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MOLECULAR MICROBIAL ECOLOGY OF AMMONIA OXIDATION IN COASTAL SEDIMENTS

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A thesis submitted to the National University of Ireland Galway for the degree of:

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February 2017
# Table of Contents

**Ammonia Oxidation in Coastal Sediments**

## Acknowledgements

## Abstract

1) **Chapter 1: General Introduction**

1.1 Nitrogen cycle

1.1.1 Importance of the nitrogen cycle in coastal ecosystems

1.1.2 Human impact

1.2 Nitrification

1.3 Nitrifiers

1.3.1 Ammonia Oxidising Bacteria (AOB)

1.3.2 Ammonia Oxidising Archaea (AOA)

1.4 The influence environmental factors have on ammonia oxidisers

1.4.1 Ammonia substrates

1.4.2 pH

1.4.3 Temperature

1.4.4 Oxygen availability

1.4.5 Salinity

1.5 Scope of thesis

1.5.1 Aims and objectives

1.5.2 Hypothesis

2) **Chapter 2: Co-occurrence of transcriptionally active ammonia oxidising bacteria and archaea in intertidal sediments**

2.1 Introduction

2.2 Aims

2.3 Materials and Methods
Ammonia Oxidation in Coastal Sediments

2.3.1 Site description and field sampling ................................................................. 32
2.3.2 Determination of physicochemical parameters .............................................. 33
2.3.3 Potential nitrification rates (PNR) .................................................................. 34
2.3.4 DNA and RNA co-extraction ......................................................................... 35
2.3.5 cDNA synthesis .............................................................................................. 36
2.3.6 Quantitative PCR (Q-PCR) and reverse transcription (RT)-Q-PCR ....... 36
2.3.7 Terminal restriction fragment length polymorphism (T-RFLP) of AOB amoA transcripts ........................................................................................................ 40
2.3.8 Cloning and sequence analysis of bacterial and archaeal amoA genes. 40
2.3.9 Nucleotide sequence accession numbers ...................................................... 42
2.3.10 Statistical analysis ......................................................................................... 42
2.4 Results .................................................................................................................. 43
2.4.1 Site Description ................................................................................................. 43
2.4.2 Spatial and temporal variation in potential nitrification rates (PNR) and AOA/AOB amoA gene abundances ................................................................. 48
2.4.3 Abundance and community structure of amoA transcripts in Rusheen bay sediments ........................................................................................................ 53
2.4.4 amoA gene phylogeny of total and active ammonia oxidisers within Rusheen bay sediments ................................................................. 55
2.5 Discussion .............................................................................................................. 60

3) Chapter 3: in situ total archaeal, bacterial and nitrifier community changes along the salinity gradient of Kinvarra bay, Co. Galway ...................... 69

3.1 Introduction ........................................................................................................... 69
3.2 Aims ....................................................................................................................... 70
3.3 Materials and Methods ...................................................................................... 71
3.3.1 Site description and field sampling ................................................................. 71
3.3.2 Determination of physicochemical parameters .............................................. 72
3.3.3 DNA and RNA co-extraction ......................................................................... 73
Ammonia Oxidation in Coastal Sediments

3.3.4 cDNA synthesis ................................................................. 73
3.3.5 Illumina MiSeq amplicon sequencing of 16S rRNA gene from archaea and bacteria ......................................................................................................................... 73
3.3.6 Potential nitrification rates (PNR) .............................................. 74
3.3.7 Quantitative PCR (Q-PCR) .......................................................... 75
3.3.8 Cloning and sequence analysis of bacterial and archaeal amoA genes in situ and active fractions ........................................................................................................ 77

3.4 Results: .......................................................................................... 78
3.4.1 Site Description ........................................................................... 78
3.4.2 16S rRNA Bacterial and Archaeal diversity ............................... 80
3.4.3 Nitrifier community dynamics in Kinvarra Bay Sediments .......... 94
3.4.4 Ammonia oxidiser community dynamics, PNR and functional gene abundances across a salinity gradient ................................................................. 99

3.5 Discussion ....................................................................................... 107

4) Chapter 4: Growth and activity response of ammonia oxidisers along an estuarine salinity gradient in sediment microcosms ........................................ 116

4.1 Introduction .................................................................................... 116
4.2 Aims .............................................................................................. 118
4.3 Materials and Methods ................................................................. 119
4.3.1 Site description and field sampling ........................................... 119
4.3.2 Sediment microcosm incubation ................................................. 119
4.3.3 Potential nitrification rates (PNR) ................................................. 121
4.3.4 DNA and RNA co-extraction ..................................................... 121
4.3.5 Quantitative PCR (Q-PCR) ......................................................... 121
4.3.6 Fractionation .............................................................................. 124
4.3.7 Denaturing gradient gel electrophoresis (DGGE) ...................... 125
4.3.8 Cloning and sequence analysis of bacterial and archaeal amoA genes in situ and active fractions ................................................................. 126

III
4.3.9 Statistical analysis.................................................................126

4.4 Results ....................................................................................127

Part I: Sediment Microcosm Experiment...........................................127

4.4.1 Sediment nitrification activity and growth of AOA and AOB
community along a natural salinity gradient.......................................127

4.4.2 Sediment nitrification activity and growth of AOA and AOB
community when salinity is varied to a lower or higher salinity than in situ
measurements..................................................................................131

Part II: Who are the active ammonia oxidisers? .................................138

4.4.3 Active nitrifying community within freshwater sediment site K1 as
revealed by SIP..........................................................138

4.5 Discussion...............................................................................145

4.5.1 Nitrifier activity at various salinities .................................145

4.5.2 Effect of Salinity on AOA .......................................................146

4.5.3 Autotrophic activity of AOB ..................................................148

4.5.4 Conclusions..................................................................150

5) Chapter 5: General Conclusions & Future Directions ............... 151

5.1 AOB versus AOA: Who is the dominant driver of ammonia oxidation in
intertidal sediments?.................................................................151

5.2 Temporal and spatial changes in AO dynamics within intertidal bays ....153

5.3 What is driving ammonia oxidation dynamics in intertidal sediments? ....156

5.4 The Importance of ecotypes in AO communities..........................159

5.5 Future work and conclusions....................................................165

6) References ...............................................................................168
Ammonia Oxidation in Coastal Sediments

DECLARATION

I, Aoife Duff, certify that this thesis is my own work and that I have not obtained a degree in this University or elsewhere on the basis of any of this work.
Ammonia Oxidation in Coastal Sediments

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This thesis focuses on the processes of nitrification in three coastal bays, specifically, aerobic ammonia oxidation which is the rate limiting step of nitrification and is mediated by ammonia oxidising bacteria and/or archaea (AOB/AOA). Rusheen, Clew and Kinvarra bay are located on the Atlantic coast of Ireland. The aim of this project is to combine *in situ* field data with experimental approaches, addressing temporal and spatial changes in ammonia oxidiser (AO) activity, abundance and diversity across environmental gradients in coastal bay ecosystems, in order to elucidate the key AO and environmental drivers within dynamic intertidal sediment ecosystems. Using this approach we report for the first time transcriptionally active AOB and AOA were present within Rusheen bay intertidal sediments, bringing us a step closer to confirming actively nitrifying co-occurring AOB and AOA in intertidal sediments. In chapter three we observe the huge diversity that can be found in coastal sediments. We show that using 16S rRNA transcripts enables us to identify low abundant or rare OTUs that were not picked up by sequencing DNA alone. Finally in chapter four through the use of experimental sediment microcosm incubations combined with SIP, we were able to demonstrate the growth and activity of *Nitrosoarchaeum* like AOAs in a low salinity habitat originating from intertidal coastal sediment. In addition throughout all three chapters we saw the impact environmental conditions such as nutrient concentrations, salinity, pH and temperature have on driving the distribution of communities within intertidal sediments.
Ammonia Oxidation in Coastal Sediments

ABBREVIATIONS

**Chemical formulas**

- $N_2$: Dinitrogen
- $NH_3$: Ammonia
- $NH_4^+$: Ammonium
- $NO_2^-$: Nitrite
- $NO_3^-$: Nitrate
- $N_2O$: Nitrous oxide
- $NO$: Nitric oxide
- $CO_2$: Carbon dioxide
- $HCO_3^-$: Bicarbonate
- $O_2$: Oxygen

**Microorganisms**

- AO: Ammonia oxidisers
- AOA: Ammonia oxidising archaea
- AOB: Ammonia oxidising bacteria
- NOB: Nitrite oxidising bacteria
- SOB: Sulfur oxidising bacteria

**Enzymes**

- NIR: Nitrite reductase
- NOR: Nitric oxide reductase
- NXR: Nitrite oxidoreductase
- AMO: Ammonia monoxygenase
- HAO: Hydroxylamine oxidoreductase
- RuBisCO: ribulose 1,5-bisphosphate carboxylase/oxygenase
- MCO: Multicopper oxidase
- HNO: Nitroxyl hydride
- NXOR: Nitroxyl oxidoreductase

**Pathways**

- DNRA: Dissimilatory nitrate reduction to ammonium
- HP/HB: Hydroxypropionate/hydroxybutyrate

**Physicochemical measurements**

- TOC: Total organic carbon
- DOC: Dissolved organic carbon
- TN: Total nitrogen
- DIN: Dissolved inorganic nitrogen
- TON: Total organic nitrogen
- LOI: Loss on ignition
Molecular terms

DNA  Deoxyribonucleic acid
RNA  Ribonucleic acid
mRNA  Messenger RNA
cDNA  Complementary DNA
PCR  Polymerase chain reaction
Q-PCR  Quantitative PCR
RT-Q-PCR  Reverse transcription-Q-PCR
DEPC  Diethylpyrocarbonate
dNTP  Deoxynucleoside triphosphate
DTT  Dithiothreitol
NTC  No-template controls
T-RFLP  Terminal restriction fragment length polymorphism
TRF  Terminal restriction fragment
OTU  Operational taxonomic unit

General abbreviations

EPS  Extracellular polymeric substances
ANOVA  Analysis of variance
ANCOVA  analysis of covariance
CCA  Canonical correspondence analysis
PCoA  Principle coordinate analysis
PNR  Potential nitrification rates
SIP  Stable Isotope Probing
ASW  Artificial sea water
SD  Standard deviation
SE  Standard error
PW  Porewater
LSD  Low salinity DNA
MSD  Medium salinity DNA
HSD  High salinity DNA
LSR  Low salinity RNA
MSR  Medium salinity RNA
HSR  High salinity RNA
UV  Ultraviolet
Ammonia Oxidation in Coastal Sediments

Figure 1.1: Schematic representation of the nitrogen cycle in coastal sediments................................................................. 1
Figure 1.2: Schematic representation of the nitrification step via (i) AOB and (ii) AOA along with nitrous oxide formation......................... 7
Figure 1.3: Phylogenetic 16S rRNA trees of AOB (a) taken directly from Purkhold et al., (2000) and AOA (b) taken directly from Pester et al., (2011). ................................................................. 10
Figure 1.4: Process urea undergoes after entering cell of an ammonia oxidiser................................................................. 18
Figure 2.1: Location of sampling sites................................................................. 33
Figure 2.2: Spatial and temporal variation in Potential Nitrification Rates (PNR)................................................................. 49
Figure 2.3: Spatial and temporal variation in AOA and AOB amoA gene abundances in Rusheen and Clew bay sediments..................... 51
Figure 2.4: Log_{10} AOA and AOB transcript numbers g^{-1} sediment for February 2014 in Rusheen bay............................................ 53
Figure 2.5: Canonical correspondence analysis illustrates the separation of AOB cDNA amoA gene TRFs based on physical/chemical parameters measured................................................................. 55
Figure 2.6: Neighbour-joining phylogenetic tree of AOB amoA genes at protein level (≥ 97% similarity)................................................................. 57
Figure 2.7: Neighbour-joining phylogenetic tree of AOA amoA genes at protein level (≥ 97% similarity)................................................................. 59
Figure 3.1: Location of sampling sites in Kinvarra bay................................................................. 72
Figure 3.2: Species richness and evenness of sites K1 to K3 DNA and RNA at 97% similarity................................................................. 82
Figure 3.3: Mean relative abundance of 16S rRNA Bacteria and Archaea at phylum level................................................................. 84
Figure 3.4: Diversity of archaeal 16S rRNA gene sequences at DNA and RNA level from Kinvarra bay sediments showing phylum, class and order level diversity................................................................. 86
Figure 3.5: Heatmap representing phylum level of 16S rRNA data of all sites at both DNA and RNA level

Figure 3.6: Venn diagram representing shared or unique OTUs

Figure 3.7: Venn diagram representing shared or unique OTUs

Figure 3.8: Principle coordinate analysis plots showing differences in the phylogenetic diversities in DNA samples across a natural salinity gradient

Figure 3.9: Principle coordinate analysis plots showing differences in the phylogenetic diversities in cDNA samples across a natural salinity gradient

Figure 3.10: Nitrifier operational taxonomic units

Figure 3.11: Principle coordinate analysis plots of bray curtis distances showing differences in the phylogenetic diversities samples across a natural salinity gradient

Figure 3.12: Potential nitrification rates (PNR) in situ in Kinvarra bay

Figure 3.13: AOB and AOA gene abundances from Kinvarra site K1, K2 and K3

Figure 3.14: Neighbour-joining phylogenetic tree of AOB amoA genes at protein level (≥ 97% similarity)

Figure 3.15: Neighbour-joining phylogenetic tree of AOA amoA genes at protein level (≥ 97% similarity)

Figure 3.16: CCA illustrates the separation of AO community amoA gene clones for AOA and AOB based on physicochemical parameters measured

Figure 4.1: Flow diagram of sediment microcosm experimental setup for i) natural salinity gradient and ii) forced salinity gradient

Figure 4.2: Schematic of amoA gene
Ammonia Oxidation in Coastal Sediments

Figure 4.3: Change in nitrification activity and amoA gene abundance over a natural salinity gradient in sediment microcosms over a period of 29 days................................................................. 129

Figure 4.4: Potential nitrification rates (PNR) in Kinvarra bay............. 131

Figure 4.5: Change in nitrification activity in freshwater site K1 and amoA gene abundance in sediment microcosms incubated with different salinities over a period of 29 days......................................................... 133

Figure 4.6: Change in nitrification activity in intermediate salinity site K2 and amoA gene abundance in sediment microcosms incubated with different salinities over a period of 29 days.............................. 135

Figure 4.7: Change in nitrification activity in full salinity site K3 and amoA gene abundance in sediment microcosms incubated with different salinities over a period of 29 days........................................... 137

Figure 4.8: K1 2 psu day 29 DNA fractions quantified from light to heavy buoyant density................................................................. 139

Figure 4.9 Dendrogram based on presence absence of DGGE bands....... 141

Figure 4.10: DGGE archaeal amoA gene banding pattern of K1C day 29 2 psu fractions 4 -7................................................................. 142

Figure 4.11: Neighbour-joining phylogenetic tree of AOA amoA genes at nucleotide level (≥ 97% similarity)............................................. 144

Figure 5.1: Neighbour-joining phylogenetic tree of AOB amoA genes at protein level (≥ 97% similarity).............................................. 161

Figure 5.2: Neighbour-joining phylogenetic tree of AOA amoA genes at protein level (≥ 97% similarity).............................................. 164
List of Tables

Table 1.1: An example of characterisation of nitrifying bacteria and archaea, their phylogeny and distribution in the environment........ 9
Table 1.2 Comparison of kinetic constants between Nitrosomonas europaea (AOB) and Nitrosopumilus maritimus (AOA) data taken from Martens-Habbena et al., (2009) unless specified otherwise........ 14
Table 1.3: Percentage of un-ionised ammonia (NH₃) at 18-22% salinity at different pH and temperatures values from Bower & Bidwell, 1978................................................................. 16
Table 2.1: Suite of primers used in this study................................. 38
Table 2.2: Q-PCR standard curve descriptors................................. 39
Table 2.3 (A): Rusheen bay Environmental Parameters..................... 44
Table 2.3 (B): Clew bay Environmental Parameters.......................... 46
Table 2.4: Significant Spearman Rank Correlations carried out on all time-points in Rusheen bay dataset (temporally)............................. 52
Table 2.5: Significant Spearman Rank Correlations carried out on February 2014 dataset (spatially)................................................. 54
Table 2.6: Number of clones sequenced per library, and identity at OTU level................................................................. 56
Table 3.1: Suite of primers used in this study................................. 76
Table 3.2: Q-PCR standard curve descriptors................................. 77
Table 3.3: Physicochemical properties of sediments used in this study 79
Table 3.4: Significant Pearson Correlations carried out on Kinvarra April 2015 dataset ................................................................. 81
Table 4.1: Suite of primers used in this study .................................. 122
Table 4.2: Q-PCR standard curve descriptors................................. 123
Table 4.3: Number of clones sequenced per fraction, and identity at OTU level................................................................. 143
1) CHAPTER 1: GENERAL INTRODUCTION

1.1 NITROGEN CYCLE

The Nitrogen Cycle is arguably one of the most important biogeochemical cycles on the planet. It is directly involved in, and is often the limiting factor of, primary productivity due to the element being found in all living things i.e. nitrogen is found in DNA, amino acids and subsequently proteins which are vital for cellular function. However, not all nitrogen forms are available to living organisms (Herbert, 1999). The atmosphere is composed of 80% dinitrogen gas (N₂) that is unavailable to most living organisms due to the strong triple bond between the N atoms (Bianchi, 2007). Therefore dinitrogen must be “fixed” by a group of microorganisms called the nitrogen fixing bacteria (NFB) into a more bio-available form ammonia/ammonium (NH₃/NH₄⁺; Pathway 1. Fig. 1.1). From this point forward nitrogen can be cycled through its various forms through the process of oxidation and reduction, this cycle is one of the most complex biogeochemical cycles as it is stable across 8 oxidation states (+5 to -3). Numerous different types of microorganisms are responsible for mediating these compounds (Bianchi, 2007).

