetic survey of widely contrasting environments, anammox was discovered in periphyton dominated aerobic sediment. In addition, Schmid *et al*. (2007) detected the presence of anammox cells in the subsurface layer of marine sediments, yet failed to explain the co-occurrence of these organisms with oxygen (The Fresian Front, The North Sea). Woebken *et al*. (2007), however, put forward an argument for the existence of

anammox in oxygen depleted microniches (in planktonic snow of the Namibian shelf waters). Essentially, in such environments, a protective layer of microorganisms (consuming oxygen) shield anammox bacteria from the external environment. The oxygen concentrations measured in the subsurface of the sediment at Site MBM are well above those reported in the Nambian shelf waters (25µM) (Woebken *et al*. 2007). This suggests that the slow growth rate and anaerobic physiology of anammox does not limit their survival under dynamic environmental conditions.

This study confirms the presence of anammox organisms in the Medway Estuary and therefore adds to the limited knowledge of anammox in estuarine environments. Anammox organisms were detected at all sites and throughout the sediment substrata. The distribution of these organisms and depth specific values of *ra* are not, however, necessarily linked. To determine whether this is the result of a low number of 'highly active' anammox organisms, future studies should focus on resolving the depth specific rate measurements per cell. This could be achieved by quantitative FISH and measurements of depth specific anammox activity. Given the non-translucent properties of sediment samples, however, volume based quantification of the total organisms may prove challenging. Alternatively, if the *in situ* diversity cannot be fully explored using current molecular techniques, future studies should focus on the development of new, anammox specific probes/ primers.

Intriguingly, measurements of CO_2 production verified a positive correlation between the significance of anammox and sediment metabolism. The yield of ^{15}N labelled gas, from $^{15}NO_3^-$, revealed a fourfold decrease in denitrification relative to an increase in anammox (at higher sediment metabolisms). This could implicate DNRA as an important pathway for the delivery of NO_2^- to the anammox community. Studies addressing the role of DNRA in the regulation of anammox are, however, relatively scarce.

Surprisingly, the greatest potential for anammox is associated with unfavourable conditions due to incursions of oxygen as a result of bioturbating activity. Hence, this implies that anammox organisms are more flexible than enrichment culture studies suggest (Strous *et al*., 1999). Future studies could focus on determining the tolerance of environmental strains of anammox to oxygen and the effects of bioturbating invertebrates. Investigations including techniques such as microautoradiography combined with FISH could facilitate cell specific substrate uptake patterns of anammox bacteria under aerobic conditions (Daims *et al*., 2001; Nielsen *et al*., 2003; Kindaichi *et al*., 2004)

In conclusion, it is clear that further studies are required to fully understand the dynamics of anammox in the environment, particularly with regard to the role of DNRA and the formation of stable communities under otherwise unfavourable conditions. Enrichment culture studies provide some insight into the physiological requirements for anammox, but fail to reflect the dynamics of the environment.

10. INTRODUCTION

Anammox is the anaerobic oxidation of NH_4^+, coupled to NO_2^- reduction, to form di-nitrogen gas (N_2) (Van de Graaf *et al*., 1995; Mulder *et al*., 1995; Dalsgaard *et al*., 2003). Environmental studies clearly demonstrate that anammox is critical to N removal and constitutes up to 67-79% of total N_2 production in marine sediments, yet the total number of organisms affiliated with this process, form just 2-8% of the prokaryotic population (Thamdrup and Dalsgaard, 2002; Thamdrup *et al*., 2005; Schmid *et al*., 2007). Although the occurrence of anammox is well documented in a diverse range of marine sediments, studies specifically addressing the factors that regulate the distribution, abundance and significance of this process, are relatively scarce.

In contrast to estuarine ecosystems, the availability of organic matter in marine environments is highly reduced. This is particularly evident in deep, offshore waters where a large portion of organic matter is mineralised (to NH_4^+) before reaching the sediment surface. Essentially, this severely limits the accessibility of heterotrophic microorganisms to suitable electron donors and thus reduces the capacity for denitrification in marine sediments (Canfield *et al*., 1993). In the absence of organic carbon, the overall competition for electron acceptors, such as NO_3^- and NO_2^-, is further reduced and therefore enhances availability of interstitial NO_2^- (Trimmer *et al*., 2005; Dalsgaard *et al*. 2005). Given that the production of NO_2^- is 4 x greater than NO_2^- consumption in the Skagerrak, this could explain the comparative importance of this process (relative to denitrification) in deep, offshore waters (Thamdrup and Dalsgaard,

2002). It is also important to note that the anammox reaction is saturated at low concentrations of interstitial NO_2^- (K_m <3μM) (Thamdrup and Dalsgaard, 2002; Trimmer *et al*., 2005). In an environment where the *in situ* supply of NO_2^- is abundant, and the affinity of anammox organisms for this nutrient is low, this could sustain a comparatively large community of anammox organisms (relative to estuarine environments). To date, information regarding the relationship between the significance of anammox and the total abundance of anammox organisms is limited to just one marine study (Schmid *et al*., 2007).

The overall availability of NO_3^- is prerequisite to NO_2^- production, and therefore essential to the formation of a stable anammox community. In terms of bottom water NO_3^-, Risgaard-Petersen *et al*., (2005) demonstrated that the penetration of NO_3^- into the sediment substrata was crucial to sustaining anammox activity. This was evident in Norsminde Fjord where the combined affect of low water column NO_3^-, and the presence of benthic microalgae (through active uptake), essentially inhibited the diffusion of NO_3^- into the NO_3^- reducing zone (Risgaard-Petersen *et al*., 2005). Consequently, this was used to explain the absence of anammox activity from these sites and demonstrates that the anammox process is not ubiquitous to the environment.

In contrast to conditions at Norsminde Fjord, the delivery of sufficient NO_3^-, to the NO_3^- reducing zone, promotes the formation of interstitial NO_2^-. In turn, this was shown to enhance the capacity for anammox (Meyer *et al*., 2005). This was clearly the case in

Randers Fjord, where the concentration of NO_3^- at the oxic-suboxic interface (23μM) sustained values of ra of up to 22%. The relationship between anammox and NO_3^- availability has been further corroborated by both marine and estuarine studies (Risgaard-Petersen *et al*., 2003; Rysgaard *et al*., 2004; Meyer *et al*., 2005; Nicholls and Trimmer, 2009).

In contrast to hypereutrophic estuarine sediments, the low overall availability of organic matter in marine environments implies that concentrations bottom water NO_3^- may play a key role in regulating significance of anammox. The extent to which NO_3^- penetrates the sediment could cause changes in the portion of the sediment that constitutes NO_3^- reduction zone. Consequently, this may alter the depth specific potential for anammox and ultimately the size of the anammox community.

The purpose of this study was to compare the depth integrated potential for anammox with the distribution of these organisms in the sediments of the North Atlantic and Irish Sea. The zone of potential anammox activity was characterised using porewater oxygen profiles and ^{15}N tracer experiments to investigate the depth specific potential for anammox. In addition, FISH analysis was used to map the distribution of anammox organisms with depth.

11. MATERIALS AND METHODS

11.1. Study sites

The sediment characteristics and site locations are described in section 3.1.1., Table 3.1. and Figure 3.1.1. (Chapter II).

11.2. Sediment collection, storage and sample preparation

Samples were retrieved from Site 1 in the North Atlantic and Sites 4 and 5 in the Irish Sea (March 2006). The collection, storage and preparation of sediment samples was performed according to the methods described in section 3.2., Chapter II. All samples were sliced at intervals of -3mm to a total depth of -36mm. 6-7 depth intervals were selected for the purpose of subsequent analysis.

11.3. Porewater oxygen profiles, end-point incubation experiments and FISH analyses

Subsequent porewater oxygen profiles, end-point incubation experiments and FISH analyses were performed according to the methods described in sections 7.3., 7.4. and 7.6. (Chapter III). FISH analysis was conducted using manual counts.

12. RESULTS

12.1. Porewater oxygen profiles

Dissolved oxygen profiles were measured in each of the in-tact sediment cores retrieved from the North Atlantic (Site 1) and Irish Sea (Sites 4 and 5) (Figure 12.1.1.). The position of the oxic-suboxic interface was on average 12.4mm at Site 1, 9.8mm at Site 4 and 17.0mm at Site 5. The uniformity of each of the oxygen profiles, measured across all sites, demonstrates the partitioning of the sediment substrata into well defined oxic and suboxic regions. At site 5, however, the oxygen penetration depth is intriguingly extensive (Figure 12.1.1.A). Jaeschke *et al.*, 2009 suggested that this could be attributed to re-working of the sediment strata, by the Anthropod *'Nephrops norvegicus*'. Ultimately this may have resulted in an extension of the oxic zone which was in stark contrast to the shallow oxygen penetration depths measured at other coastal sites (Jaeschke *et al*., 2009).

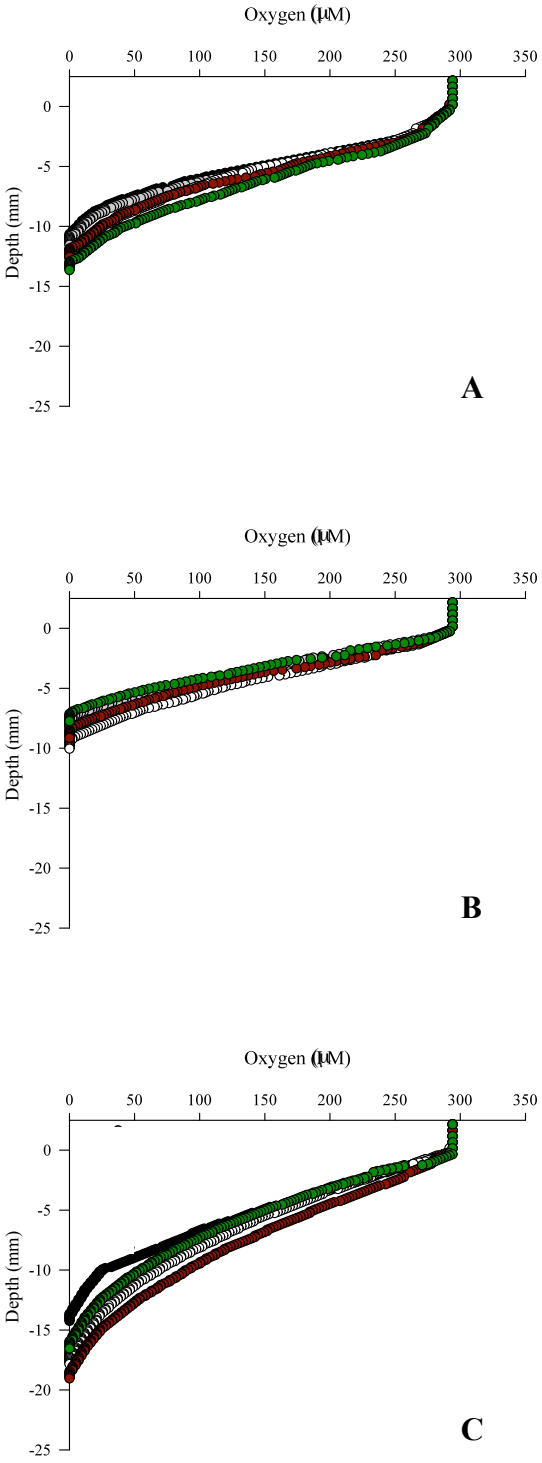


Figure 12.1.1. Multiple dissolved oxygen profiles as measured in intact sediment cores from (A) Site 1, (B) Site 4 and (C) Site 5. The uniform decrease in oxygen represents the separation of the sediment substrata into defined oxic and suboxic zones (n=5). Each profile represents 1 core.