Figure 1.1: Schematic representation of the nitrogen cycle in coastal sediments. Various steps in the nitrogen cycle are numbered 1 – 5. Dashed line
indicates separation of oxic and anoxic phase in coastal sediments. Dashed arrows indicate gaseous diffusion from sediments into the atmosphere.

Ammonia/ammonium is converted to nitrate (NO$_3^-$) via nitrification by a one or two-step process. The one-step process of nitrification, recently described by Daims et al., (2015); van Kessel et al., (2015) is where *Nitrospira*, a chemolithoautotrophic microorganism, can complete nitrification of ammonia to nitrate by itself. In two-step nitrification, the first step is mediated by ammonia oxidising bacteria and/or archaea (AOB and AOA). In the second step, nitrite (NO$_2^-$) is oxidised to nitrate by nitrite oxidising bacteria (NOB). Nitrate is also a bioavailable nutrient that plants can assimilate (Herbert, 1999). Nitrate does not adhere to soil or sediment particles as well as ammonia/ammonium (NH$_3$/NH$_4^+$), and so nitrate can leach further down into the sediment to the anoxic layer. Nitrate is reduced via the denitrification, dissimilatory nitrate reduction to ammonium (DNRA) and anammox pathways all mediated by microorganisms under anaerobic conditions (pathway 3, 4 and 5; Fig 1.1). Denitrification is the reduction of inorganic N such as nitrate and nitrite to dinitrogen gas mediated by facultative anaerobic bacteria. Intermediates such as nitric oxide (NO) and nitrous oxide (N$_2$O) are produced as a result of denitrification before the final product N$_2$ (Dong et al., 2009; Smith et al., 2015). Nitrite can also be reduced to dinitrogen by anoxic autotrophic bacteria mainly the Planctomycetes via anammox where NO$_2^-$ and NH$_4^+$ are simultaneously converted to N$_2$ (Dong et al., 2009; Strous et al., 1999; Smith et al., 2015). Dinitrogen is either lost to the atmosphere or it can be assimilated back to ammonia and kept within the biologically available pool of nitrogen. Finally, NO$_3^-$ and NO$_2^-$ can also be converted to NH$_4^+$ via DNRA, one of the least understood nitrogen processes. DNRA is found in sediments with high organic content, and contributes to retaining nitrogen in the biologically available pool (Fig. 1.1; Dong et al., 2009; Smith et al., 2015). Nitrate is the main product of nitrification where ammonia oxidation is often the rate limiting step. Nitrate and nitrite feed into three anaerobic pathways (Fig 1.1), therefore, factors that stimulate nitrification can subsequently affect the three major anaerobic pathways later on in the cycle. An
example of this is the tight coupling found between nitrification and denitrification (Jenkins and Kemp, 1984).

Furthermore, the nitrogen cycle is known to be closely linked to the carbon cycle since the Redfield ratio in 1958 (Redfield, 1958). Key pathways in the nitrogen cycle such as nitrogen fixation and nitrification are mediated by phytoplankton and autotrophs which directly fix CO$_2$. In addition, heterotrophic microorganisms found in nitrate reduction pathways and anammox can be indirectly influenced by changes in CO$_2$ levels (Hutchins et al., 2009). Nitrogen can be a limiting factor for the overall productivity of the ocean as it is often the limiting factor of primary productivity (Redfield, 1958). The nitrogen cycle can help reduce carbon dioxide in the atmosphere by microorganisms fixing carbon dioxide during nitrogen fixation and nitrification (Schindler and Bayley, 1993). Ammonium is often found at low levels in the ocean; however it supplies plants and algae with nitrogen and enables photoautotrophic carbon fixation. It is assimilated into organic matter. If the ocean is deficient in nitrogen, productivity is minimised and less carbon is bound in biomass (Gruber, 2004). On the other hand, high levels of biological activity in the water results in more biomass and the carbon from dead animals or plants that sink to the bottom of the ocean and slowly decay will be degraded to CO$_2$ again. Nevertheless, during primary productivity, CO$_2$ is withdrawn from the atmosphere for very long periods of time (Schindler & Bayley 1993).

1.1.1 IMPORTANCE OF THE NITROGEN CYCLE IN COASTAL ECOSYSTEMS

Nitrogen is a key element in primary productivity (Howarth, 1988). While nitrate-limited environments suffer from low primary productivity (Vitousek and Howarth, 1991), excess nitrate in the environment is problematic as it leads to eutrophication, hypoxia (NRC, 2000) and pollution of groundwater. Groundwater pollution can lead to health problems like methemeglobin in infants and carcinogenic compounds such as nitrosamines. Another problematic compound produced during nitrification and denitrification is nitrous oxide a
greenhouse gas which has a global warming potential that is 298 times higher than CO₂ (van Groenigen et al., 2011).

Coastal ecosystems are particularly vulnerable to excess nitrogen loads as they are transition regions between terrestrial and marine environments, where land, streams, groundwater and rivers meet the sea. These waters are increasingly laden with nitrogen originating from terrestrial and anthropogenic sources. For example, 10.1% of transitional waters in Ireland are categorised as eutrophic in the latest publication from the EPA 2007-2009 (McGarrigle et al., 2010). Anthropogenic nitrogen originating from agricultural and urban runoff can often lead to altered or loss of biodiversity, shifts in trophic dynamics and general habitat destruction of coastal ecosystems (Bricker et al., 2008). However, microbial processes within coastal ecosystems mediate the nutrient load entering coastal waters via the various steps of the nitrogen cycle, including nitrification, denitrification, DNRA and anammox (Fig 1.1; Smith et al., 2014, 2015; Trimmer and Nicholls, 2009). In fact, it is estimated that more than 50% of anthropogenic dissolved inorganic nitrogen (DIN) inputs to coastal ecosystems are removed by microbial transformations of the nitrogen cycle (Lohse et al., 1993). Coastal ecosystems must be considered as a valuable ecosystem from a human’s perspective. The areas are used for recreation, food, non-renewable and renewable resources such as power transportation and waste disposal. A recent paper by Costanza et al., (2014) values ecosystems based on benefits they provide to people. Coastal ecosystems were the highest valued ecosystem, with wetlands a close second. Coastal zones cover only 3102 ha of the earth’s surface. Making them all the more important to protect.

1.1.2 HUMAN IMPACT

This is the era of Anthropocene (Crutzen, 2006); ‘Only microbes and human activities control the amount of biologically available N in the biosphere’ as stated by Galloway & Cowling (2002). Unfortunately human activities have significantly impacted the nitrogen cycle transformations. Human production of nitrogen fertilisers totals 80 Tg of nitrogen per year. The combustion of fossil
fuels which releases nitrogen from long term storage releases 20 Tg per year into the atmosphere. Vegetation that host nitrogen fixing bacteria such as peas, soybeans and other crops contributes approximately 40 Tg per year. Furthermore, the release of nitrogen from long term storage in organic matter and soil is sped up by the activity of humans (Galloway 1998). Estimates have concluded that about 140 Tg of new nitrogen is naturally fixed in ecosystems, while another 140 Tg of new nitrogen is being released due to human activity (Galloway & Cowling 2002). This may impact the rates and locations of nitrification, denitrification and nitrogen fixation through the influence of increased nitrogen on microbial transformations in the nitrogen cycle (Chindler et al., 1997; Galloway and Cowling, 2002). Human activity is also impacting the nitrogen cycle by increasing CO$_2$ in the atmosphere through the burning of fossil fuels and agriculture. This contributes to climate change and ocean acidification and will impact the nitrogen cycle directly because of its tight links with the carbon cycle (section 1.1.1).

Other consequences of human perturbations in the nitrogen cycle include the release of nitrous oxide (N$_2$O), a greenhouse gas contributing to global warming as well as depleting the ozone layer (Ravishankara et al. 2009). Nitric oxide (NO) plays a role in creating brown smog. It can also react with other oxides to produce nitric acid which is a large part of acid rain (Vitousek et al. 1997). Excess nitrogen impacts ecosystem functioning caused by nitrogen saturation in soils (Aber et al. 1991) and aquatic systems e.g. eutrophication (Ryther & Dunstan 1971). In fact in marine systems such as coastal ecosystems nitrogen inputs are a primary cause of pollution.

These problems are now being recognised by policy makers. The EU have set out to combat these problems by implementing a number of different directives such as the nitrates directive (91/676/EEC), national emission ceilings directive (2001/81/EC), integrated pollution, prevention and control directive (2008/1/EC), ground water directive (2006/118/EC) and marine strategy framework directive (2008/56/EC). These have all been implemented to reduce nitrogen pollution and slow and reverse some of the negative impacts
anthropogenic activities have already caused. It is imperative to protect and conserve our environment to prevent long term decline in coastal fisheries and loss of biodiversity and ecosystem functioning.

1.2 NITRIFICATION

Nitrification has a central role in the nitrogen cycle, it provides an important link between decomposition of organic matter and the removal of anthropogenic nitrogen that has found its way into coastal waters. It is tightly linked with denitrification removing biologically available nitrogen which is helpful in combatting eutrophication and pollution. In addition, it is important for nitrogen mineralisation in the marine environment. It provides nitrogen for primary productivity as ammonia and nitrate are more readily available forms for primary producers.

Nitrification is made up of two steps: ammonia oxidation and nitrite oxidation. The first step is the rate limiting step of nitrification where ammonia is oxidised to nitrite under aerobic conditions. This step can be oxidised by two groups of microorganisms AOB and AOA who are phylogenetically distinct microorganisms but carry out the same function. However, the AOB pathway has been proposed for quite some time while the AOA pathway has a few different possible scenarios with the most recent depicted in Fig. 1.2 (Kozlowski et al., 2016; Suzuki et al., 1974). The ammonia oxidation process is discussed in more detail in section 1.3.1 and 1.3.2. Both AOA and AOB oxidise ammonia to nitrite but contribute to nitrous oxide pollution in different ways. AOB complete the ammonia oxidation step and any excess nitrite is converted to NO using nitrite reductase (NIR). NO is further reduced to N₂O using nitric oxide reductase (NOR). In contrast AOA does not have the NOR enzyme, thus NO accumulates and feeds back into the ammonia oxidation step as NO is a necessary intermediate in the AOA pathway. However, the exact mechanism for this requires further investigation. Finally, excess NO diffuses into the water or atmosphere and spontaneously hybridises with H₂O to form N₂O (Fig 1.2; Kozlowski et al., 2016).
AO also live in close association with nitrite oxidising bacteria (NOB) and form a symbiotic relationship with them where AO supply nitrite to NOB. Subsequently, the NOB detoxify \( \text{NO}_2^- \) from the environment using nitrite oxidoreductase (NXR) to produce \( \text{NO}_3^- \), a less toxic form used by primary producers. Both steps in nitrification occur simultaneously preventing a build up of \( \text{NO}_2^- \) in the environment. In addition, NOB are also helpful in providing alternative ammonia sources for AOA and AOB that they can not provide for themselves i.e. some AO lack urease and cyanase enzymes. *Nitrospira* have the ability to convert cyanate and urea to \( \text{NH}_4^+ \) using a cyanase and urease enzyme. This provides \( \text{NH}_3 \) for AO and contributes to reciprocal feeding as NOB supply \( \text{NH}_3 \) and AO supply \( \text{NO}_2^- \) (Koch et al., 2015; Palatinszky et al., 2015).

Figure 1.2: Schematic representation of the nitrification step via (i) AOB and (ii) AOA along with nitrous oxide formation. Nitrification composed of 1: ammonia oxidation and 2: nitrite oxidation. Dashed arrows indicate gaseous diffusion from sediments into the atmosphere. Uppercase letters above arrows refer to enzymes. AMO – ammonia monooxygenase; HAO- hydroxylamine oxidoreductase; Cu- novel enzyme – reduces NO and \( \text{NH}_2\text{OH} \); NIR – nitrite reductase; NOR – nitric oxide reductase; NXR - nitrite oxidoreductase.
Chapter 1

1.3 NITRIFIERS

Nitrifying bacteria are made up of two groups of bacteria, ammonia oxidisers (AO) and nitrite oxidising bacteria (NOB). Nitrifying bacteria belong in the *proteobacteria* phylum (Fig 1.3 (a); Table 1.1). *Proteobacteria* are the largest and most metabolically diverse of all bacteria. They are all gram negative and have a wide diversity of energy-generating mechanisms and physiologies. For example there are chemolithotrophs, chemoorganotrophic and phototrophic species and also anaerobic or facultative aerobic species (Madigan et al., 1997). Phylogenetically, *proteobacteria* are divided into different classes; namely, alpha, beta, gamma, delta and epsilon (Head et al., 1993). Nitrifying bacteria are found in all sub classes except epsilon (Fig. 1.3 (a)).
### Table 1.1: An example of characterisation of nitrifying bacteria and archaea, their phylogeny and distribution in the environment.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Phyla</th>
<th>Genera</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidise ammonia</td>
<td><em>Beta</em>-proteobacteria</td>
<td><em>Nitrosomonas</em></td>
<td>Soil, Sewage, Freshwater, Marine</td>
</tr>
<tr>
<td>Oxidise ammonia</td>
<td><em>Gamma</em>-proteobacteria</td>
<td><em>Nitrosococcus</em></td>
<td>Freshwater, Marine</td>
</tr>
<tr>
<td>Oxidise ammonia</td>
<td><em>Thaumarchaeae</em> - group III</td>
<td><em>Nitrosocaldus</em></td>
<td>Hot water extremophile</td>
</tr>
<tr>
<td>Oxidise ammonia</td>
<td><em>Thaumarchaeae</em> - group I.Ib</td>
<td><em>Nitrososphaera</em></td>
<td>Soil and other environments</td>
</tr>
<tr>
<td>Oxidise ammonia</td>
<td><em>Thaumarchaeae</em> - SAGMGC-1</td>
<td><em>Nitrosotalea</em></td>
<td>Ubiquitous cluster</td>
</tr>
<tr>
<td>Oxidise ammonia</td>
<td><em>Thaumarchaeae</em> - group I.Ia</td>
<td><em>Nitrosopumilus</em></td>
<td>Marine and other environments</td>
</tr>
<tr>
<td>Oxidise nitrite</td>
<td><em>Alpha</em>-proteobacteria</td>
<td><em>Nitrobacter</em></td>
<td>Soil, Freshwater, Marine</td>
</tr>
<tr>
<td>Oxidise nitrite</td>
<td><em>Gamma</em>-proteobacteria</td>
<td><em>Nitrococcus</em></td>
<td>Marine</td>
</tr>
<tr>
<td>Oxidise nitrite</td>
<td><em>Nitrospinae</em></td>
<td><em>Nitrospina</em></td>
<td>Marine</td>
</tr>
<tr>
<td>Oxidise nitrite</td>
<td><em>Nitrospira</em> group</td>
<td><em>Nitrospira</em></td>
<td>Marine, Soil</td>
</tr>
</tbody>
</table>

The *Nitrospira* and *Nitrospinae* form their own phyla of bacteria because they are only related to other nitrifying bacteria in a metabolic sense (Lück et al., 2013; Off et al., 2010).

Ammonia oxidising archaea (AOA) belong to the *Thaumarchaeota* phylum which was first identified from group I.Ia archaeon *Nitrosopumilus*.
Chapter 1

*maritimus* (Fig. 1.3 (b); Venter et al., 2004; Brochier Armanet et al., 2008; Könneke et al., 2005). Not all *Thaumarchaea* are known to perform ammonia oxidation, and several of the groups have no isolated representative (Brochier-Armanet et al., 2012). Only four different groups have isolates with proven AO (Table 1.1, Fig 1.3 (b); Pester et al., 2011). There is a considerable amount to learn about how to classify them as currently there are only a handful of isolates.

Figure 1.3: Phylogenetic 16S *rRNA* trees of AOB (a) taken directly from Purkhold et al., (2000) and AOA (b) taken directly from Pester et al., (2011).

Figure was removed due to copyright
Chapter 1

The AOB tree is a neighbour-joining tree with a 50% conversion filter of both AOB and non-AOB reference sequences. The AOA tree is a majority consensus tree of both 16S rRNA and amoA gene using maximum likelihood, maximum parsimony and distance methods.

This thesis will focus on ammonia oxidation which is often considered the rate limiting step of nitrification where ammonia is oxidised to nitrite (Fig. 1.2). Nitrite is subsequently oxidised to nitrate by the NOB. AOB belong to the *Proteobacteria* phylum (Table 1.1, Fig. 1.3 (a)) and the recently discovered AOA belonging to the *Thaumarchaeota* phylum (Venter et al., 2004; Brochier Armanet et al., 2008; Table 1.1, Fig. 1.3 (b)).