12.2. The depth specific significance of anammox relative to denitrification

The potential contribution of anammox to total N_2 production was on average 49% at Site 5, 32% at Site 1 and 13% at Site 4. There was no significant difference in the depth specific values of *ra* at either Site 1 or Site 4, although distinct variations were evident at Site 5 (1 way ANOVA, F=12.65, P=0). This is clearly reflected by the depth specific values of *ra* across all sites.

The contribution of anammox to total N_2 production at Site 1 peaked at -27mm where *ra* was equal to 40% (Figure 12.2.1.). This is well below the oxic-suboxic interface (9.8mm) although there is little variation either side of this value with depth. Measurements of *ra* at the sediment surface (-3mm) indicate the potential for anammox within the oxic zone. Sub surface peaks in anammox were common to all sites, and in this case, the value of *ra* at the sediment surface (43%) is comparable to the maximum value at depth (Figures 12.2.1, 12.2.2. and 12.2.3.). Similarly the potential for anammox peaks in the first few mm of the sediment at Site 4. Here, the significance of anammox is equal to 19% of the overall N_2 production (Figure12.2.2.). Below the sediment surface there is little variation with depth and no obvious peak in the potential for anammox. Interestingly, values of *ra* vary significantly throughout the sediment at Site 5 (12.2.3.). A peak in the capacity for anammox is apparent in the first 3mm of the sediment (65%) followed by a secondary peak between -9 and -18mm (62%). Below this depth the significance of anammox decreases to 35% at -36mm. Although the potential for anammox is highly variable, the capacity for this process is clear throughout both the oxic and suboxic zones. Moreover, the sediment collected from Site 5 shows the greatest capacity for anammox relative to all other sites and depth intervals.

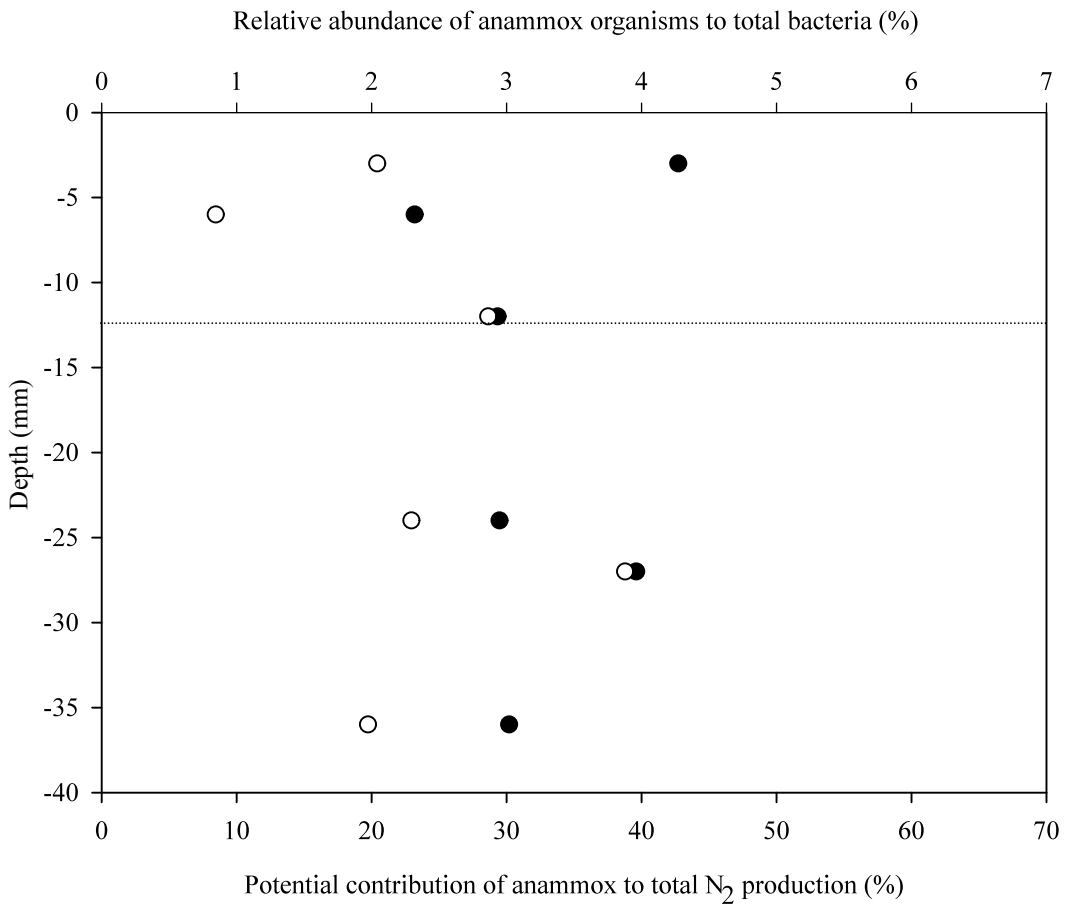


Figure 12.2.1. The depth specific distribution of *ra* (black dots) and the relative abundance of anammox organisms to total bacteria (white dots) at Site 1. The dotted line marks the position of the oxic-suboxic interface (-12.4mm). Each data point represents the mean of 5 (*ra*) and 20 replicates (relative abundance).

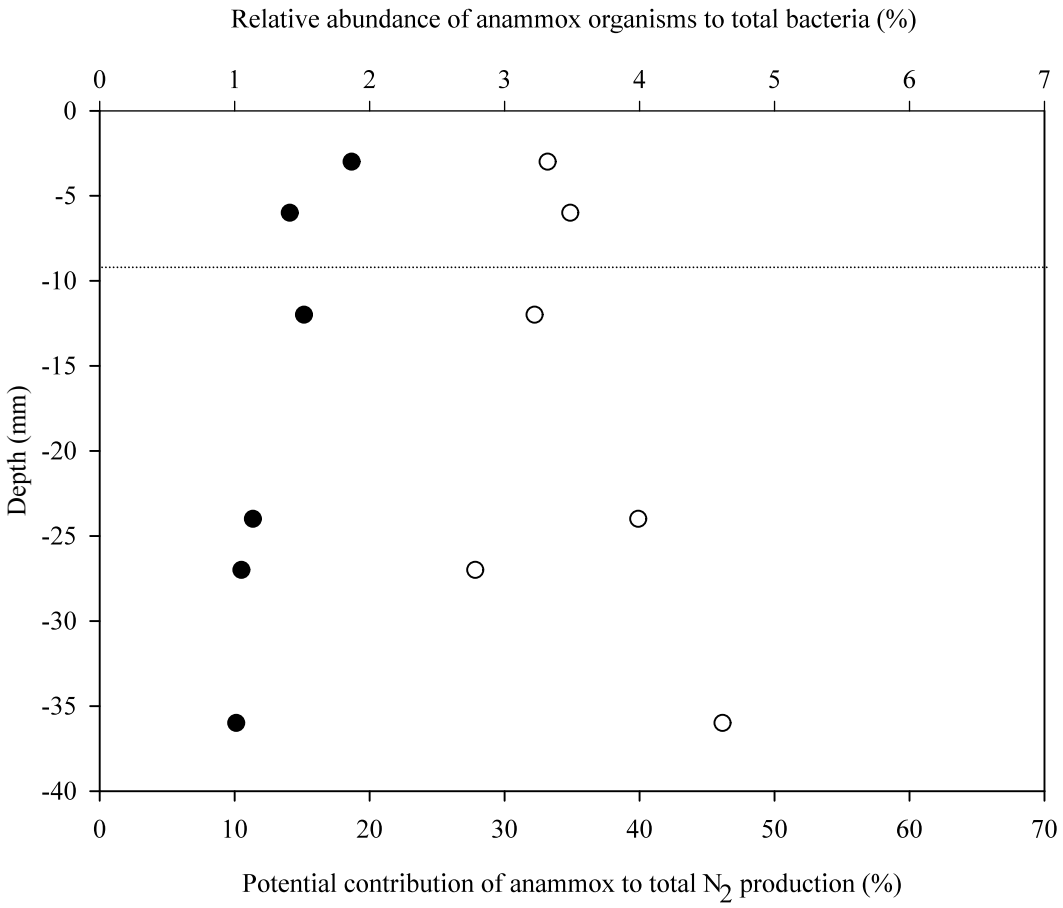


Figure 12.2.2. The depth specific distribution of *ra* (black dots) and the relative abundance of anammox organisms to total bacteria (white dots) at Site 4. The dotted line marks the position of the oxic-suboxic interface (-9.8mm). Each data point represents the mean of 5 (*ra*) and 20 replicates (relative abundance).

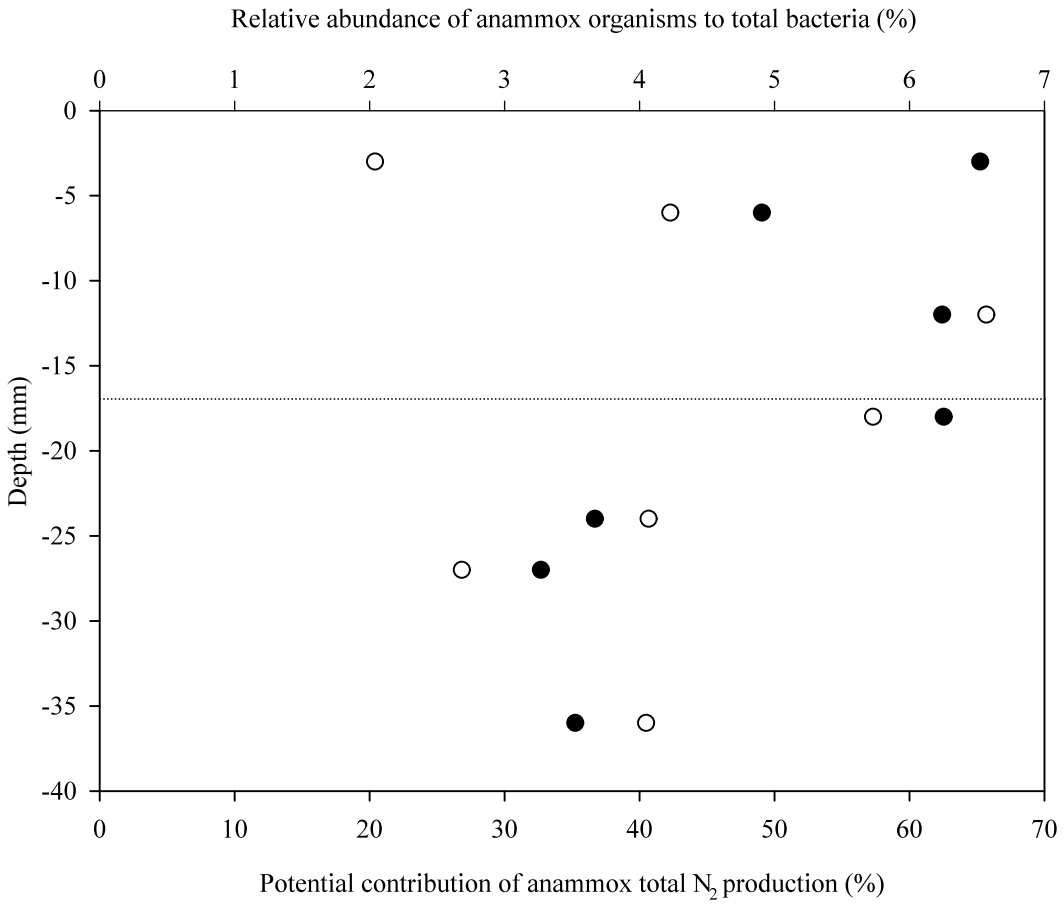


Figure 12.2.3. The depth specific distribution of *ra* (black dots) and the relative abundance of anammox organisms to total bacteria (white dots) at Site 5. The dotted line marks the position of the oxic-suboxic interface (-17.0mm). Each data point represents the mean of 5 (*ra*) and 20 replicates (relative abundance).