### 1.3.1 AMMONIA OXIDISING BACTERIA (AOB)

AOB have two enzymes that are tightly coupled to carry out the process of oxidising ammonia to nitrite (Suzuki et al., 1974). Ammonia monoxygenase (AMO) binds to the substrate NH₃ in the presence of O₂ and converts it to hydroxylamine (NH₂OH). Hydroxylamine is then simultaneously oxidised to nitrite (NO₂⁻) through the hydroxylamine oxidoreductase enzyme (HAO; Fig 1.2). The intermediate product hydroxylamine is rarely detected due to the tight coupling of AMO and HAO enzymes. This is one of the reasons why AMO has not been purified yet. The genes that encode the AMO enzyme, *amoC* *amoA* and *amoB*, are found on the same operon. *amoA* is frequently used as a functional gene marker to detect, quantify and identify AOA and AOB in the environment (Francis et al., 2005; Rotthauwe et al., 1997). *amoA* is present as a single copy in AOA (Berg et al., 2015; Jung et al., 2011; Walker et al., 2010) and 1-3 copies in AOB depending on the phylotype (Norton et al., 2002).

AOB are obligate aerobes and chemolitho-autotrophs (Bodelier et al., 1996), where most of the energy they produce from oxidising ammonia goes towards fixing carbon dioxide. According to Baas-Becking and Parks, (1927) as much as 35 molecules of ammonia must be oxidised in order to generate enough energy to fix one carbon dioxide molecule. The pathway that is required to do this is called the Calvin-Bassam-Benson cycle and is catalysed by ribulose 1, 5-
bisphosphate carboxylase/oxygenase (RuBisCO) enzyme. Arp et al., (2007) suggested, based on genome analysis, that because AOB are so dependent on O₂ they may have evolved by losing the ability to process alternative energy resources, including organics, due to adaptation to environmental conditions. They also state AOB can be grouped into four major ecotypes based on genetic data consisting of freshwater, soil, wastewater and marine. AOB have evolved specifically to those environmental conditions e.g. environments where you have high ammonia and high CO₂ concentrations such as wastewater and coastal sediments. Factors such as salinity, pH and substrate concentration are all environmental drivers that can potentially differentiate AOB into different ecotypes. This will be discussed further in section 1.4.

1.3.2 AMMONIA OXIDISING ARCHAEA (AOA)

AOA have a similar metabolism to AOB although it is not exactly the same. AOA also contain similar genes to amoA, B and C which encode a homolog of AMO. However instead of containing an iron electron transfer system it relies on a copper containing electron transfer system. Furthermore, AOA do not encode a similar enzyme to HAO in bacteria. This means they either have a different enzyme that allows them to oxidise hydroxylamine to nitrite by utilising one of the periplasmic multi copper oxidase (MCOs) i.e. acting like a copper alternative to HAO (Walker et al., 2010), or they could operate an entirely different pathway whereby hydroxylamine is not an intermediate product. Walker et al., 2010 proposed a pathway where nitroxyl hydride (HNO) would act as the intermediate product instead and the second enzyme involved would be a nitroxyl oxidoreductase (NXOR) converting HNO and H₂O to 2 protons and nitrite. Recently Kozlowski et al., (2016) has shown NO to be a necessary intermediate in AOA ammonia oxidation, strengthening the idea that there is a novel copper containing enzyme that has the ability to oxidise hydroxylamine and nitric oxide to nitrite, the proposed pathway is depicted in Fig 1.2.
AOA are also chemolitho-autotrophs and oxidise 10 NH\textsubscript{3} molecules for every 1 CO\textsubscript{2} molecule fixed (Berg et al., 2015). However, there is a possibility that some AOA are mixotrophic and switch from autotrophic to heterotrophic metabolism (Agogué et al., 2008; Ingalls et al., 2006; Ouverney and Fuhrman, 2000; Qin et al., 2014). Important differences are observed between AOB and AOA in how they fix carbon. AOA use the hydroxypropionate/hydroxybutyrate (HP/HB) cycle to fix carbon. *Thaumarchaeota* have evolved from *Crenarchaeota* to carry out this cycle more efficiently and save energy. Könneke et al., (2014) show how small modifications in this cycle have made the *Thaumarchaeal* HP/HB cycle the most efficient at fixing carbon compared to both the crenarchaeal HP/HB cycle and the Calvin Benson cycle used by AOBs. This provides an explanation on how AOA can survive in more oligotrophic environments such as the open ocean. In addition the difference in cycles for AOA and AOB also suggests the use of different carbon species. AOA fix bicarbonate (HCO\textsubscript{3}\textsuperscript{-}) whereas AOB fix CO\textsubscript{2} (Hatzenpichler, 2012). The HP/HB cycle also gives AOA the ability to co-assimilate various organic compounds which may be an explanation as to why mixotrophy has been observed in *Thaumarchaeota* (Hatzenpichler, 2012).

Table 1.2 shows an example of how AOA and AOB kinetics vary. *N. maritimus* has a much higher substrate affinity than *N. europaea* however it also has a much smaller cell size (Table 1.2) and seems to be tailored to oligotrophic environments (Martens-Habbena et al., 2009). Nevertheless, *N. maritimus* and *N. europaea* like organisms have also been detected in coastal sediments (Fan et al., 2015) which have much higher nutrient inputs than the oligotrophic ocean waters, this shows that perhaps AOA and AOB have the ability to occupy separate niches within an ecosystem.
Table 1.2 Comparison of kinetic constants between *Nitrosomonas europaea* (AOB) and *Nitrosopumilus maritimus* (AOA) data taken from Martens-Habbena et al., (2009) unless specified otherwise

<table>
<thead>
<tr>
<th></th>
<th><em>Nitrosomonas europaea</em></th>
<th><em>Nitrosopumilus maritimus strain SCM1</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum specific cell activity ($V_{max}$)</td>
<td>122.07 μM N mg protein$^{-1}$ h$^{-1}$</td>
<td>23.77 μM N mg protein$^{-1}$ h$^{-1}$</td>
</tr>
<tr>
<td>Saturation constant for activity ($K_m$)</td>
<td>553 μM NH$_4^+$</td>
<td>0.132 μM NH$_4^+$</td>
</tr>
<tr>
<td>Maximum specific growth rate ($\mu_{max}$)</td>
<td>Not determined</td>
<td>0.027 h$^{-1}$</td>
</tr>
<tr>
<td>Maximum specific biomass activity</td>
<td>30–80 nmol NH$_3$ g protein$^{-1}$ h$^{-1}$</td>
<td>51.9 nmol NH$_3$ g protein$^{-1}$ h$^{-1}$</td>
</tr>
<tr>
<td>Cell size</td>
<td>120 fg protein cell$^{-1}$</td>
<td>10.2 fg protein cell$^{-1}$</td>
</tr>
<tr>
<td>Inhibition constant ($K_i$)</td>
<td>400 mM NH$_4^+$ (Koops et al., 1991)</td>
<td>2 mM NH$_4^+$</td>
</tr>
</tbody>
</table>

These differences in metabolic ability between AOA and AOB may provide an explanation for co-occurrence of AOA and AOB in the same environments.

1.4 ENVIRONMENTAL FACTORS INFLUENCING AMMONIA OXIDISERS THROUGH CONTROLLING AMMONIA AVAILABILITY AND NICHE DIFFERENTIATION

In addition to the metabolic differences between AOA and AOB described above, there are also environmental factors to consider that regulate and control niche differentiation between AOA and AOB. AOA and AOB have a specialised metabolism, one that relies on substrate concentration but also can be sensitive to environmental changes whether biological, physical or chemical.

As a result of ammonia oxidiser sensitivity to both known and potentially unknown variables in the environment, ecological questions are best answered with *in situ* based experiments or field studies combined with hypothesis-testing laboratory experiments to link community structure with function and activity of
AOA and AOB. This allows us to get one step closer to determining their activity under certain conditions. Carefully controlled experiments allow us to gain a better understanding of how ammonia oxidisers are affected by at least a subset of the environmental stimuli.

### 1.4.1 AMMONIA SUBSTRATES

The balance of NH$_4^+$ to NH$_3$ is crucial as high NH$_3$ concentrations can be toxic while low NH$_3$ concentrations can be substrate limiting to AOA and AOB (Bollmann et al., 2005; Koops et al., 2006; Martens-Habbena et al., 2009; Nakagawa and Stahl, 2013). NH$_3$ rather than NH$_4^+$ is known to be the substrate ammonia oxidisers utilise (Suzuki et al., 1974). There are a number of parameters that control NH$_3$ availability such as pH, temperature and salinity; that can vary substantially in the environment (Christman et al., 2011; Puthiya Veettil et al., 2015). These parameters can interact to control ammonia availability. For example, using the formula below (equation 1) the combined effects of temperature and salinity on free ammonia can be determined.

\[
F = \frac{1}{1 + 10^{pK_s - pH - S}}
\]

\[
pK_s = (2792.92/T_C + 273.16) + 0.09018
\]  

**equation (1)**

Where $F$ = free ammonia fraction of NH$_3$; $T_C$ = temperature in °C; $S$ = salinity factor (Bower and Bidwell, 1978; Groeneweg et al., 1994). An example of the effect these interacting factors have on the availability of ammonia is shown in Table 1.3.
Table 1.3: Percentage of un-ionised ammonia (NH$_3$) at 18-22% salinity at different pH and temperatures values from Bower & Bidwell, (1978).

Table was removed due to copyright

From Table 1.3 it is clear that a simple variation in pH, temperature and salinity can change the percentage of un-ionised ammonia substantially, the red colour indicates ammonia availability decreasing as pH and temperature goes down, while the orange colour indicates ammonia availability increasing as pH and temperature goes up.

From the current literature, there is certainly mixed data on how temperature and salinity influence ammonia oxidiser communities as seen in section 1.4.3 and 1.4.5; yet, both parameters contribute to the ammonia availability. pH is the strongest parameter in driving change in ammonia oxidising communities (section 1.4.2). In low pH environments availability of NH$_3$ is decreased to negligible levels as shown in Table 1.3. An alternative ammonia containing substrate, urea, can be utilised by some AO. Burton and Prosser, 2001 explained the importance of urea hydrolysis at low pH by comparing the growth of known monocultures (AOB capable of growing at low pH) and co-cultures (AOB and acidophilic NOB). From this they showed that
when urea was used as a substrate, growth of ammonia oxidisers was possible, as urea can pass through the cell membrane and be hydrolysed by the microorganism. The NH$_3$ produced from the reaction stays within the cell to be further oxidised to nitrite and the excess NH$_3$ is released into the medium and ionised (NH$_4^+$) due to the low pH in the surrounding environment. In contrast, there was never any growth in the medium containing ammonium sulphate below pH 7. Ammonia oxidisers would require active transport to allow NH$_4^+$ across the cell membrane at low pH which would require a lot of energy (Chain et al., 2003). This makes urea a potential substrate for AO that carry the urease gene as it can diffuse across the cell membrane (Mobley and Hausinger, 1989; Fig. 1.4).

Lu et al., (2012) proved that AOA were able to grow and utilise ammonia with urea as a substrate. They did not know whether AOA were directly or indirectly utilising urea as a substrate as they were unsure whether AOA could hydrolyse urea. In this study, they showed that AOB were not found in soils with low pH at 3.75 and 5.4 while archaeal amoA and crenarchaeal 16S rRNA genes increased in proportion with nitrification activity. These findings further support the discovery of Alonso-Saez et al., (2012) who showed firstly, that the majority of Thaumarchaea are ammonia oxidisers and secondly they have a urease gene giving them the potential to hydrolyse urea. Therefore, it is important to measure the concentration of urea in the environment too, as this may be an alternative substrate of some ammonia oxidisers. Furthermore, the hydrolysis of urea not only supplies NH$_3$ to the ammonia oxidiser but also CO$_2$ (Fig. 1.4).
1.4.2 PH

pH is an environmental parameter that directly affects ammonia availability because of the impact pH has on the NH$_4^+$: NH$_3$ ratio; the higher the pH the more NH$_3$ is available for ammonia oxidation as mentioned above (Suzuki et al., 1974). A primary concern for the coastal and open ocean ecosystems into the future is ocean acidification. The oceans pH is currently decreasing worldwide due to carbon dioxide dissolution (Caldeira and Wickett, 2005). This reduction will directly affect the ammonia available for ammonia oxidisers which will in turn reduce the amount of nitrate produced and consequently reduce primary production. This affect is one of many impacts ocean acidification may have on AO.

FIELD STUDIES

Many studies have tested the impact of low pH on nitrification in soils (Lu et al., 2012; Wang et al., 2015a; Xia et al., 2011; Zhang et al., 2012). Fewer studies have been carried out on the effect pH has in marine environments (Gao et al.,

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Figure 1.4: Process urea undergoes after entering cell of an ammonia oxidiser.
Chapter 1

2012; Isnansetyo et al., 2011; Jones and Hood, 1980; Kitidis et al., 2011; Laverock et al., 2014; Zheng et al., 2014). Studies on ocean acidification in the water column have shown that nitrification rates drop dramatically with a decrease in pH (6.5) (Huesemann et al., 2002; Kitidis et al., 2011). In contrast, Kitidis et al., (2011) also looked at the effect of pH on AO in surface sediments and found a decrease in pH had no effect; while Allison and Prosser, 1993 saw a decrease in activity by 50% when pH was reduced to 5 in soils. However, Kitidis et al., (2011) did not measure the pH of the actual sediments, only the overlying water which signifies the pH of the sediments may still have been high. There is evidence in the literature that sediments are not affected by pH of overlying water unless it is exposed for a long time (20 weeks) due to pH buffering in the pore water (Widdicombe et al., 2009). Other studies have suggested that in low pH environments there may be areas of microenvironments and/or biofilms with higher pH protecting AOA and AOB microorganisms and allowing them to nitrify (Allison and Prosser, 1993). However, this hypothesis has been challenged through recreating a microenvironment by growing a nitrifier biofilm on chalk particles versus glass beads in a low pH medium (Gieseke et al., 2006). Chalk dissolution in an acidic medium provides an increase in pH at the chalk surface. However, when measuring pH and O$_2$ concentrations within the biofilms on both surfaces, they showed that there was no evidence for high pH microenvironments within the biofilm and furthermore there were no significant differences in biofilm performance on either surface. Further studies on the extracellular polymeric substances (EPS) found in biofilms are required to understand how AO and other microorganisms in the biofilm have adapted to these acidic conditions. Additionally, sediment environments are complex and are impacted by macrofauna too. Laverock et al., (2014) showed *Upogebia deltaura* bioturbation significantly increases ammonia oxidation in sediments at pH 8, but when the pH decreased *U. deltaura* behaved differently and in some cases died, consequently reducing ammonia oxidation significantly in sediments.
Lehtovirta-Morley et al., (2011) enriched and cultivated *Nitrosotalea devanaterra* the first obligate acidophile ammonia oxidiser. This provides evidence of niche specialisation as this particular AOA has adapted solely to low pH environments. From isolating two strains of *N. devanaterra*, Lehtovirta-Morley et al., 2014 was able to test the physiology and activity of these microorganisms over a range of conditions such as temperature and pH. One of the strains had a broader range in pH (4 - 6.1 vs. 4.2 – 5.6) and each strain grew at a different optimal temperature (35 and 25°C, respectively). This study showed how two closely-related strains had different physiological responses to pH.

1.4.3 TEMPERATURE

At temperate latitudes, seasonal and daily temperature fluctuations influence ecosystems. Does temperature have a significant impact on ammonia oxidisers?

FIELD STUDIES

In marine coastal sediments, Zheng et al., (2014) found negative correlations between AOB abundances and temperature. While in coastal ocean water samples Smith et al., (2014a), reported negative correlations between the abundance of two AOA ecotype and temperature. In contrast, there was no significant difference in AOA and AOB community compositions between winter and summer in coastal sediments where temperatures varied from 2.4-3.6°C in winter and 29.1 to 33.5°C in summer with the exception of one out of seven sites, where AOA was significantly different in winter and summer (Zheng et al., 2014). Following on from this, another study on coastal water samples showed AOA *amoA* gene and transcript abundances peaked in August consistently over two years. There was no strong seasonal difference in AOB; however, transcripts were lower in winter (Hollibaugh et al., 2014). Similarly AOA and AOB *amoA* gene abundances were found to peak in August in North
Sea coastal sediments. Here AOA amoA transcripts were not present in the sediments during winter whereas AOB transcripts were lower than August values (Lipsewers et al., 2014). A review of the current literature on temporal variation does not reveal a clear pattern that defines the impact of temperature on AO. Further, disentangling temperature from other factors, such as NH$_4^+$, salinity and pH is required to gain a better understanding (Equation 1; Table 1.3). As we have seen in section 1.4.1, simple variation in pH, temperature and salinity can change the percentage of un-ionised ammonia substantially. Most studies measure concentrations of unionised ammonia rather than ionised ammonia (the ammonia oxidiser substrate); therefore, we cannot say that temperature is not having a significant effect on communities, as we don’t know whether the communities are limited by un-ionised ammonia. The effect of temperature is therefore difficult to untangle from the interacting effects of pH and salinity, in particular in ecosystems where each parameter may vary, such as estuaries. In some instances, there are significant differences seen between high and low temperatures (Urakawa et al., 2014). In these cases temperature has more than likely had a significant impact on the AO communities and the change was not limited by NH$_3$.