Site	Depth (mm)	*ra* (%)	SEM	Relative abundance of anammox organisms to total bacteria (%)	SEM
1	0-3	42.7	6.4	2.0	0.5
	3-6	23.2	8.7	0.8	0.4
	9-12	29.3	5.3	2.8	0.8
	21-24	29.5	6.3	2.3	0.2
	24-27	39.6	7.5	3.9	0.9
	33-36	30.2	5.4	2.0	0.4
4	0-3	18.7	1.5	3.3	0.5
	3-6	14.1	1.1	3.5	0.5
	9-12	15.1	2.5	3.2	0.8
	21-24	11.5	0.5	4.0	0.8
	24-27	9.9	0.6	2.8	0.6
	33-36	10.3	1.4	4.4	1.4
5	0-3	65.2	0.7	2.0	0.7
	3-6	49.1	0.8	4.2	0.8
	9-12	62.4	1.8	6.6	1.8
	15-18	62.5	0.9	5.7	0.9
	21-24	36.7	1.0	4.1	1.0
	24-27	32.7	0.4	2.7	0.4
	27-30	35.2	1.0	4.0	1.0

Table 12.2. The potential contribution (*ra*%) and relative abundance of anammox organisms (%) at 3 sites in the North Atlantic and Irish Sea. Each value represents the mean of 5 (*ra*) and 20 replicates (relative abundance of anammox organisms). All values are 1 +/- SEM.

12.3. *In situ* detection and the depth distribution of anammox organisms

Anammox organisms were detected at all sites throughout the North Atlantic and Irish Sea using FISH. Triple hybridisation of the probes Amx 0368 (CY3), Sca-1309/ BS 820 (fluos) and Pla 46 (CY5) with target cells produced clear signals, pointing towards the presence of '*Scalindua*-like' organisms (Schmid *et al* 2003; Kuypers *et al*., 2003 and Neef *et al*., 1998). The observed cells were typically doughnut shaped and fell within the expected size range (for anammox organisms) (Figure 12.3.1.). Only cells that positively hybridised with these probes were included in anammox specific counts.

The relative abundance of anammox organisms to total bacteria was 5% at Site 5, 4% at Site 4 and 1% at site 1. Statistical analyses indicated that there was no significant difference between either the abundance of anammox organisms or total bacteria with depth. This is in line with previous findings where both the abundance of anammox organisms varies between 2-8% and there is no depth specific variation in the prokaryotic population (Schmid *et al*., 2007). Interestingly, the relative abundance of anammox organisms to total bacteria follows the same overall trend as the depth specific distribution of *ra*. This is clearly evident both at sites 1 and 5 (Table 12.2, Figures 12.2.1 and 12.2.3.). At Site 1 the relative abundance of anammox organisms to total bacteria peaks at -27mm where the anammox community represents 4% of the prokaryotic population and 40% of N_2 production. Either side of this peak, however, there is little variation in the abundance of anammox organisms, clearly reflecting the depth specific distribution of N_2 produced via anammox. Moreover anammox bacteria were detected throughout the oxic

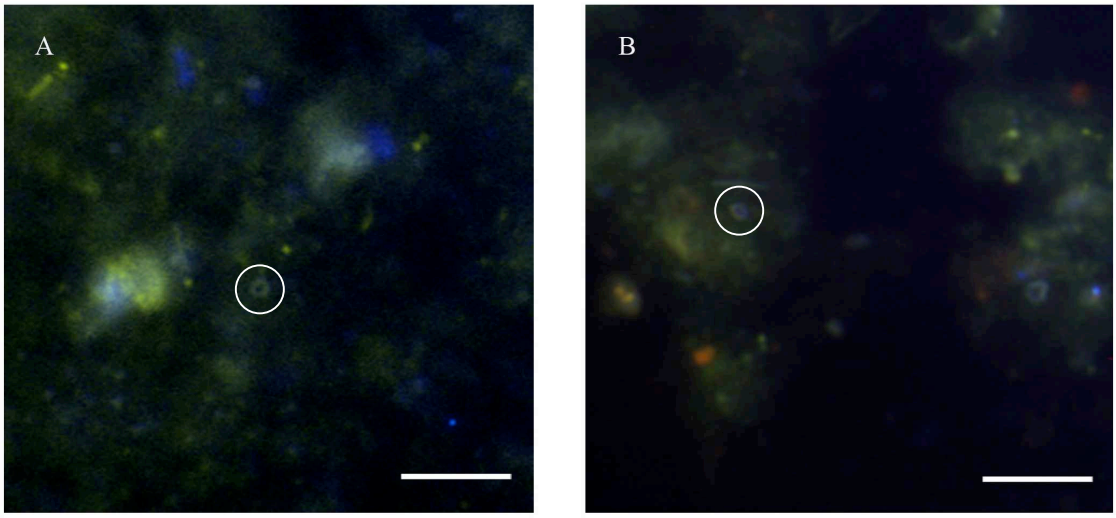
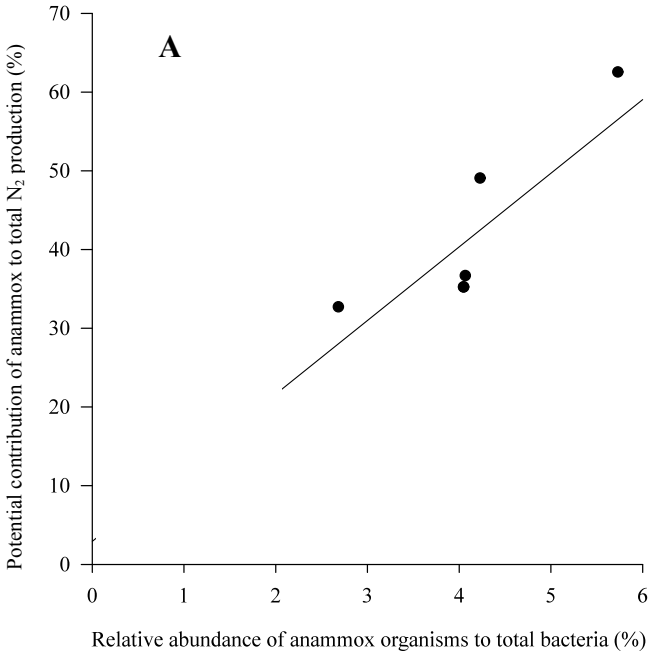