**EXPERIMENTAL APPROACH**

Urakawa et al., (2014) developed a model to elucidate the average single cell ammonia oxidation rate of AOA and AOB by using ammonia oxidation activity measured and single cell protein content through lipid analysis of the cell. The AOA and AOB contribution in the water column along the Hood Canal was determined. They found the contribution of AOB was minor compared to AOA the majority of the time, except at the shallow site (15 m depth) where the AOB single cell ammonia oxidation rate dropped significantly when temperature changed from 11.5 °C (October) to 8.7 °C (December). In contrast the AOA single cell ammonia rates stayed consistent. This shows that AOB responded to temperature more than AOA and became a more competitive player in this environment.
Horak et al., (2013) looked at finding a solution to experimental limitations when measuring ammonia oxidation rates using temperatures that normally deviate from in situ temperatures. This study was carried out on water samples from the Hood canal stratified water column with an in situ temperature of 8.5°C. Ammonia oxidation temperature sensitivity was investigated on four separate cruises on the hood canal water column, microcosms were established using ex situ temperatures ranging from 4 to 23 °C. A $Q_{10}$ temperature coefficient correction factor was used to measure the rate of change in a biological system as a consequence of a 10°C increase. The appropriate $Q_{10}$ correction factor was applied to all temperatures in the microcosm experiment. Only AOA transcripts were detected. AOA amoA transcripts and ammonia oxidation rates were significantly affected only when incubation temperatures were above or below the 8 year temperature range (8-20°C). Therefore, as long as temperature ranges are within the average seasonal range, temperature should not control AOA growth and activity throughout the year which agrees with the findings of Urakawa et al., (2014).

An experiment carried out without limiting conditions in the Ariake Sea sediments found nitrification rates responded in a bell curve with the optimum temperature for nitrification found to be 29.3°C. This implies that temperature did affect AO when in non-limiting ammonia and oxygen conditions (Isnansetyo et al., 2011; Kim et al., 2008). However that was the optimum temperature for that specific community found in the Ariake Sea.

1.4.4 OXYGEN AVAILABILITY

Oxygen concentration is another important parameter as it is an additional requirement for ammonia oxidisers to oxidise ammonia. Therefore, it is important to understand the depth oxygen can penetrate in coastal sediments before sampling. Oxygen penetration in coastal sediments usually ranges between 1 – 8 mm deep (Kemp et al., 1990; Louati et al., 2013). Oxygen penetration of sediment is directly related to the ability of the sediments to consume oxygen. Hence, when oxygen is rapidly consumed it won’t penetrate as
deep in the sediments. The main driver of oxygen consumption is organic decomposition (Wang et al., 2015; references therein). Therefore, sediment that has a higher oxygen consumption rate would also have higher organic carbon content. Although oxygen concentrations are shown to penetrate only a few mm deep, nitrification has been observed up to 10 cm deep, into the anoxic layer (Beman et al., 2012). A few possible explanations for this include: bioturbation from different macrofauna such as *Upogebia deltaura* that can create oxic and anoxic microenvironments by burrowing through the sediments and creating air pockets where nitrification and denitrification can be coupled in close proximity (Gilbert et al., 1998; Laverock et al., 2014). Secondly, in a paper by Park et al., (2010) AOA was grown successfully by co-culturing with sulfur oxidising bacteria (SOB). Thiosulfate was used as an electron donor in SOB. This may be because SOB produces factors essential for AOA to grow. SOB activity resulted in a large reduction of dissolved oxygen from 250 µM to 30 µM. Nevertheless AOA performed nitrification at this low oxygen concentration at a maximum growth rate of 0.6 per day. In contrast Erguder et al., (2009) claimed that AOA were able to compete with AOB because of niche differentiation in relation to both ammonia and oxygen. AOA can perform nitrification at lower ammonium levels than AOB and also at lower oxygen concentrations. This explains why oxygen minimum zones contain relatively high numbers of AOA (Erguder et al., 2009).

### 1.4.5 SALINITY

Salinity is an obvious environmental parameter that separates marine and terrestrial ecosystems. Salinity affects ammonia oxidisers in two ways: firstly it facilitates ammonia release or binding to sediments, otherwise known as ammonia or benthic fluxes (Weston et al., 2010). High salinity releases the ammonium bound to sediments, whereas low salinity increases the adsorption of ammonium to sediments (Boatman and Murray, 1982; Rysgaard et al., 1999; Seitzinger et al., 1991). This aids in providing varying concentrations of ammonia to ammonia oxidisers (Dollar et al., 1991). Secondly, salinity can add environmental stress to cells such as osmotic stress and cell toxicity. AOA and
AOB phylotypes have different ways of dealing with these stresses leading to some phylotypes being better adapted than others at withstanding the pressure of salinity (Roeßler and Müller, 2001). As a result of this, salinity affects changes in communities of AOA and AOB (Bollmann and Laanbroek, 2002; Zheng et al., 2014).

FIELD STUDIES

Numerous field studies have been carried out recording AOA and AOB dynamics at different salinities. AOB were more abundant in 10 out of 14 samples in the Yangtze estuary, China. *Nitrosomonas I* to *V* were more abundant at lower salinity (0.7 to 1.5 psu) whereas *Nitrosomonas VI* were more abundant at a higher salinity (6.2 to 14.8 psu). AOA were still present and clustered in two major branches of marine group I.I A and soil group I.I B. But, there was no correlation between AOA and salinity except for a trend indicating higher AOA gene abundance at the low salinity site (Zheng et al., 2014). AOA are consistently more abundant than AOB in San Francisco bay (US) at the low salinity sites (Damashek et al., 2015; Mosier and Francis, 2008). In contrast AOB were found to be more abundant in low salinity sites of the Cochin estuary (India) and throughout the Colne estuary (UK) at all salinity sites (Li et al., 2015; Puthiya Veettil et al., 2015). Interestingly, AOA marine group I.I A correlated strongly with potential nitrification rates (PNR; a measure of ammonia oxidising activity; Zheng et al., 2014); a trend similarly noted by Caffrey et al., (2007). The opposite is true according to Bernhard et al., (2010), where AOA were always more abundant along the salinity gradient of the Plum Island estuary. However, PNR correlated more strongly with AOB abundance. They suggested a couple of reasons for this. Firstly, AOA may have heterotrophic or mixotrophic metabolism (Agogué et al., 2008; Ingalls et al., 2006; Ouverney and Fuhrman, 2000), secondly, they may have been inhibited by agitation (Martens-Habbena et al., 2009), and thirdly, that they may have been inhibited by high NH$_4^+$ concentration, though this is most unlikely as they used in situ NH$_4^+$ concentrations.
Ammonia oxidiser communities have also been shown to tolerate variations in salinity with the highest potential nitrification rates found at 10 to 15 psu (ex situ salinity) the next highest rate was exhibited under in situ salinity (0 or 30 psu) from samples taken along a natural salinity gradient in a New England estuary. The Duoro estuary sediments incubated with salinities at 0, 15 and 30 psu also showed highest nitrification rates at 15 psu (Bernhard et al., 2007; Magalhães et al., 2005). Caffrey et al., (2007) found highest PNR rates were recorded when salinity, bottom water dissolved oxygen and pore water sulphide were low and sediment chlorophyll A and AOA abundance were high. They concluded that environmental variables are more important than gene abundances in controlling PNR rates.

(II) LINKING NITRIFICATION ACTIVITY TO FUNCTION

Field studies are necessary to unravel the diversity and abundance of AO in the environment as mentioned above. However, identifying the group and phylotypes of nitrifiers contributing to the observed/measured nitrifier activity is challenging.

Some studies struggle to detect AOB and AOA transcript abundances in situ as the AO can be a small percentage of the overall community. Further, it is difficult to extract fully intact mRNA from environmental samples. DNA and RNA approaches can also be biased due to inherent PCR biases (Smith and Osborn, 2009). Despite these difficulties, it is especially useful to quantify transcripts as it brings us a step closer to identifying the active microorganisms than gene quantification alone. An example on how targeting transcripts can be useful in linking nitrification activity to function was carried out by Zhang et al., (2015b). They grew AO in sediment microcosms under varying salinities and measured the transcriptional activity of both AOA and AOB to see who the active nitrifiers were. They found that AOB were not sensitive to salinity changes, however, they exhibited lower transcriptional activity as salinity increased and AOA had highest transcriptional activity at intermediate salinity. The active community was made up of five major clusters that were related to Nitrosopumilus maritimus. To date, the literature has provided contrasting
evidence on how AOA and AOB respond to salinity, making it all the more important to back up field studies with laboratory based experiments.

Stable Isotope Probing (SIP) has been used in various studies from resolving mechanisms of *Escherichia coli* removal using C\textsuperscript{13}-labelled glucose through sand filters, to looking at the diversity of active methanotrophs in a forest soil using C\textsuperscript{13} labelled CH\textsubscript{3}OH (Haig et al., 2014; Radajewski et al., 2002). In the case of nitrifiers, SIP is a useful approach to identify active nitrifiers. SIP tracks the incorporation of heavy labelled isotopes such as C\textsuperscript{13} and N\textsuperscript{15} into genomic DNA or RNA. Usually when studying nitrifiers this is achieved by exploiting the autotrophic nature of ammonia oxidisers and using C\textsuperscript{13} in the form of carbon dioxide to feed the microorganisms in the microcosms (Neufeld et al., 2007a). C\textsuperscript{13} is a heavy labelled carbon isotope found in small quantities in the environment while C\textsuperscript{12} is the more abundant isotope found in natural systems. If C\textsuperscript{13} is incorporated into the ammonia oxidisers genome it confirms first of all that they are autotrophic and secondly that they are actively growing (Freitag et al., 2006). Whitby et al., (2001) employed SIP to identify active autotrophic AOB in freshwater sediments, they found that *Nitrosospira* species dominated freshwater *in situ*, however, when looking at the active heavy labelled DNA fractions from a SIP experiment, *Nitrosomonas* was found to be active not *Nitrosospira*. N\textsuperscript{15} can be used and incorporated into ammonia substrate which the ammonia oxidisers utilise, but successful separation of N\textsuperscript{15} and N\textsuperscript{14} is limited due to the maximum buoyant density shift being quite small (0.016 g ml\textsuperscript{-1}) compared to the maximum shift in buoyant density of C\textsuperscript{13} and C\textsuperscript{12} (0.036 g ml\textsuperscript{-1}; Buckley et al., 2007). To my knowledge, Freitag and Prosser (2006), is the only SIP study carried out on testing whether AOB activity is influenced by salinity along an estuarine gradient. DGGE revealed contrasting communities along the salinity gradient; SIP was used to determine who were active at these sites. They found *Nitrosospira* to be the most abundant AOB species close to the estuary mouth and *Nitrosomonas oligotropha* were most abundant in the freshwater sites. SIP then confirmed that *Nitrosomonas* species Nm143 were active within estuarine sediments. AOA were only just discovered at this time (Venter et al., 2004) and not included in the Freitag and Prosser study.
1.5 SCOPE OF THESIS

Nitrification has a central role in the nitrogen cycle which is essential for all life on earth. Ammonia oxidising bacteria and archaea take part in the rate limiting step of this process which essentially contributes to both nitrogen removal and nitrogen accumulation in the environment. They are also inherently linked to the carbon cycle signifying their role in the contribution to global warming and climate change. The ecological importance of AO is indisputable. Therefore it is imperative to understand the ecology of these microorganisms and how they respond to different environmental conditions, especially high nutrient loads which are often found in coastal ecosystems. Recently discovered differences in metabolic ability in AOA and AOB may provide an explanation for co-occurrence of AO in the same environment, however further studies are required to confirm this. Other factors that can contribute to the co-occurrence of AOA and AOB are environmental factors that regulate and control niche differentiation between AOA and AOB such as substrate concentration, pH, temperature, oxygen availability and salinity. From reviewing the literature it is clear that it is difficult to untangle the influence environmental parameters have on AO communities. Temperature and salinity influence on AO communities seem to be the most contradictory in field studies and require more experimental studies to elucidate their impact on AOA and AOB growth and activity in situ. The main question this thesis will address is how do AO respond to environmental parameters in their environment? There are only a handful of isolated representatives of both AOA and AOB because of the difficulty and length of time it takes to grow them. We have seen evidence that although two strains may be closely related, it does not necessarily mean they will have the same physiological response. There is a dire need for more isolates in this field to help us elucidate the basic physiology of the microorganisms. Nevertheless, it is also important to study microorganisms in their natural environment and so we can use alternative methods to study microorganisms in situ and address the knowledge gaps. This project combines molecular methods like Q-PCR, RT-Q-PCR and sequence data along with in situ environmental parameter and nutrient
Chapter 1

measurements, rate process measurements and SIP to provide a link between community structure and activity with ecosystem function.

1.5.1 AIMS AND OBJECTIVES

This thesis, combining in situ field data with experimental approaches, addresses temporal and spatial changes in AO activity, abundance and diversity across environmental gradients in coastal bay ecosystems, in order to elucidate the key AO and environmental drivers within dynamic intertidal sediment ecosystems.

To do this, the study described in chapter 2 aimed to determine temporal and spatial dynamics of inorganic nitrogen, AO activity, AOA and AOB abundances across varying environmental conditions (temperature, sediment type, pH, salinity) within the intertidal sediments of two bays (Rusheen and Clew bay) on the west coast of Ireland. Furthermore one time-point was chosen to measure the activity, abundance and diversity of AOB and AOA within intertidal sediments across Rusheen bay, and further quantify and identify active ammonia oxidisers in situ by targeting amoA gene transcripts.

In chapter 3, salinity as an environmental driver of AOA and AOB contribution to ammonia oxidation was explored in further detail. For this, three sites spanning a natural salinity gradient created by groundwater inputs into Kinvarra Bay, Galway were studied. Microbial diversity, nitrifier activity and AOA and AOB gene abundances and diversity were determined in situ at one time-point in a similar fashion to chapter 2.

Finally chapter 4 expands the in situ study in chapter 3, examining the influence of salinity on AO activity in experimental nitrifying microcosms. Twenty-nine day sediment microcosm incubations from each site along the bay were incubated at natural and forced salinities to identify active ammonia oxidisers under these conditions using a combination of activity measurements, Q-PCR and SIP.
1.5.2 HYPOTHESIS

**Hypothesis I:** AOB verses AOA as the dominant driver of ammonia oxidation in intertidal sediments.

Ammonia concentration is one of the main drivers influencing AOA and AOB dynamics and niche differentiation in situ. In general, AOB prefer higher ammonia concentrations to AOA (Martens-Habbena et al., 2009). Environmental parameters such as salinity, pH and temperature all contribute to ammonia availability and also may cause stress to AOA and AOB cells thereby impacting their growth and activity (Section 1.4). Intertidal sediments are at the interface of land and sea buffering nutrient loads reaching coastal waters. As such, they receive high inorganic nitrogen loads created by anthropogenic activity. Therefore within intertidal bays, it is hypothesised that AOB will be more abundant that AOA.

**Hypothesis II:** Temporal and spatial changes in AO dynamics within intertidal bays

There is a general trend in the literature that nitrification rates drop when pH decreases (Suzuki et al., 1974). Additionally, there is no clear pattern that defines the impact of temperature and salinity on AOA and AOB derived from field studies alone (section 1.4.3 & section 1.4.5). However, laboratory based experiments have shown that AOB responded to temperature fluctuations more than AOA (Horak et al., 2013; Urakawa et al., 2014). Therefore, we hypothesise seasonal increases in nitrifier activity and AOB gene abundances but not AOA. Salinity does not appear to have a significant effect on AOB transcription; however AOA transcription was significantly higher at intermediate salinities (Zhang et al., 2015b). It is hypothesised that high and low salinity will not affect AOB activity, but will affect AOA activity.

It is clear from the evidence seen in various studies in coastal sediments, estuarine sediments and the open ocean (Abell et al., 2010; Bernhard et al., 2010; Caffrey et al., 2007; Li et al., 2015; Magalhães et al., 2009; Moin et al., 2009; Mosier and Francis, 2008; Santoro et al., 2008; Wankel et al., 2011) that
fluctuating environmental parameters influence AO activity, community structure and function. These variations in environmental parameters create ecotypes which are distinct species of microorganisms (in this case ammonia oxidisers) that are adapted to occupying a specific set of environmental conditions. Hence, it is further hypothesised that AOA and AOB ecotypes will shift due to several environmental parameters that differ at each site including salinity.

**Hypothesis III: Linking activity and function to phylogeny of active AO**

Ammonia oxidisers are not readily cultivated; another way of studying their activity under certain conditions is now possible with SIP and transcript measurements. Using a SIP-based approach, Whitby et al., 2001 showed that the dominant nitrifier *in situ* was not the most active nitrifier (Whitby et al., 2001). We hypothesise that SIP will reveal low abundant ecotypes, that were not normally considered abundant or major players in ammonia oxidation, to be the major drivers in their community.

Numerous studies have identified ecotypes of AOA/AOB within sediments at DNA level (Bernhard et al., 2005b; Li et al., 2015; Smith et al., 2014a; Zheng et al., 2013, 2014), yet few at transcript level (Fan et al., 2015). We hypothesise that numerically dominant AO are transcriptionally active, and therefore will be represented at the transcriptionally active AO.