Figure 12.3.1. Detection of anammox organisms with Fluorescence in situ hybridisation from (A) Site 1, -36mm and (B) Site 5, -9mm; the white circles mark the position of anammox cells. This is the result of the triple hybridisation of probes Amx 0368 (CY3), BS 820 (fluos) and Pla 46 (CY5) with target cells. The scale bar represents 5µm.

zone, including the first 3mm of the sediment (2%). At this depth, the average concentration of porewater oxygen was 270μM. This is well above the described concentrations of oxygen in which anammox is known to occur in the environment (Kuypers *et al*., 2005; Woebken *et al*., 2007). Similarly, the presence of anammox organisms was verified throughout the oxic zones of sites 4 and 5. Within the first 3mm of the sediment at Site 5 *ra* is equal to 65% of total N_2 production, yet the abundance of anammox organisms is just 2% of the total prokaryotic population. This discrepancy only occurs at the sediment surface where ra tends to peak disproportionately relative to the abundance of anammox organisms across all sites. Below the first 3mm of the sediment, the depth specific abundance of anammox organisms tends to reflect the trend in *ra*. The secondary peak in *ra*, between -9 and -18mm (62%), is linked to a peak in the abundance of anammox organisms representing 6-7% of the total population. Below this depth the significance of anammox decreases to 35% at -36mm where the anammox community is proportional to 4% of the total population. To explore this relationship further, the potential contribution of anammox to total N_2 production was scattered against the abundance of anammox cells relative to total bacteria (Figure 12.3.2.). This revealed a clear linear relationship between relative abundance of anammox organisms and *ra*, where r^2 = 0.865 (P=0.024) and 0.84 (P=0.011) at sites 1 and 5 respectively. No significant relationship, however, was observed between relative abundance of anammox organisms and *ra* at Site 4.



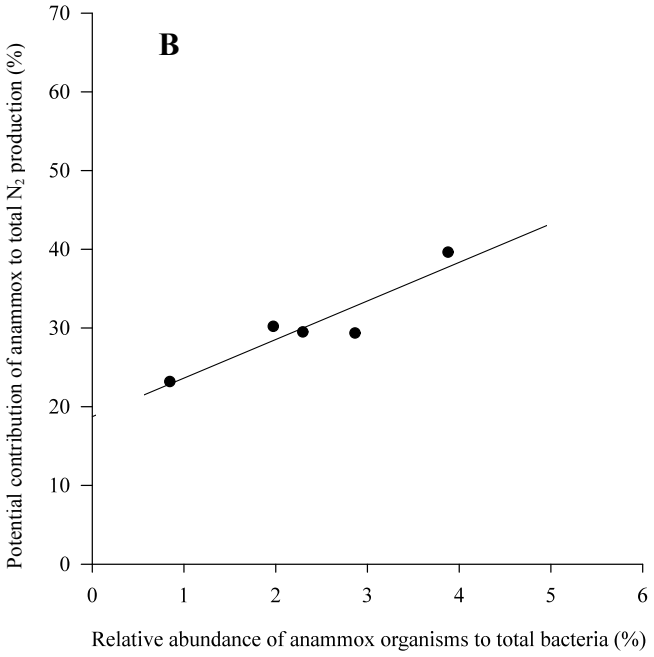


Figure 12.3.2. The contribution of anammox to total N_2 production as a function of the relative abundance of anammox organisms at (A) Site 1(where r^2=0.86, P=0.024) and (B) Site 5 (r^2=0.84, P=0.011). Mean values of *ra* are plotted against the mean abundance of anammox organisms relative to total bacteria.

13. DISCUSSION

Anammox is highly significant in marine sediments, yet the factors that regulate the distribution, abundance and significance of this process are largely unexplored (Van de Graaf *et al*., 1995; Mulder *et al*., 1995; Dalsgaard *et al*., 2003). According to current knowledge, anammox is reversibly inhibited by less than 1.1μM oxygen and is dependant on the availability of NO_2^- as a prerequisite to N_2 production (Strous *et al*., 1997; Thamdrup and Dalsgaard, 2002). Theoretically, this would place anammox organisms well within the suboxic zone of the sediment, coinciding with the location of NO_3^- reduction and the absence of oxygen (Meyer *et al*., 2005). The purpose of this study was to characterise the potential zone of anammox activity in order to investigate links between the depth specific potential and distribution of anammox organisms.

Strong evidence for the presence and distribution of anammox organisms was confirmed by FISH analysis and ^{15}N isotope labelling experiments. This was apparent across all sites investigated in the sediments of the North Atlantic and Irish Sea. According to previous marine investigations, the potential contribution of anammox to total N_2 production (*ra*) positively correlates with increased sediment depth (Thamdrup and Dalsgaard, 2002; Thamdrup *et al*., 2005). This coincides with a reduction in organic loading and therefore rates of sediment mineralisation. On average, the potential contribution of anammox to total N_2 production was 49%, 32% and 13% at Sites 5, 1 and 4 respectively. Given that the mean value of *ra* at Site 5 (100m) exceeds values of *ra* at Site 1 (2000m), this would suggest that the data are not in line with previous findings.