Finally, if gene abundances are measured, it will reflect the active communities in both Rusheen and Kinvarra bay; although, the entire community is not expected to be active at all times.
CHAPTER 2: CO-OCCURRENCE OF TRANSCRIPTIONALLY ACTIVE AMMONIA OXIDISING BACTERIA AND ARCHAEA IN INTERTIDAL SEDIMENTS

2.1 INTRODUCTION

Using the amoA gene as a functional gene marker approach as mentioned in section 1.2.1, the abundance of AOA and AOB has been extensively surveyed in a range of coastal and estuarine environments to infer the distribution of AOA and AOB (Abell et al., 2010; Bernhard et al., 2010; Caffrey et al., 2007; Li et al., 2015; Magalhães et al., 2009; Moin et al., 2009; Mosier and Francis, 2008; Santoro et al., 2008; Wankel et al., 2011). While AOA and AOB are both present, niche differentiation is often apparent (Hatzenpichler, 2012; Martens-Habbena et al., 2009; Prosser and Nicol, 2012) and abiotic factors such as ammonia concentration, oxygen, temperature, pH and salinity have been shown to influence the abundances of AOA and AOB (Freitag et al., 2006; Ke et al., 2015; Laverock et al., 2014; Urakawa et al., 2014; Zhang et al., 2015b). Within coastal sediments, in some cases AOB have been reported as more abundant (Damashek et al., 2015; Li et al., 2015; Mosier and Francis, 2008) whilst other studies report AOA as dominant (Lipsewers et al., 2014; Wuchter et al., 2006). Generally a trend of greater AOA dominance at low ammonia (Martens-Habbena et al., 2009; Sauder et al., 2012) verses AOB dominance at high ammonia concentrations is evident (Baolan et al., 2012; Di et al., 2010; Jia and Conrad, 2009; Verhamme et al., 2011). What is not understood is if gene abundance equates to activity. For this, the detection and quantification of amoA mRNA transcripts would be more informative than DNA alone (Philippot et al., 2005), and would be a significant step forward from the current understanding of active nitrifiers.

To date, the majority of studies in coastal sediments have been conducted over large spatial scales and primarily focus on gene abundances only. Nitrifier dynamics over smaller spatial scales and active AO are not well understood. To this end, we investigated nitrifier activity and ammonia oxidisers across a range
of sediment types and salinities within two small intertidal bays (~ 1 km²) on the west coast of Ireland over an annual period (Fig. 2.1). Contrasting bays were selected, one located in a rural and the other in an urban setting (approximately 100 km apart).

2.2 AIMS

The objectives of the study were to monitor nitrification potential in addition to AOA and AOB amoA gene abundances within and between bays over an annual cycle. Furthermore, we aimed to quantify and identify active ammonia oxidisers in situ by targeting amoA gene transcripts within a single bay and time-point. Intertidal bays are highly complex and dynamic ecosystems. We therefore hypothesised that nitrifier activity and amoA gene abundances would vary spatially and temporally within and between bays due to fluctuating environmental parameters. Furthermore, we postulated that AOB would be more abundant than AOA due to elevated ammonia concentrations. Finally, that those abundant ammonia oxidisers’ amoA transcripts would reflect abundant amoA genes within intertidal sediments.

2.3 MATERIALS AND METHODS

2.3.1 SITE DESCRIPTION AND FIELD SAMPLING

The study was conducted in two small (~ 1 km²) intertidal coastal bays - Rusheen and Clew bay located on the west coast of Ireland (Fig. 2.1). Rusheen bay (53° 25.5894’N, -9° 11.9532’W) is a sheltered marsh intertidal mud/sand flat, situated along the north side of Galway bay on the edge of Galway city (population > 75,000). It is sheltered by a mixed sand and storm beach. In addition, two sites at the mouth of the River Corrib, Galway city centre, were selected as low salinity sites (Fig. 2.1). Clew bay (53° 78.6962’N, -9° 64.9515’W) is an intertidal mud/sand flat situated in a rural area surrounded by agricultural land, mainly sheep farming, approximately 100 km north-west of Rusheen Bay. A beach shelters the bay on its seaward side. Numerous groundwater upwelling and freshwater streams enter the bay. In both bays,
intertidal sediments (top 0-5 cm) were collected at low tide from 7 sites, incorporating sandy to muddy sediment types and low salinity sites (Rsed_6,7 and Csed_6). Three biological replicates were collected from each site. Each replicate was composed of 10 random samples collected within a 10 m² area. Each bay was sampled in May, August, and November 2013, and in February 2014. Sediment samples were sub-sampled from each replicate into 0.5 g aliquots and immediately stored at -80°C for subsequent molecular analysis. Samples for PNR measurements and physicochemical analysis were stored at 4°C upon analysis.

![Figure 2.1: Location of sampling sites](image)

**Figure 2.1: Location of sampling sites** within A: Rusheen Bay (sites R Sed_1 to R Sed_5) and at the Corrib estuary mouth (R sed_6 & 7), B: Clew Bay (sites C Sed_1 to C Sed_6) and C: Location of Rusheen and Clew Bay on the West Atlantic coast of Ireland.

### 2.3.2 Determination of Physicochemical Parameters

Sediment temperature was measured at each site with a mercury thermometer. Salinity of site seawater was determined with a refractometer
(Coral Farm, Ireland). pH was determined by mixing sediments with deionised water at a ratio of 1:2 and measured with an Orion pH meter, model 420A (Cole Parmer, Ireland). Water content was calculated via the weight loss of a known amount of sediment dried at 105°C until a constant value was reached. The Mastersizer 2000 Laser Particle Sizer (Malvern, UK) was used to determine particle sizes <1 mm. Results were analysed using Gradistat v8, excel version 14 (Blott, 2010). Particles above 1 mm were analysed using a dry sieving technique (Rowden et al., 1998). Ammonium (NH$_4^+$) and nitrate (NO$_3^-$) were extracted from the sediment by incubating 5 g sediment with 30 ml of 1 M KCl for 1 h at 15°C on a shaker. Samples were then filtered with grade 52 Whatmann filter paper (Fisher scientific, Ireland) and frozen at -20°C until further analysis. Nitrite (NO$_2^-$) was extracted by incubating 30 ml 2 M KCl for 1 h at 15°C. The Hach powder pillow pack Nitriver 3 (Hach-Lange, Ireland) was used as a colorimetric method to measure nitrite concentration, according to manufacturer’s instructions. NO$_3^-$ and NH$_4^+$ were measured using a colorimetric method described in Bollmann et al., (2010). Total organic carbon (TOC) content was determined by incinerating dry sediment for 12 h in a furnace (450°C) (Rowden et al., 1998).

2.3.3 POTENTIAL NITRIFICATION RATES (PNR)

PNRs were carried out in triplicate from each replicate at all sites. Five g sediment (wet weight) was added to a 500 ml bottle containing 30 ml site water amended with 24 µM sodium azide (NaN$_3$, nitrite oxidation inhibitor; Sigma, Ireland; Ginestet et al., 1998) and two concentrations of (NH$_4$)$_2$SO$_4$ (Sigma, Ireland) per microcosm (0 µM, 250 µM). Microcosms were incubated in the dark on a shaker at 90 r.p.m. at 15°C for 24 h. After incubation, NO$_2^-$ was extracted with 2 M KCl. Nitrite was measured as described above. PNR rate was calculated using the following formula:

$$\frac{(mg/l \text{ Nitrite} \times 60 \text{ ml})}{(5 \text{ g sediment})} = PNR \text{ per g sediment per day}$$
2.3.4 DNA AND RNA CO-EXTRACTION

DNA and RNA extractions from sediment samples were carried out using the Griffith method (Griffiths et al., 2000). All reagents were made with sterile Diethylpyrocarbonate (DEPC) water, glassware (baked at 180°C overnight) and plasticware (soaked in RNase Away; Ambion, Ireland) were treated to ensure they were RNase free. DNA and RNA were extracted from 0.5-0.7 g fresh sediment using 0.5 ml 5% CTAB and 0.5 ml phenol:chloroform:isoamyl (25:24:1). Bead beating was carried out for 2.5 min on a Vortex Genie (Scientific Industries, Ireland) followed by centrifugation for 20 min at 12,000 x g at 4°C. The supernatant was removed and placed in a fresh tube and mixed with 0.5 ml chloroform: isoamyl (24:1). The mixture was centrifuged at 16,000 x g for 5 min at 4°C and the supernatant was removed and precipitated with 2 volumes 30% PEGG for 2 h. The precipitate was pelleted at 16,000 x g for 30 min at 4°C. The pellet was washed with 70% ethanol twice and left to air dry. Nucleic acids were re-suspended in 50 µl DEPC water. The integrity of extracted DNA and RNA was checked on a 1% agarose gel. For samples collected from Rusheen bay, February 2014, a 25 µl aliquot of DNA/RNA was removed to prepare RNA using TURBO DNase (Ambion, Ireland) according to the manufacturer’s instructions. The absence of DNA from the RNA fraction was confirmed by no amplification of the 16S rRNA gene using primers F63 (5’-CAG GCC TAA CAC ATG GCA AGT C-3’) and 518R (5’-ATT ACC GCG GCT GCT GG-3’) (Marchesi et al., 1998; Muyzer et al., 1993). RNA was diluted from neat to 10⁻² and 2 µl of RNA template was added to a 50 µl PCR mixture containing 5 µl PCR buffer including MgCl₂ (Sigma Aldrich, Ireland), 0.2 mM of each deoxynucleoside triphosphate (dNTP; Sigma Aldrich, Ireland), 0.25 µM of each primer (Eurofins, Ireland), and 2.5 units of Taq polymerase (Sigma Aldrich, Ireland). The reaction was initially denatured at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min, with a final extension step at 72°C for 7 min. The absence of a 16S rRNA gene band was visually confirmed on a 1% agarose gel. DNA and RNA were stored at -80°C until further analysis.
2.3.5 CDNA SYNTHESIS

RNA was converted to cDNA using Superscript III (Life Science, USA). Gene specific two-step reverse transcription PCR (RT-PCR) amplification was performed on RNA targeting AOA amoA gene and AOB amoA gene. The initial RT reaction mixtures contained 8 µl of environmental RNA, 2 mM of the appropriate reverse primer, ArchamoAR (5’- GCG GCC ATC CAT CTG TAT GT -3’) (Francis et al., 2005) or BacamoA-2R (5’- CCC CTC KGS AAA GCC TTC TTC – 3’) (Rotthauwe et al., 1997), and 10 mM of each dNTP, making the total volume 10 µl. The mixture was denatured at 65°C for 5 min and transferred to ice for 1 min. 4 µl 5X first-strand buffer, 1 µl 0.1 mM dithiothreitol (DTT), and 200 units SuperScript III were added to the reaction mixture and incubated at 50°C for 50 min, followed by inactivation of the reaction at 72°C for 15 min. Samples were stored at -80°C until further analysis.

2.3.6 QUANTITATIVE PCR (Q-PCR) AND REVERSE TRANSCRIPTION (RT)-Q-PCR

2.3.6.1 STANDARD CURVE CONSTRUCTION

DNA and RNA standard curves were constructed by amplifying the gene of interest (Table 2.1) according to Smith et al., 2006. Briefly, target PCR amplicons were cloned using pGem-T-Easy Vector Systems (Promega, Ireland) according to the manufacturer’s instructions. White colonies were screened by PCR using vector M13 forward primer (5’- TGTAAAACGACGGCCAGT-3’) and gene specific reverse primer (i.e. ArchamoAR or BacamoA-2R) to select gene targets in the antisense orientation for subsequent in vitro RNA transcription. Purified PCR products were quantified on the Nanodrop 2000C (Thermo Scientific, Ireland) and gene copy numbers (copies µl⁻¹) were calculated (Smith et al., 2006). A one-in-five dilution series over an eight point dynamic range from 5³ to 5¹⁰ was made and used as template for the standard curve. cDNA standard curves were constructed from in vitro transcribed RNA of the target antisense DNA template using the Megascript T7 transcription kit reaction
Chapter 2

(Ambion, Ireland) according to the manufacturer’s instructions. RNA was re-suspended in 30 µl RNA-later solution and quantified on the Nanodrop 2000C (Thermo Scientific, Ireland). An RT reaction, as described above, was performed on the neat quantified in vitro transcribed RNA, with appropriate reverse primer (Table 2.1) to create cDNA template. This was serially diluted one-in-five and used as the RT-Q-PCR standard curve.
Table 2.1: Suite of primers used in this study

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer sequence (5’-3’)</th>
<th>Target gene</th>
<th>Thermal Profile</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>F63</td>
<td>CAG GCC TAA CAC ATG GCA AGT C</td>
<td>bacterial 16S rRNA gene</td>
<td>95 °C, 5 min; 30 x (95°C, 30 s; 57°C, 30 s; 72°C, 1 min) 72 °C, 7 min</td>
<td>(Marchesi et al., 1998; Muyzer et al., 1993)</td>
</tr>
<tr>
<td>R518</td>
<td>ATT ACC GCG GCT GCT GG</td>
<td>bacterial amoA gene</td>
<td>Touchdown- 94°C, 3 min; 10X (94°C, 30 s; 62°C (AOB)/60°C (AOA), 45 s; -0.5°C at 3 °C/s; 72°C, 1 min) 30X (94°C, 30 s; 57°C (AOB)/55°C (AOA), 45 s; 72°C, 1 min) 72°C, 10 min</td>
<td>(Francis et al., 2005)</td>
</tr>
<tr>
<td>Arch-amoAF</td>
<td>STA ATG GTC TGG CTT AGA CG</td>
<td>archaeal amoA gene (635 bp)</td>
<td>Q-PCR - 95°C, 3 min; 40×(95°C, 30 s; 55°C(Bac)/ 53°C (Arch) 30s; 72°C, 1 min, 81°C with plate read); Melt curve 65°C to 95°C, increment 0.5°C, 0:05+ plate read</td>
<td>(Rotthauwe et al., 1997)</td>
</tr>
<tr>
<td>Arch-amoAR</td>
<td>GCG GCC ATC CAT CTG TAT GT</td>
<td>bacterial amoA gene (435 bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEX-BacamoA-1F</td>
<td>GGG GTT TCT ACT GGT GGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BacamoA-1F</td>
<td>GGG GTT TCT ACT GGT GGT</td>
<td>bacterial amoA gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BacamoA-2R</td>
<td>CCC CTC KGS AAA GCC TTC TTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13F</td>
<td>TGT AAA ACG ACG GCC AGT</td>
<td>vector primers</td>
<td>95°C, 5 min; 30×(95°C, 30 s; 57°C, 30 s; 72°C, 1 min) 72°C, 10 min</td>
<td>(Invitrogen, Ireland)</td>
</tr>
<tr>
<td>T7F</td>
<td>TAA TAC GAC TCA CTA TAG GG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13R</td>
<td>CAG GAA ACA GCT ATG AC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Wobbles included: S: G/C; K: G/T; Y: C/T
AOA and AOB *amoA* genes (Table 2.1) were amplified from triplicate sediments collected from each site in Rusheen and Clew bay from May to February. Q-PCR assays for each gene target were amplified on a single Q-PCR plate for each sampling time-point (i.e. May, August, November, and February). Triplicate no-template controls (NTC) and appropriate standard curve were included in each assay. Each 20 µl EVA Green Q-PCR reaction mixture contained 10 µl EVA Green master mix, 0.4 µl of each primer (10 mM), and 2 µl template DNA (10⁻¹ dilution). The specificity of the products was confirmed by melting curve analysis at the end of each RT-Q-PCR experimental run. The PCR conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 53°C for 30 s (AOA) or 55°C for 30 s (AOB), 1 min at 72°C, and finally an acquisition temperature 81°C (AOA) or 83°C (AOB). Melting curve analysis was performed at 95°C for 5 s, 65°C for 1 min, and 97°C continuous.

*amoA* transcripts were quantified from Rusheen Bay, February 2014 sediments. RT-Q-PCR conditions were the same as for DNA, but gene specific environmental and standard curve cDNA was used as template. The slope, y intercept, and $r^2$ values of DNA and cDNA curves are reported (Table 2.2).

**Table 2.2: Q-PCR standard curve descriptors**

<table>
<thead>
<tr>
<th>Nucleic Acid</th>
<th>Genetic Target</th>
<th>Std. Curve Descriptions</th>
<th>NTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Archaea <em>amoA</em></td>
<td>Slope: 3.60, Efficiency: 89.5, Y Intercept: 38.99, $r^2$: 0.99</td>
<td>32.5</td>
</tr>
<tr>
<td>DNA</td>
<td>Bacteria <em>amoA</em></td>
<td>Slope: 3.52, Efficiency: 92.5, Y Intercept: 37.68, $r^2$: 0.99</td>
<td>30.75</td>
</tr>
<tr>
<td>RNA</td>
<td>Archaea <em>amoA</em></td>
<td>Slope: 3.43, Efficiency: 96, Y Intercept: 42.45, $r^2$: 0.99</td>
<td>35</td>
</tr>
<tr>
<td>RNA</td>
<td>Bacteria <em>amoA</em></td>
<td>Slope: 3.94, Efficiency: 79, Y Intercept: 53.01, $r^2$: 0.99</td>
<td>30</td>
</tr>
</tbody>
</table>
2.3.7 TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM (T-RFLP) OF AOB AMOA TRANSCRIPTS

cDNA from AOB amoA transcripts were created from all sites in Rusheen bay, February 2014, as described above. AOB amoA cDNA was PCR amplified with a HEX labelled forward primer HEX-BacamoA-1F (5’- GGG GTT TCT ACT GGT GGT -3’) and BacamoA-2R (5’- CCC CTC KGS AAA GCC TTC TTC-3’) (Rotthauwe et al., 1997) reverse primer. Each 50 µl reaction mix contained 1 µl amoA AOB gene specific cDNA, 20 µM forward and reverse primers and 25 µl 2X MyTaq mix (Bioline, Ireland). A touchdown PCR amplification was carried out on a Mastercycler Gradient PCR machine (Eppendorf, Germany) as follows: 94°C for 3 min, 10X (94°C for 30 s; 62°C for 45 s; -0.5°C at 3 °/s; 72°C for 1 min) 30X (94°C for 30s; 57°C (AOB) for 45s; 72°C for 1 min) with a final elongation step 72°C for 10 min. PCR products were purified using SureClean Plus (Bioline, Ireland) purification kit according to manufacturer’s instructions and re-suspended in 15 µl of sterile water. Three enzymes, Taq I, Hha I and Alu I, (Thermo Fisher Scientific, Ireland) were used to independently digest 5 µl PCR product per sample according to manufacturer’s instructions. Samples were sent to Source Bioscience (Tramore, Waterford) for T-RFLP analysis. Terminal Restriction Fragments (TRF) were sized against a ROX-genescan 500 internal size standard using Peakscanner software v1.0 (Applied Biosystems, Country). T-RFLP profiles from individual enzymes were combined into a single profile and distinguished from each other by adding 1000 to TaqI TRFs and 2000 to HhaI TRF’s for analysis purposes; AluI TRFs remained unaltered. The combined T-RFLP profiles among sites were aligned using T-Align (Smith et al., 2005).

2.3.8 CLONING AND SEQUENCE ANALYSIS OF BACTERIAL AND ARCHAEAL AMOA GENES

AOA and AOB amoA genes and transcripts from replicate samples from sites Rsed_1, Rsed_5, Rsed_6 & Rsed_7 (Fig. 2.1) in Rusheen Bay February 2014 were cloned and sequenced. cDNA was reverse transcribed with gene
specific primers as described above. 5 µl DNA (10⁻¹) or cDNA (neat) was amplified as follows: 10X 1.5 mM MgCl₂ PCR buffer (Sigma Aldrich, Ireland), 0.2 mM of each dNTP (Sigma, Ireland), 0.25 µM of each primer ArchamoAF (5’-STA ATG GTC TGG CTT AGA CG-3’), and ArchamoAR (5’- GCG GCC ATC CAT CTG TAT GT -3’) (Francis et al., 2005), or BacamoA1F (5’- GGG GTT ACT GGT GGT -3’) and BacamoA2R (5’- CCC CTC KGS AAA GCC TTC TTC-3’) (Rotthauwe et al., 1997; Eurofins, Ireland), and 2.5 units of Taq polymerase (Sigma, Ireland). PCR conditions were as follows: 94°C for 3 min, 10X (94°C for 30 s, 62°C (AOB)/60°C (AOA) for 45 s, -0.5°C at 3 °/s, 72°C for 1 min), 30X (94°C for 30 s, 57°C (AOB)/55°C (AOA) for 45 s, 72°C for 1 min) with a final elongation step 72°C for 10 min. Correct sized amplicons were gel purified using a Gel Purification Kit (Qiagen, Ireland) according to manufacturer’s instructions and re-suspended in 15 µl sterile water. PCR products were cloned using PGem-T cloning kit (Promega, Ireland) according to manufacturer’s instructions. Transformants were selected on Luria-Bertani agar plates containing 100 mg ml⁻¹ ampicillin, 20 mg ml⁻¹ X-Gal (Sigma, Ireland) and 0.0238 mg ml⁻¹ IPTG (Sigma, Ireland). White colonies were screened using a vector PCR with primers T7F (5’- TAA TAC GAC TCA CTA TAG GG -3’) / M13F (5’- GTT TTC CCA GTC ACG AC -3’) (10 mM) and M13R (5’- CAG GAA ACA GCT ATG AC -3’) (10 mM). Clones containing inserts of the correct size were sent for sequencing using primers M13F/T7F (Macrogen, Amsterdam or Source Bioscience, Tramore). amoA nucleotide sequences were translated into protein sequences using the Translate tool on the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (http://us.expasy.org/tools/dna.html). Nucleotide and protein sequences were compared to entries in GenBank using BlastN and BlastP, respectively (Altschul et al., 1990). Protein sequence alignments were constructed using ClustalX program (version 2.1)/Bioedit (version 7.2) (Thompson et al., 1997; Hall, 1999). amoA gene sequences displaying more than 97% similarity were grouped into a single operational taxonomic unit (OTU) using DOTUR software by the furthest neighbour approach (Schloss & Handelsman, 2005). Distance matrices were calculated using the PROTDIST program in PHYLIP (Felsenstein, 1989).
Phylogenetic trees were created from the distance matrices by using the neighbour-joining method (Saitou & Nei, 1987) and Kimura substitution algorithm (Kimura, 1983) using MEGA 6 (Tamura et al., 2013). Consensus trees were calculated after bootstrapping (1,000 replicate trees).

2.3.9 NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

AOA accession numbers range from KX664728 - KX664820. AOB DNA accession numbers range from KX673291 - KX673360, while AOB cDNA accession numbers range from KX690304 - KX690497.

2.3.10 STATISTICAL ANALYSIS

All data was tested for normality using Kolmogorov-Smirnov. When data was not normal, it was log-transformed (Bartlett, 1947). Variation in potential nitrification rates or gene abundances or environmental variables such as TOC and inorganic nitrogen measurements between sites, bays and time-points were analysed using a three-way analysis of variance (ANOVA) followed by a post-hoc Bonferroni test (Dunn, 1961) in SPSS v21 (IBM, USA). A one-way ANOVA was used on environmental variables such as TOC and inorganic nitrogen measurements between sites in both bays at each time-point in SPSS v21. Analysis of covariance (ANCOVA) was carried out between Q-PCR assays for AOA and AOB. Each assay was carried out by time-point in graph pad prism v6. Differences were considered significant in these tests when p < 0.05.

Possible relationships between community structure, environmental parameters, temperature (T), pH, soil, inorganic N concentrations (NH$_4^+$, NO$_2^-$ and NO$_3^-$), TOC, salinity and grain size were explored using canonical correspondence analysis (CCA) using the software CANOCO 5 (http://www.canoco5.com). Unimodal response model gave better approximation of AOB community structure relationship with the explanatory variables. Explanatory value of environmental factors was determined using forward selection (tested by 499 Montecarlo permutations). Differences and correlation
coefficients were considered significant at $P < 0.05$ unless otherwise stated in the text. Only significant explanatory variables were plotted.

### 2.4 RESULTS

#### 2.4.1 SITE DESCRIPTION

Both bays were sampled every three months from May (2013) to February (2014) (Fig. 2.1). Temperatures ranged from 8 – 22°C, salinity from 0 - 32.08 ppt. Sediments were classified as sandy (>90% between 1 mm and 63 µm), gravelly (>1 mm), muddy (<63 µm), muddy gravelly sand (30-50% mud and gravel and at least 50% sand) and sandy mud (30-50% sand: 30-50% mud). TOC ranged from 0.5 – 20.03%; pH from 6.65-8.43, increasing from May to November (One-way ANOVA; $P < 0.05$).

$\text{NH}_4^+$ concentrations were higher in Rusheen (Range: 0 - 1166.2; Average: 283.55 ± SD 38.68 µM$^{-1}$ g$^{-1}$ fs$^{-1}$) and lower in Clew bay in April and November ranging from 0- 269.11 µM$^{-1}$ g$^{-1}$ fs$^{-1}$ with an average of 63.76 ± SD 16.99 µM$^{-1}$ g$^{-1}$ fs$^{-1}$ (One-way ANOVA $P < 0.05$). There was an increase in $\text{NH}_4^+$ and pH in Rusheen bay in November (909.68 ± 43.34 $\text{NH}_4^+$ µM$^{-1}$ g$^{-1}$ fs$^{-1}$; pH 8.06 ± 0.13). (Table 2.3(A) & 2.3(B)).
## Table 2.3 (A): Rusheen bay Environmental Parameters

<table>
<thead>
<tr>
<th>Time</th>
<th>Site</th>
<th>$\text{NO}_3^-$ (µM) (± s.d.)</th>
<th>$\text{NO}_2^-$ (µM) (± s.d.)</th>
<th>$\text{NH}_4^+$ (µM) (± s.d.)</th>
<th>pH (± s.d.)</th>
<th>TOC (%) (± s.d.)</th>
<th>Temp (°C)</th>
<th>Salinity (ppt)</th>
<th>Gravel (%) (± s.d.)</th>
<th>Sand (%) (± s.d.)</th>
<th>Mud (%) (± s.d.)</th>
<th>Eunis Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>APRIL</td>
<td>RSed_1</td>
<td>2.26 ± 0.78</td>
<td>0.33 ± 0.06</td>
<td>40.44 ± 16.39</td>
<td>7.48 ± 0.04</td>
<td>17.98 ± 1.4</td>
<td>11</td>
<td>32.08</td>
<td>0</td>
<td>42.2 ± 0.04</td>
<td>57.8 ± 0.04</td>
<td>mS- Sandy mud</td>
</tr>
<tr>
<td></td>
<td>RSed_2</td>
<td>2.26 ± 0.78</td>
<td>0.22 ± 0.07</td>
<td>152.44 ± 68.32</td>
<td>7.7 ± 0.4</td>
<td>16.49 ± 0.4</td>
<td>11</td>
<td>32.08</td>
<td>2.0 ± 0.006</td>
<td>85.4 ± 0.025</td>
<td>14.6 ± 0.025</td>
<td>mS- muddy sand</td>
</tr>
<tr>
<td></td>
<td>RSed_3</td>
<td>1.81 ± 0.78</td>
<td>0.1 ± 0.16</td>
<td>68.44 ± 17.68</td>
<td>7.79 ± 0.07</td>
<td>17.5 ± 1.6</td>
<td>11</td>
<td>32.08</td>
<td>0.1 ± 0.0003</td>
<td>98.5 ± 0.003</td>
<td>1.5 ± 0.003</td>
<td>(m)S- slightly muddy sand</td>
</tr>
<tr>
<td></td>
<td>RSed_4</td>
<td>1.80 ± 1.56</td>
<td>0.07 ± 0.095</td>
<td>12.44 ± 7.12</td>
<td>7.96 ± 0.03</td>
<td>12.57 ± 0.6</td>
<td>11</td>
<td>33.84</td>
<td>18.7 ± 0.05</td>
<td>66.4 ± 0.33</td>
<td>30.2 ± 0.3</td>
<td>gmS- gravelly muddy sand</td>
</tr>
<tr>
<td></td>
<td>RSed_5</td>
<td>0.90 ± 1.56</td>
<td>0.14 ± 0.045</td>
<td>203.78 ± 15</td>
<td>7.92 ± 0.02</td>
<td>17.8 ± 4.2</td>
<td>11</td>
<td>32.08</td>
<td>2.3 ± 0.01</td>
<td>94.7 ± 0.03</td>
<td>5.2 ± 0.03</td>
<td>(g)mS- slightly gravelly muddy sand</td>
</tr>
<tr>
<td></td>
<td>RSed_6</td>
<td>4.52 ± 2.82</td>
<td>0.14 ± 0.07</td>
<td>42 ± 9.33</td>
<td>7.71 ± 0.03</td>
<td>15.24 ± 1.05</td>
<td>11</td>
<td>0.92</td>
<td>3.4 ± 0.006</td>
<td>99.2 ± 0.005</td>
<td>0.8 ± 0.005</td>
<td>(g)S- slightly gravelly sand</td>
</tr>
<tr>
<td></td>
<td>RSed_7</td>
<td>0.90 ± 0.78</td>
<td>0.28 ± 0.1</td>
<td>113.56 ± 112.42</td>
<td>7.92 ± 0.008</td>
<td>17.14 ± 1.3</td>
<td>11</td>
<td>3.53</td>
<td>0.5 ± 0.003</td>
<td>99.4 ± 0.004</td>
<td>0.6 ± 0.004</td>
<td>S- Sand</td>
</tr>
<tr>
<td>AUGUST</td>
<td>RSed_1</td>
<td>2.26 ± 0.78</td>
<td>4.43 ± 0.52</td>
<td>141.56 ± 88.34</td>
<td>7.63 ± 0.02</td>
<td>6.74 ± 1.06</td>
<td>22</td>
<td>16.66</td>
<td>11.6 ± 0.015</td>
<td>49.7 ± 0.02</td>
<td>38.7 ± 0.03</td>
<td>gmS- gravelly muddy sand</td>
</tr>
<tr>
<td></td>
<td>RSed_2</td>
<td>1.35 ± 2.74e-16</td>
<td>4.00 ± 0.40</td>
<td>96.44 ± 40.23</td>
<td>7.72 ± 0.01</td>
<td>2.06 ± 0.08</td>
<td>22</td>
<td>16.66</td>
<td>0.4 ± 0.002</td>
<td>84.7 ± 0.02</td>
<td>14.9 ± 0.02</td>
<td>mS- muddy Sand</td>
</tr>
<tr>
<td></td>
<td>RSed_3</td>
<td>RSed_4</td>
<td>RSed_5</td>
<td>RSed_6</td>
<td>RSed_7</td>
<td>N</td>
<td>O</td>
<td>V</td>
<td>E</td>
<td>B</td>
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<td></td>
<td>ST</td>
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<td></td>
<td></td>
<td></td>
<td>S</td>
<td>NOVEMBER</td>
<td>M</td>
<td>E</td>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>1.80 ± 0.78</td>
<td>3.65 ± 0.26</td>
<td>85.56 ± 5.39</td>
<td>7.74 ± 0.01</td>
<td>2.65 ± 1.7</td>
<td>22</td>
<td>16.66</td>
<td>0.4 ± 0.002</td>
<td>97.9 ± 0.002</td>
<td>1.7 ± 0.002</td>
<td>(m)S- slightly muddy sand</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.451 ± 0.78</td>
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<td>48.22 ± 2.69</td>
<td>7.99 ± 0.05</td>
<td>6.97 ± 6.5</td>
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<td>16.66</td>
<td>8.6 ± 0.03</td>
<td>91.3 ± 0.03</td>
<td>0.1 ± 0.0001</td>
<td>gS - gravelly sand</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.35 ± 2.74e-16</td>
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<td>96.44 ± 31.07</td>
<td>7.66 ± 0.03</td>
<td>1.46 ± 0.27</td>
<td>22</td>
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<td>3.6 ± 0.03</td>
<td>89.4 ± 0.03</td>
<td>7 ± 0.02</td>
<td>(g)mS- slightly gravelly muddy sand</td>
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</tr>
<tr>
<td></td>
<td>1.35 ± 2.74e-16</td>
<td>2.96 ± 0.6</td>
<td>73.11 ± 2.69</td>
<td>7.87 ± 0.04</td>
<td>0.96 ± 0.2</td>
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<td>4.2</td>
<td>2.5 ± 0.008</td>
<td>97.3 ± 0.008</td>
<td>0.2 ± 0.0002</td>
<td>(g)S- slightly gravelly sand</td>
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<td>3.16 ± 0.78</td>
<td>0.17 ± 0.3</td>
<td>304.89 ± 2.69</td>
<td>7.87 ± 0.03</td>
<td>0.83 ± 0.2</td>
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<td>20.05</td>
<td>0.3 ± 0.001</td>
<td>99.5 ± 0.001</td>
<td>0.2 ± 0.0005</td>
<td>S- Sand</td>
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<tr>
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<td>3.42 ± 3.88</td>
<td>2.87 ± 0.26</td>
<td>894.42 ± 120.84</td>
<td>7.95 ± 0.05</td>
<td>15.02 ± 8.3</td>
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<td>56.4 ± 0.12</td>
<td>24.3 ± 0.05</td>
<td>19.3 ± 0.07</td>
<td>msG- muddy sandy gravel</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.69 ± 3.72</td>
<td>2.87 ± 0.9</td>
<td>843.91 ± 49.63</td>
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<td>2.65 ± 0.29</td>
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<td>32</td>
<td>2.1 ± 0.009</td>
<td>87.1 ± 0.02</td>
<td>10.7 ± 0.008</td>
<td>(g)mS- slightly gravelly muddy Sand</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.31 ± 2.3</td>
<td>3.04 ± 0.15</td>
<td>1107.6 ± 37.96</td>
<td>8.14 ± 0.08</td>
<td>1.5 ± 0.07</td>
<td>8</td>
<td>32</td>
<td>3.2 ± 0.002</td>
<td>96.6 ± 0.002</td>
<td>0</td>
<td>(g)S- slightly gravelly sand</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.61 ± 1.07</td>
<td>2.43 ± 1.34</td>
<td>689.04 ± 12.98</td>
<td>8.27 ± 0.5</td>
<td>2.14 ± 0.67</td>
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<td>32</td>
<td>8.9 ± 0.042</td>
<td>85.7 ± 0.04</td>
<td>5.4 ± 0.01</td>
<td>gmS - gravelly muddy sand</td>
<td></td>
</tr>
<tr>
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<td>0.98 ± 0.98</td>
<td>3.04 ± 0.54</td>
<td>1166.20 ± 80.86</td>
<td>7.91 ± 0.02</td>
<td>1.75 ± 0.08</td>
<td>8</td>
<td>32</td>
<td>6.3 ± 0.02</td>
<td>90.4 ± 0.016</td>
<td>3.3 ± 0.004</td>
<td>(m)gS- slightly muddy gravelly sand</td>
<td></td>
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<td>2.91 ± 3.72</td>
<td>4.17 ± 1.14</td>
<td>885.21 ± 123.96</td>
<td>7.94 ± 0.02</td>
<td>1.06 ± 0.1</td>
<td>8</td>
<td>20</td>
<td>0.2 ± 0.0007</td>
<td>99.7 ± 0.0006</td>
<td>0.1 ± 0.0008</td>
<td>S - Sand</td>
<td></td>
</tr>
<tr>
<td>FEBRUARY</td>
<td>RSed_7</td>
<td>1.06 ± 1.64</td>
<td>2.78 ± 0.4</td>
<td>781.4 ± 37.4</td>
<td>8.13 ± 0.02</td>
<td>0.69 ± 0.4</td>
<td>8</td>
<td>10</td>
<td>1.3 ± 0.003</td>
<td>98.7 ± 0.003</td>
<td>0</td>
<td>(g)S- slightly gravelly sand</td>
</tr>
<tr>
<td>-----------</td>
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<td>----------------------------</td>
</tr>
<tr>
<td>RSed_1</td>
<td>3.21 ± 0.37</td>
<td>1.13 ± 1.96</td>
<td>0 ± 0</td>
<td>7.8 ± 0.04</td>
<td>8.43 ± 2.2</td>
<td>10</td>
<td>24</td>
<td>37.5 ± 0.08</td>
<td>44.5 ± 0.08</td>
<td>17.9 ± 0.003</td>
<td>mgS- muddy gravelly sand</td>
<td></td>
</tr>
<tr>
<td>RSed_2</td>
<td>2.18 ± 0.42</td>
<td>3.91 ± 5.9</td>
<td>19.94 ± 34.54</td>
<td>7.88 ± 0.03</td>
<td>3.78 ± 0.02</td>
<td>10</td>
<td>24</td>
<td>1.3 ± 0.003</td>
<td>77.4 ± 0.02</td>
<td>21.4 ± 0.014</td>
<td>(g)mS- slightly gravelly muddy Sand</td>
<td></td>
</tr>
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<td>RSed_3</td>
<td>2.53 ± 0.49</td>
<td>0.61 ± 1.05</td>
<td>15.44 ± 13.39</td>
<td>7.78 ± 0.05</td>
<td>1.33 ± 0.06</td>
<td>10</td>
<td>24</td>
<td>0</td>
<td>99.8 ± 0.001</td>
<td>0.2 ± 0.001</td>
<td>S- sand</td>
<td></td>
</tr>
<tr>
<td>RSed_4</td>
<td>0.37 ± 0.14</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>7.15 ± 0.1</td>
<td>1.16 ± 0.02</td>
<td>10</td>
<td>24</td>
<td>0.8 ± 0.007</td>
<td>99.2 ± 0.008</td>
<td>0</td>
<td>S- Sand</td>
<td></td>
</tr>
<tr>
<td>RSed_5</td>
<td>3.92 ± 1.71</td>
<td>0.61 ± 0.84</td>
<td>56.56 ± 57.26</td>
<td>7.62 ± 0.04</td>
<td>1.49 ± 0.14</td>
<td>10</td>
<td>24</td>
<td>0.1 ± 0.0002</td>
<td>95.4 ± 0.005</td>
<td>4.5 ± 0.005</td>
<td>(m)S- slightly muddy sand</td>
<td></td>
</tr>
<tr>
<td>RSed_6</td>
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<td>0.43 ± 0.75</td>
<td>0.39 ± 0.14</td>
<td>6.65 ± 0.14</td>
<td>1.59 ± 0.16</td>
<td>10</td>
<td>0</td>
<td>1.6 ± 0.005</td>
<td>98.4 ± 0.005</td>
<td>0</td>
<td>(g)S- slightly gravelly sand</td>
<td></td>
</tr>
<tr>
<td>RSed_7</td>
<td>3.18 ± 1.13</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>7.99 ± 0.02</td>
<td>0.94 ± 0.04</td>
<td>10</td>
<td>9</td>
<td>0.2 ± 0.0009</td>
<td>99.8 ± 0.0009</td>
<td>0</td>
<td>S- Sand</td>
<td></td>
</tr>
</tbody>
</table>
### Table 2.3 (B): Clew bay Environmental Parameters