Measurements of the accumulation of ^{29}N, from ^{15}N labelled NH_4^+ and $^{14}NO_3^-$, are in good agreement with the above values of *ra*. Jaeschke *et al*. (2009) reported generally consistent values of ^{29}N across all sites with the exception of Site 5, where a peak in ^{29}N production (25.7 nmol ml-1 wet sediment) was clearly evident. Although these findings corroborate high values of *ra* at this site, it is important to note that only the top 2 cm of the sediment was sampled. If this reaction occurs at depth, this potentially excludes portions of the active anammox zone that could significantly contribute to N_2 production. The conditions, however, that govern the significance of anammox, may relate to local environmental characteristics and may not strictly reflect those described by general correlations (Nicholls and Trimmer, 2009).

In terms of the depth specific potential for anammox, this reaction is theoretically limited by environmental conditions that sustain an *in situ* supply of NO_2^- in the absence of oxygen. With the exception of peaks in *ra* within the first few mm of the sediment, this was evident at Sites 1 and 4. Although there was no significant difference in depth specific values of *ra*, the greatest contribution of anammox to total N_2 production peaked well below the oxic zone at Sites 1 (-27mm, *ra*=40%) and 4 (-12mm, *ra*=15%) (Figures 12.2.1 and 12.2.2.).

A similar trend was observed at Site 4 where a general decrease in the potential contribution of anammox to total N_2 production occurred. This was typical of sites in the Irish Sea where peaks in the potential for anammox were associated with the oxic-suboxic interface (Sites 4 and 5). At this depth, moderate organic loading (TOC=0.82-

13.6%), abundant NH_4^+ and an accumulation of NO_3^- (8-10 μM) could provide suitable conditions for the formation of a stable anammox community (Jaeschke *et al*., 2009). Furthermore, NO_3^- reducers become NO_3^- limited at 2-3 times lower than their K_m value (Dalsgaard *et al*., 2005). This implies that NO_3^- reduction is limited by ≤2-3μM NO_3^- (Dalsgaard and Bak, 1994). In turn, this suggests that the potential zone of anammox activity encompasses the first 3cm of the sediment in the Irish Sea. This is clearly reflected in the depth specific values of *ra* at Sites 4 and 5.

The potential contribution of anammox to total N_2 production varied significantly with sediment depth at site 5 (1 way ANOVA, F=12.65, P=0). Although peaks in *ra* (62%) coincide with the position of the oxic-suboxic interface (-17mm) the potential contribution of anammox to total N_2 production is evident throughout both the oxic and suboxic zones (Figure 12.2.3.). 'Physical reworking' of the sediment by the anthropod 'Nephrops norvegicus' is perhaps the cause of this 'mixed signal' and associated with extension of the oxic zone (Aller *et al*., 2004, Jaescke *et al*., 2009). (The presence of these invertebrates was noted during sampling). It was clear, however, that at all sites both *ra* and the physical presence of anammox organisms in the oxic zone occurred. This was subsequently confirmed by FISH analysis.

The anammox process is inhibited by <1.1μM oxygen yet a peak in subsurface (-3mm) N_2 production was common across all sites (Strous *et al*., 1997) (Figures 12.2.1, 12.2.2 and 12.2.3.). At this depth, the concentration of oxygen varied between 200-210μM O_2. Although anammox activity has been observed at ambient concentrations of 25μM O_2,

the concentration of O_2 at the sediment surface is at least 10 x greater than those reported in the Namibian Shelf waters (Woebken *et al*., 2007). This demonstrates that anammox organisms in the Irish Sea and North Atlantic are not necessarily constrained by environmental conditions given that FISH analysis revealed the presence of these organisms in all cases. This is additionally noted in the highly bioturbated sediments described in detail in Chapter III.

Positive FISH signals demonstrated the presence of anammox organisms in sediments of the North Atlantic and Irish Sea. Schmid *et al*. (2007) reported an average abundance of anammox organism within the range of 2-8% in marine systems. Given that anammox constitutes 67% of the total N_2 production in deep marine sediments, this indicates that a relatively small fraction of the prokaryotic population is required for significant N - removal (Thamdrup and Dalsgaard, 2002). This is clearly reflected by the average abundance of anammox organisms across all sites. Essentially, the abundance of anammox organisms peaks at Site 5 (5%) and subsequently decreases with sediment depth (Site 4 = 4%, Site 1=1%).

In terms of the positive correlation of anammox with increased sediment depth (Thamdrup and Dalsgaard, 2002), this does not coincide with expected trend. In sediments where the postulated significance of anammox is high, the expected correlation should theoretically coincide with an increase in the anammox community with sediment depth. In terms of the depth specific significance, however, the relative abundance of anammox organisms with sediment depth, coincides with depth specific values of *ra*.

This excludes Site 4, but is evident at Sites 1 and 5 (Figure 12.3.2.). This supports the theory that a low number of anammox organisms are responsible for a significant portion of N_2 production in marine systems (Kuypers *et al*., 2003; 2005; Hamersley *et al*., 2007; Schmid *et al*., 2007).

This study confirms the presence of anammox organisms in an additional marine system and consequently adds to limited knowledge of the distribution of these organisms in sediments. The overall significance of significance of anammox organisms is not clearly reflected by depth integrated values of *ra,* although points towards the confinement of anammox activity to the sediment surface (Sites 4 and 5). The limitations of the sampling potentially excluded a significant portion of depth integrated values of *ra* in combination with a major portion of the microbial population (Site 1). In addition, it is also clear that anammox organisms are not inextricably constrained by environmental conditions. Investigations including the tolerance of anammox to oxygen, and the effects of bioturbation are required (see Chapter III).