<table>
<thead>
<tr>
<th>Time</th>
<th>Site</th>
<th>NO\textsubscript{3}⁻ (µM) ± s.d.</th>
<th>NO\textsubscript{2}⁻ (µM) ± s.d.</th>
<th>NH\textsuperscript{4}⁺ (µM) ± s.d.</th>
<th>pH ± s.d.</th>
<th>TOC (% ± s.d.)</th>
<th>Temp (°C)</th>
<th>Salinity (ppt) ± s.d.</th>
<th>Gravel (%) ± s.d.</th>
<th>Sand (%) ± s.d.</th>
<th>Mud (%) ± s.d.</th>
<th>Eunis Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>APRIL</td>
<td>CSed_1</td>
<td>0.45 ± 0.78</td>
<td>2.96 ± 0.4</td>
<td>269.11 ± 31.07</td>
<td>7.53 ± 0.02</td>
<td>20.3 ± 0.23</td>
<td>11</td>
<td>32.13 ± 0.02</td>
<td>0</td>
<td>48.3 ± 0.02</td>
<td>51.7 ± 0.02</td>
<td>sM- sandy mud</td>
</tr>
<tr>
<td></td>
<td>CSed_2</td>
<td>1.35 ± 1.35</td>
<td>3.13 ± 0.26</td>
<td>45.11 ± 16.39</td>
<td>7.60 ± 0.2</td>
<td>14 ± 0.8</td>
<td>11</td>
<td>30.69 ± 0.0001</td>
<td>97.3 ± 0.006</td>
<td>2.7 ± 0.006</td>
<td>(m)S- slightly muddy sand</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CSed_3</td>
<td>0.45 ± 0.78</td>
<td>2.43 ± 0.15</td>
<td>7.78 ± 9.71</td>
<td>8.18 ± 0.04</td>
<td>14.59 ± 0.8</td>
<td>11</td>
<td>32 ± 0.0015</td>
<td>99.8 ± 0.0003</td>
<td>0.2 ± 0.0003</td>
<td>S- Sand</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CSed_4</td>
<td>No Sampling was carried out at this time point</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CSed_5</td>
<td>1.806 ± 0.78</td>
<td>2.52 ± 0.66</td>
<td>126 ± 36.44</td>
<td>7.56 ± 0.2</td>
<td>13.38 ± 1.99</td>
<td>11</td>
<td>33.41 ± 0.018</td>
<td>0</td>
<td>55.4 ± 0.009</td>
<td>44.6 ± 0.009</td>
<td>mS- muddy sand</td>
</tr>
<tr>
<td></td>
<td>CSed_6</td>
<td>1.80 ± 0.78</td>
<td>3.91 ± 0.26</td>
<td>122.89 ± 9.71</td>
<td>7.67 ± 0.02</td>
<td>15.88 ± 1.85</td>
<td>11</td>
<td>0.23 ± 0.0001</td>
<td>12.8 ± 0.01</td>
<td>89.3 ± 0.007</td>
<td>10.6 ± 0.007</td>
<td>gmS - gravelly muddy sand</td>
</tr>
<tr>
<td>AUGUST</td>
<td>CSed_1</td>
<td>1.35 ± 2.74e-16</td>
<td>2.35 ± 0</td>
<td>182 ± 51.33</td>
<td>7.87 ± 0.03</td>
<td>4.34 ± 0.2</td>
<td>22</td>
<td>34.35 ± 0.008</td>
<td>1.2 ± 0.008</td>
<td>41.7 ± 0.018</td>
<td>57.2 ± 0.02</td>
<td>(g)sM- slightly gravelly sandy mud</td>
</tr>
<tr>
<td></td>
<td>CSed_2</td>
<td>1.35 ± 2.74e-16</td>
<td>2.43 ± 0.4</td>
<td>56 ± 0</td>
<td>7.8 ± 0.16</td>
<td>0.78 ± 0.12</td>
<td>22</td>
<td>34.35 ± 0.0001</td>
<td>0.1 ± 0.0001</td>
<td>98.9 ± 0.006</td>
<td>0.4 ± 0.0008</td>
<td>S- Sand</td>
</tr>
<tr>
<td>CSed_1</td>
<td>0.6 ± 0.52</td>
<td>0</td>
<td>45.48 ± 55.38</td>
<td>7.68 ± 0.04</td>
<td>4.78 ± 0.67</td>
<td>9</td>
<td>36</td>
<td>27.5 ± 0.03</td>
<td>36.4 ± 0.05</td>
<td>36.1 ± 0.02</td>
<td>gmS- gravelly muddy sand</td>
<td></td>
</tr>
<tr>
<td>CSed_2</td>
<td>0.67 ± 0.71</td>
<td>0.522 ± 0.69</td>
<td>0 ± 0</td>
<td>8.03 ± 0.06</td>
<td>0.5 ± 0.09</td>
<td>9</td>
<td>36</td>
<td>0.3 ± 8.02e-5</td>
<td>99.5 ± 0.002</td>
<td>0.2 ± 0.0016</td>
<td>S- Sand</td>
<td></td>
</tr>
<tr>
<td>CSed_3</td>
<td>0.43 ± 0.76</td>
<td>0.96 ± 1.53</td>
<td>0 ± 0</td>
<td>7.89 ± 0.1</td>
<td>0.98 ± 0.2</td>
<td>9</td>
<td>36</td>
<td>1.8 ± 0.005</td>
<td>98.2 ± 0.005</td>
<td>0</td>
<td>(g)S- slightly gravelly sand</td>
<td></td>
</tr>
<tr>
<td>CSed_4</td>
<td>1.07 ± 1.54</td>
<td>1.04 ± 1.3</td>
<td>0 ± 0</td>
<td>8.03 ± 0.07</td>
<td>0.74 ± 0.03</td>
<td>9</td>
<td>36</td>
<td>0.5 ± 0.001</td>
<td>99.5 ± 0.001</td>
<td>0</td>
<td>S- Sand</td>
<td></td>
</tr>
<tr>
<td>CSed_5</td>
<td>0.56 ± 0.13</td>
<td>1.22 ± 0.92</td>
<td>95.15± 25</td>
<td>7.76 ± 0.03</td>
<td>3.18 ± 0.83</td>
<td>9</td>
<td>36</td>
<td>19.8 ± 0.03</td>
<td>61.4 ± 0.08</td>
<td>18.7 ± 0.04</td>
<td>gmS - gravelly muddy sand</td>
<td></td>
</tr>
<tr>
<td>CSed_6</td>
<td>1.58 ± 0.68</td>
<td>1.65 ± 2.64</td>
<td>0 ± 0</td>
<td>7.96 ± 0.04</td>
<td>2.74 ± 0.9</td>
<td>9</td>
<td>0</td>
<td>55.9 ± 0.03</td>
<td>38.9 ± 0.02</td>
<td>5.1 ± 0.008</td>
<td>msG- muddy sandy gravel</td>
<td></td>
</tr>
</tbody>
</table>

| CSed_3 | 0.90 ± 0.78 | 2.35 ± 0 | 62.22 ± 2.69 | 8.13 ± 0.03 | 0.87 ± 0.04 | 22 | 34.35 | 0.7 ± 0.0009 | 99.2 ± 0.001 | 0.1 ± 0.0002 | S- Sand |
| CSed_4 | 1.35 ± 0 | 2.26 ± 0.91 | 68.44 ± 9.71 | 8.26 ± 0.015 | 0.64 ± 0.06 | 22 | 34.35 | 0.3 ± 0.001 | 99.3 ± 0.0005 | 0.4 ± 0.0005 | S- Sand |
| CSed_5 | 3.16 ± 3.13 | 2.35 ± 0.52 | 158.67 ± 20.34 | 7.82 ± 0.03 | 2.58 ± 0.38 | 22 | 34.35 | 0.8 ± 0.004 | 60.2 ± 0.03 | 39 ± 0.03 | mS- muddy sand |
| CSed_6 | 1.80 ± 0.78 | 3.83 ± 0.8 | 108.89 ± 15 | 7.83 ± 0.02 | 1.45 ± 0.14 | 22 | 0.07 | 31.4 ± 0.03 | 62.9 ± 0.03 | 5.7 ± 0.003 | gmS - gravelly muddy sand |

<p>| N O V E M B E R | CSed_1 | 0 ± 0 | 1.57 ± 0.45 | 0 ± 0 | 7.85 ± 0.03 | 13.68 ± 4 | 8 | 34 | 22 ± 0.08 | 52.6 ± 0.07 | 25.4 ± 0.03 | gmS- gravelly muddy sand |
| CSed_2 | 0.1 ± 0.01 | 0.87 ± 0.6 | 0 ± 0 | 8.28 ± 0.042 | 0.54 ± 0.18 | 8 | 34 | 0.1 ± 0.0005 | 99.9 ± 0.0005 | 0 | S- Sand |</p>
<table>
<thead>
<tr>
<th>CSed_3</th>
<th>U A R Y</th>
<th>0.48 ± 0.84</th>
<th>1.22 ± 0.54</th>
<th>0 ± 0</th>
<th>7.75 ± 0.05</th>
<th>0.58 ± 0.09</th>
<th>8</th>
<th>34</th>
<th>2.2 ± 0.02</th>
<th>97.8 ± 0.02</th>
<th>0</th>
<th>(g)S- slightly gravelly sand</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSed_4</td>
<td>U A R Y</td>
<td>0.42 ± 0.73</td>
<td>4.43 ± 1.59</td>
<td>0 ± 0</td>
<td>8.43 ± 0.07</td>
<td>0.76 ± 0.03</td>
<td>8</td>
<td>34</td>
<td>0.1 ± 0.0003</td>
<td>98.1 ± 0.009</td>
<td>1.8 ± 0.009</td>
<td>(m)S- slightly muddy sand</td>
</tr>
<tr>
<td>CSed_5</td>
<td>U A R Y</td>
<td>0 ± 0</td>
<td>2.35 ± 0.78</td>
<td>182.44 ± 29.84</td>
<td>8.35 ± 0.02</td>
<td>12.99 ± 8.5</td>
<td>8</td>
<td>34</td>
<td>0.2 ± 0.0009</td>
<td>26.4 ± 0.05</td>
<td>73.4 ± 0.05</td>
<td>sM- Sandy mud</td>
</tr>
<tr>
<td>CSed_6</td>
<td>U A R Y</td>
<td>0.58 ± 0.34</td>
<td>1.39 ± 0.66</td>
<td>0 ± 0</td>
<td>8.05 ± 0.1</td>
<td>1.56 ± 0.34</td>
<td>8</td>
<td>0</td>
<td>10.7 ± 0.05</td>
<td>78.4 ± 0.07</td>
<td>10.9 ± 0.03</td>
<td>gmS - gravelly muddy sand</td>
</tr>
</tbody>
</table>
2.4.2 SPATIAL AND TEMPORAL VARIATION IN POTENTIAL NITRIFICATION RATES (PNR) AND AOA/AOB AMOA GENE ABUNDANCES

PNR is a measure of the nitrifying communities’ capacity to oxidise ammonia to nitrite. PNR was quantified to inform of changes in the activity of the nitrifying communities within and between bays temporally, reflecting potential changes in in situ nitrification rates. PNR in Rusheen (range 0 to 12.65 µg fs\(^{-1}\) d\(^{-1}\)) was up to a log-fold greater than Clew bay (range 0 to 2.14 µg fs\(^{-1}\) d\(^{-1}\)) (Fig. 2.2; Three-way ANOVA P < 0.001). Within Rusheen bay, PNR varied spatially increasing towards the muddy-sediments where PNR was highest (range 0.06 to 12.65 µg fs\(^{-1}\) d\(^{-1}\); P < 0.05), in all months except November. Within Clew bay PNR did not vary spatially with the exception of the freshwater site (CSed_6) which was significantly higher than all other sites in August (0.54 µg fs\(^{-1}\) d\(^{-1}\)) and November (0.97 µg fs\(^{-1}\)d\(^{-1}\); One-way ANOVA P < 0.05). Temporally, within each bay, there was no difference in PNR between April, August and November; however, PNR was higher in Rusheen bay, but lower in Clew bay in February than all other time-points (Fig. 2.2; Three-way ANOVA P < 0.05).
Figure 2.2: Spatial and temporal variation in Potential Nitrification Rates (PNR) (PNR NO$_2$ µg$^{-1}$ g$^{-1}$ day$^{-1}$) in Rusheen and Clew bay sediments (mean n=3, standard deviation indicated by error bars). PNR in Rusheen bay are located on the left y-axis, Clew bay is on the right y-axis. Sites are arranged in order of sandy sediments to muddy sediments. Four time points include rates measured in April, August, November 2013 and February 2014. Letters indicate significant differences between sites within a bay (One-way ANOVA P < 0.05), Greek
letters indicate significant differences between time points and the asterisk symbols indicate significant differences between bays (Three-way ANOVA $P < 0.05$).

AOB $amoA$ gene abundances ranged from $1.89 \times 10^5$ to $2.5 \times 10^7$ total average $3.81 \times 10^6 \pm SD 1.5 \times 10^7$ and AOA from $1.21 \times 10^4$ to $6.29 \times 10^7$ (average $2.27 \times 10^6 \pm SD 1.14 \times 10^6$) copies per gram of sediment (Fig. 2.3). In contrast to the lower PNR in Clew bay (Three-way ANOVA $P < 0.001$), AOB $amoA$ gene abundances did not vary between bays (Three way ANOVA $P = 0.180$), although some variation in AOA $amoA$ gene abundances was observed (Three-way ANOVA $P < 0.05$). Spatially, within Rusheen bay, some variation in AOB $amoA$ gene abundance was seen (One-way ANOVA $P < 0.05$) but there was no general trend. AOA $amoA$ gene abundances were similar within Rusheen bay, with the exception of a small number of sites; for example, AOA $amoA$ gene abundances tended to be significantly lower in the sandier than the muddier sediment sites over several time points. Spatially within Clew bay, AOA and AOB $amoA$ gene abundances were similar across the bay, with some differences observed between the sandier and muddier sites (Fig. 2.3; One way ANOVA $P < 0.05$).