14. CONCLUSIONS

Anammox is the oxidation of NH_4^+ with NO_2^- to di-nitrogen gas (Van de Graaf *et al*., 1995; Mulder *et al*., 1995; Dalsgaard *et al*., 2003). This process is widely documented in the environment and circumvents coupled denitrification to provide an alternative nitrogen removal pathway in natural systems (Kuypers *et al*., 2003; Rysgaard *et al*., 2005; Schubert *et al*., 2006; Thamdrup and Dalsgaard, 2003; Trimmer *et al*., 2003; 2005). Previous studies indicate that whilst anammox occurs in a vast range of environments, the diversity of these organisms is limited to just two candidate species (Nakajima *et al*., 2008; Penton *et al*., 2006; Schmid *et al*., 2007; Woebken *et al*., 2008). These species are known as '*Scalindua sorokinni*' and '*Scalindua arabica*' (Kuypers *et al*., 2003; Woebken *et al*., 2008).

The sequences retrieved from the North Atlantic, Irish Sea and Medway estuary cannot be directly affiliated with known species of anammox (Chapter II). Intriguingly, however, a number of unidentified *Planctomycetes* retrieved from Sites 1 and 5 (in the North Atlantic and Irish Sea) form two phylogenetic clusters close to the anammox group (Chapter II, Figure 4.5.1.). These sequences share just 75% sequence similarity with '*Scalindua sorokinni*', yet exhibit common origins with the anammox lineage. Such sequences are common to environmental studies although largely disregarded (Tal *et al*., 2005; Schubert *et al*., 2006; Stevens and Ulloa, 2008; Shu and Jiao, 2008; Woebken *et al*., 2008).

The lack of sequences directly affiliated with anammox does not suggest the complete absence of these organisms from the environments investigated. Subsequent exploration confirmed the activity and presence of anammox organisms across all sites (Jaeschke *et al*., 2009; Chapters III and IV). In view of this finding, it is not unreasonable to suggest that the diversity of anammox organisms may extend well beyond the known group of 'candidate' organisms. Moreover, it is evident that future work should focus on expanding the current availability of molecular tools used in the detection of anammox. If the diversity is indeed greater than currently accepted, the development of efficient primers and detailed investigations of sequences, that are distantly related, but share common origins with anammox organisms, is essential.

Variations in the potential contribution of anammox to total N_2 production are apparent across a wide diversity of environments (Kuypers *et al*., 2003; Rysgaard *et al*., 2005; Schubert *et al*., 2006; Thamdrup and Dalsgaard, 2003; Trimmer *et al*., 2003; 2005). Details regarding the factors that regulate this process, however, are scarce. Following an exploration of the potential zone of anammox activity in the Medway estuary (Chapter III), it was evident that the relative abundance (<1-3%) and potential contribution of anammox to total N_2 production (4-35%) were in line with previous findings (Rich *et al*., 2008; Schmid *et al*., 2007; Trimmer *et al*., 2003; 2005). Direct links between the significance of anammox and changes in population size, however, were not clear.

This is illustrated by the discrepancy between the depth specific values of *ra* at Site MBM and the relative abundance of anammox organisms. Within the first cm of the

sediment, the depth specific values of *ra* vary between 35-37%, yet the corresponding abundance of anammox organisms form just 1-1.7% of the microbial population. These results were corroborated by an earlier survey where just 2% of the microbial population coincided with a value of *ra* equal to 30% (Chapter III, Figure 8.1.2.). As a consequence, this deficit could point towards organisms that are phylogenetically unrelated to anammox, yet capable of the anammox process.

Further investigation of the microbial diversity at this site was facilitated by the retrieval of a single sequence sharing 96% sequence similarity with '*Scalindua sorokinni*' (Chapter II). A fluorescent probe was designed according to this sequence and positively hybridised with candidate anammox cells (Chapter II, Figure 4.3.1.). The proportion of additional cells, however, failed to resolve the deficit between the abundance of anammox organisms and high values of *ra*.

The importance of N removal at Site MBM is substantially elevated relative to other stations (mean *ra*=28%) and coincides with the maximum rates of CO_2 production (0.0016μmol CO_2 g^{-1}). Given that the rate of CO_2 production is an indirect measurement of sediment metabolism, this finding is in line with the positive correlation between anammox and sediment reactivity (in estuarine sediments). In terms of the total yield of ^{15}N, it was evident that whilst anammox increased as a function of CO_2 production, the corresponding yield from denitrification decreased by a factor of 4. This deficit can perhaps be attributed to DNRA. In the presence of DNRA, anammox may benefit from the release of NO_2^- (released as an intermediate of this process), where as denitrification

may be suppressed (as a result of competition for NO_3^- reduced in this process). The potential relationship between anammox and DNRA however is largely unexplored, and may add to the complexity of N transformations in marine systems.

In addition to the potential role of DNRA in N transformations, the presence of bioturbating macrofauna could potentially enhance the significance of anammox by extending the zone of NO_3^- reduction and consequently increasing the availability of NO_2^- (Dalsgaard *et al*., 2005; Henriksen *et al*., 1980; Nielsen *et al*., 2004). The greatest densities of *Nereis diversicolor* and *Corophium volutator* were located at Site MBM and therefore coincide with maximum values of *ra*. The relationship between the significance of anammox and bioturbation, however, has not been investigated.

In contrast to estuarine ecosystems, depth specific values of *ra* correlate with the relative abundance of anammox organisms in continental shelf (r^2=0.86, *P*=0.024) and slope sediments (r^2=0.84, *P*=0.011) (Chapter IV). The overall significance of anammox, however, is not clearly reflected by depth specific values of *ra*, although points towards the localisation of anammox activity at the sediment surface (Sites 4 and 5). The abundance (<1-5%) and significance (13-19%) of this process is in line with previous findings (Schmid *et al*., 2007).

In conclusion, it is evident that further environmental studies are required to fully understand the factors which may regulate the distribution and significance of anammox in natural systems. This is particularly evident in the context of microbial diversity,

DNRA and bioturbation. Enrichment culture studies may provide some insight into the physiological requirements for anammox, but cannot recreate the complex dynamics that exist between anammox organisms and the environment.

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