Temporally, within Rusheen bay, AOB $amoA$ gene abundances were similar in the winter months (November and February) but were significantly different between April and August. AOA $amoA$ gene abundances varied between each time point (Two-way ANOVA $P < 0.001$). In Clew bay, while no temporal variation was observed in AOB $amoA$ gene abundances, AOA $amoA$ gene abundances varied between each sampling time point (Two-way ANOVA $P < 0.001$).
Figure 2.3: Spatial and temporal variation in AOA and AOB amoA gene abundances in Rusheen and Clew bay sediments. Black and red bars indicate log_{10} amoA gene copy numbers gene in bacteria and archaea, respectively. The black and red letters represent significant differences across sites within a bay for the AOB and AOA assays, respectively (One-way ANOVA P < 0.05). Greek letters indicate significant differences between timepoints and are beside the
corresponding bay, and the asterisk symbols indicate significant differences between bays (Two-way ANOVA $P < 0.05$).

AOB gene abundances correlated positively with PNR, *in situ* TOC and *in situ* pH ($r = 0.509, P < 0.001$; $r = 0.434, P < 0.001$; $r = 0.315 P < 0.05$); and negatively with temperature and NH$_4^+$ concentration ($r = 0.459 P < 0.001$; $r = 0.404 P < 0.001$). AOA gene abundances positively correlated with *in situ* pH and temperature ($r = 0.69 P < 0.001$; $r = 0.242 P < 0.05$) and negatively with *in situ* salinity and NH$_4^+$ concentration ($r = -0.245, P < 0.05$; $r = -0.438, P < 0.001$). PNR positively correlated with *in situ* TOC and sediment salinity ($r = 0.223, P < 0.05$; $r = 0.265, P < 0.05$; Table 2.4)

**Table 2.4: Significant Spearman Rank Correlations carried out on all time-points in Rusheen bay dataset (temporally)**

<table>
<thead>
<tr>
<th></th>
<th>PNR</th>
<th>TOC</th>
<th>Salinity</th>
<th>pH</th>
<th>Temp</th>
<th>NH$_4^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PNR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation Coefficient</td>
<td>$-$</td>
<td>0.223</td>
<td>0.265</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
</tr>
<tr>
<td>Significance (2-tailed)</td>
<td>$-$</td>
<td>0.041</td>
<td>0.015</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
</tr>
<tr>
<td><strong>AOA gene abundance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation Coefficient</td>
<td>$-$</td>
<td>$-$</td>
<td>-0.245</td>
<td>0.69</td>
<td>0.242</td>
<td>-0.438</td>
</tr>
<tr>
<td>Significance (2-tailed)</td>
<td>$-$</td>
<td>$-$</td>
<td>0.025</td>
<td>0.000</td>
<td>0.026</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>AOB gene abundance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation Coefficient</td>
<td>0.509</td>
<td>0.434</td>
<td>$-$</td>
<td>0.315</td>
<td>-0.459</td>
<td>-0.404</td>
</tr>
<tr>
<td>Significance (2-tailed)</td>
<td>0.000</td>
<td>0.000</td>
<td>$-$</td>
<td>0.004</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td>84</td>
<td>84</td>
<td>84</td>
<td>84</td>
<td>84</td>
<td>84</td>
</tr>
</tbody>
</table>

Transcript abundance was not completed for all time-points and so is not included here.
2.4.3 ABUNDANCE AND COMMUNITY STRUCTURE OF amoA TRANSCRIPTS IN RUSHEEN BAY SEDIMENTS

Rusheen bay, February 2014 was selected to further investigate the diversity and abundance of amoA DNA (total) and mRNA (active) from AOA and AOB. AOB amoA transcripts ranged from $3.25 \times 10^9$ – $6.14 \times 10^{11}$ per gram of sediment with an average ± SD of $2.86 \times 10^{11} \pm 2.57 \times 10^{11}$, were 3 - 4 orders of magnitude greater than AOA amoA transcripts ($3.84 \times 10^6$ – $1.92 \times 10^8$ per gram of sediment, $4.8 \times 10^7 \pm 1.1 \times 10^8$; Fig. 2.4).

Figure 2.4: Log$_{10}$ AOA and AOB transcript numbers g$^{-1}$ sediment for February 2014 in Rusheen bay. AOB numbers are indicated by the black squares while AOA are indicated by the red circle. The black letters represent the significant differences between sites across Rusheen bay for AOB transcripts (One-way ANOVA P < 0.001).

AOB amoA transcripts increased from the sandier to muddier sediments (One-way ANOVA P < 0.001). AOA amoA transcripts were quantified from all sites, but no spatial difference was observed (One-way ANOVA P = 0.126).
AOB amoA transcripts correlated positively with PNR ($r = 0.868$, $P < 0.001$), while AOA did not; in addition, AOB amoA transcripts and in situ nitrate concentrations were significantly correlated ($r = 0.444$, $P < 0.05$) (Spearman rank correlations; Table 2.5).

Table 2.5: Significant Spearman Rank Correlations carried out on February 2014 dataset (spatially)

<table>
<thead>
<tr>
<th></th>
<th>PNR</th>
<th>TOC</th>
<th>Sediment Salinity</th>
<th>pH</th>
<th>NO₃⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PNR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Correlation Coefficient</td>
<td>-</td>
<td>0.651</td>
<td>0.573</td>
<td>0.530</td>
<td>-</td>
</tr>
<tr>
<td>Significance (2-tailed)</td>
<td>0.001</td>
<td>0.007</td>
<td>0.014</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>-</td>
</tr>
<tr>
<td><strong>AOB gene abundance</strong></td>
<td>0.873</td>
<td>0.697</td>
<td>0.677</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Correlation Coefficient</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significance (2-tailed)</td>
<td>0.000</td>
<td>0.000</td>
<td>0.001</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>-</td>
</tr>
<tr>
<td><strong>AOA gene abundance</strong></td>
<td>0.515</td>
<td></td>
<td>0.463</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Correlation Coefficient</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significance (2-tailed)</td>
<td>0.017</td>
<td></td>
<td>0.034</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N</td>
<td>21</td>
<td></td>
<td>21</td>
<td>21</td>
<td>-</td>
</tr>
<tr>
<td><strong>AOB transcript abundance</strong></td>
<td>0.868</td>
<td>0.553</td>
<td>-</td>
<td>-</td>
<td>0.444</td>
</tr>
<tr>
<td>Correlation Coefficient</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significance (2-tailed)</td>
<td>0.000</td>
<td>0.009</td>
<td>-</td>
<td>-</td>
<td>0.044</td>
</tr>
<tr>
<td>N</td>
<td>21</td>
<td>21</td>
<td>-</td>
<td>-</td>
<td>21</td>
</tr>
</tbody>
</table>

To determine if the abundant AOB amoA transcripts represented different phylotypes among sampling sites, T-RFLP was conducted. Canonical correspondence analysis (CCA) revealed four different groupings of AOB amoA transcripts (Fig. 2.5; $r = 0.754$, $P < 0.001$). The sandy sites, Rsed_3 and Rsed_5, had similar cDNA phylotypes present ($r = 0.333$, $P < 0.05$), as did the muddier sediment sites, Rsed_1 and Rsed_2 ($r = 0.37$, $P < 0.05$). All other sites were significantly different from each other. CCA axis 1 was represented by PNR ($f = 1.59$, $P = 0.02$), mud sediment size ($f = 2$, $P = 0.002$) and TOC ($f = 2.05$, $P = 0.004$) which explained 37.6% of the variance, separating the muddier, higher PNR sites of Rsed_1 and Rsed_2. Axis 2 indicated that salinity ($f = 1.61$, $P =
explained 26.1% variance of active AOB phylotypes separating Rsed_7 (low salinity site) from Rsed_3 and Rsed_5 (high salinity sites; Fig. 2.5).

Figure 2.5: Canonical correspondence analysis illustrates the separation of AOB cDNA amoA gene TRFs based on physical/chemical parameters measured. The different colours represent an individual site. The most significant parameters are represented by the labelled vectors. The abbreviations PNR and TOC represent the potential nitrification rates and total organic carbon, respectively.

2.4.4 *amoA* gene phylogeny of total and active ammonia oxidisers within Rusheen Bay sediments

Based on CCA analysis (Fig. 2.5), one site from each cluster was selected for *amoA* AOA/AOB gene and transcript clone libraries (Rsed_1, 5, 6 & 7). In total, 68 AOB DNA and 197 cDNA *amoA* sequences and 61 AOA DNA and 32
cDNA, correctly translating to protein were recovered. From these, thirty-six DNA and 41 cDNA AOB OTUs and 19 DNA and 8 cDNA AOA OTUs at 97% similarity were identified (Wang et al., 2015a) (Table 2.6). amoA AOB transcripts were retrieved from all sites, but amoA AOA transcripts were only recovered from the sandy sediment sites RSed_5 (high salinity) and RSed_6 (variable salinity).

Table 2.6: Number of clones sequenced per library, and identity at OTU level

<table>
<thead>
<tr>
<th>Samples</th>
<th>DNA AOB</th>
<th>DNA AOA</th>
<th>cDNA AOB</th>
<th>cDNA AOA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Clones</td>
<td>% Identity</td>
<td>No. of Clones</td>
<td>% Identity</td>
</tr>
<tr>
<td>RSed_1</td>
<td>24</td>
<td>15</td>
<td>31</td>
<td>9</td>
</tr>
<tr>
<td>RSed_5</td>
<td>23</td>
<td>12</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>RSed_6</td>
<td>19</td>
<td>8</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>RSed_7</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
<td>36</td>
<td>61</td>
<td>19</td>
</tr>
</tbody>
</table>

For AOB, the majority of OTUs fell into a Nitrosomonas-like group (clusters III, IV and V; Fig. 2.6) alongside amoA sequences from globally distributed coastal and estuarine sediments. Cluster III has two sub-clusters; a DNA only cluster with sequences from three sites, and a cDNA only cluster from the high salinity mud site. Cluster IV contains DNA and cDNA from all sites, highly similar to Nitrosomonas marina, aestuarii & oligotropha. Cluster V, closely related to Nitrosomonas ureae, has the majority of AOB sequences; it contains 14 OTUs representing 72.5% of all sequences. It also contains the most abundant cDNA phylotypes (amoABacD4) recovered from all sites. Of note, this phylotype was also retrieved at DNA level, in lower abundance, from three of the four sites, but interestingly not RSed_7, which had the highest number of cDNA sequences. Cluster VI and VII are cDNA only clusters, composed of 22 OTUs from the high salinity muddy sediment site and the variable salinity sandy site. This cluster is represented only by environmental sequences from this study, and is not similar to sequences retrieved from comparable environments nor any currently isolated AOB. Cluster I contains three OTUs derived from DNA similar to Nitrosospira briensis, and cluster VIII a single OTU from the sandy sediment site.
Figure 2.6: Neighbour-joining phylogenetic tree of AOB amoA genes at protein level (≥ 97% similarity). Bootstrap values shown are greater than 70% of 1000 which are shown near nodes. Accession numbers from GenBank are shown from other studies and isolates. Circles indicate DNA sequences, triangles specify cDNA sequences. The numbers in brackets indicate the number of sequences recovered from that site. The colours maroon, yellow, blue and aqua signify the sampling sites Rsed_1, Rsed_5, Rsed_6 and Rsed_7, respectively.

AOA were less abundant and diverse than AOB (Fig. 2.7). No AOA amoA sequences were retrieved from site Rsed_7, due to insufficient amplification. The
majority of AOA fell into group I.Ia, with a single OTU from Rsed_5 falling into group I.Ib. The group I.Ib sequence was similar to AOA amoA from mangrove sediments and Can. Nitrososphaera evergladensis and gargensis. Group I.Ia, was composed of six sub-clusters, three of which contained OTUs retrieved from both DNA and cDNA. Cluster I, closely related to Can. Nitrosoarchaeum koreensis MY1, contained the majority of sequences, including DNA OTUs from all sites, and three cDNA OTUs from Rsed_6, the variable salinity site. Cluster II had a single DNA OTU from Rsed_6, similar to an environmental clone from a wastewater treatment plant. Cluster IV, also contained a single OTU from this site, this time represented by both DNA and cDNA. Cluster III contained a single DNA phylotype from Rsed_1, which clustered with Nitrosopumilus maritimus, and two candidate Nitrosopumilus sp. Cluster VI contained five DNA only OTUs from Rsed_1 and Rsed_5, in addition to three cDNA only OTUs, including the highly abundant cDNA OTU (amoAArchR1) primarily retrieved from Rsed_6, the variable salinity site, but also detected at Rsed_5 (sandy sediment site). No matching DNA phylotype was retrieved. These sequences were most closely related to AOA from intertidal sediments (Zheng et al., 2013). Clusters V and VI contained DNA OTUs from Rsed_1, highly similar to OTUs from intertidal mudflats and estuary sediments (Chen et al., 2014).
Figure 2.7: Neighbour-joining phylogenetic tree of AOA *amoA* genes at protein level (≥97% similarity). Bootstrap values shown near nodes and are greater than 70% of 1000. Accession numbers from GenBank are shown from other studies and isolates. Circles indicate DNA sequences, triangles indicate cDNA sequences. The numbers in brackets indicate the number of sequences recovered from that site. The colours maroon, yellow, blue and aqua signify different sampling sites Rsed_1, Rsed_5, Rsed_6 and Rsed_7 respectively.
2.5 DISCUSSION

Here we present AOB and AOA dynamics within two small intertidal bays over an annual period and further quantify and compare amoA from AOA and AOB at gene and transcript level from intertidal sediments.

Within both bays, at DNA level, AOB amoA gene abundances were 1 to 2 orders of magnitude greater than AOA amoA at all four time points surveyed over an annual period. amoA gene copies per cell in AOB range from 1 to 3 (Norton et al., 2002), a single copy is present in AOA (Berg et al., 2015; Walker et al., 2010; Zhalnina et al., 2014). For AOA to be considered the main contributor of ammonia oxidation, it has been suggested that AOA must be 10-fold higher than AOB gene abundances (Prosser and Nicol, 2012). In this study, AOB gene corrected for maximum amoA gene copies still outnumber AOA.

AOB amoA gene abundances from this study were similar in range to other coastal bay or intertidal sediments (Li et al., 2015; Lisa et al., 2015; Yu et al., 2016; Zheng et al., 2014). In addition, these studies found AOB to be more abundant than AOA (Li et al., 2015; Lisa et al., 2015; Yu et al., 2016; Zheng et al., 2014). In contrast, in submerged coastal and estuarine sediments and the water column AOA are more abundant than AOB (Damashek et al., 2015; Lipsewers et al., 2014; Mincer et al., 2007; Santoro et al., 2010; Zhang et al., 2015a; Zheng et al., 2013). AOA amoA gene copy numbers from this study were high but comparable to other coastal sediment studies (Santoro et al., 2008; Yu et al., 2016). amoA gene abundance surveys are conducted as proxies for cell abundance, in turn indicating the predominant driver of ammonia oxidation and molecular proxies for nitrification rates. The rationale being that numerically abundant AO are likely the primary drivers of ammonia oxidation. Our study showed that AOB were more abundant than AOA spatially and temporally in Rusheen and Clew bay. AOB gene abundances correlated significantly with PNR, potentially rendering AOB the main contributors to the nitrifying activity found in these marine intertidal coastal sediments both spatially and temporally (Fig. 2.2 and 2.3). However, in Clew bay rates of nitrification activity were not reflected by gene abundances - there was no temporal variation at all in AOB.
gene copy numbers and AOA only showed temporal variation in August and November (Fig. 2.2 and 2.3). This observation raises some interesting points about why AOB and AOA would be present in Clew bay if they are not actively nitrifying to their full capacity and if gene abundances are a suitable proxy for nitrification activity. However, as the detection of a gene does not imply function, we targeted amoA mRNA transcripts to provide a closer link to identifying active ammonia oxidisers than gene quantification alone. DNA is associated with active, dormant and dead cells, therefore the detection of mRNA expression is widely accepted as a stronger indication of activity of the associated function (Philippot et al., 2005), albeit that post-translational modifications and environmental factors may impede protein formation and ultimate activity.

AOB and AOA amoA transcripts were present in in situ samples in Rusheen bay, February 2014, indicating transcriptionally active AOB and AOA were present within the intertidal sediments (Fig. 2.4). Transcript numbers for both AOB and AOA were higher than gene copy numbers suggesting they were producing multiple transcripts per cell or the presence of highly active phylotypes. The gene and transcript clone libraries suggest the latter. Studies from oyster ground sediments and coastal microbial mats in the North sea showed AOB transcripts were more abundant than AOA transcripts (Fan et al., 2015; Lipsewers et al., 2014). On the other hand in Antarctic coastal waters AOA genes and transcripts were more abundant than AOB genes and transcripts (Tolar et al., 2016). As observed at gene level in this study, AOB transcripts were greater than AOA indicating, that within Rusheen bay gene abundance was a good proxy for activity and that AOB are the likely primary drivers of AO within these intertidal sediments. We suggest this based on consistently higher spatial and temporal gene abundances of AOB, in addition to the higher amoA transcripts numbers, and significant correlation of AOB amoA genes and transcripts with PNR, with transcript abundance resulting in a stronger correlation (gene r = 0.868, P < 0.001; transcript r = 0.873, P < 0.001; Table 2.5). While AOA amoA gene abundances also correlated significantly with PNR, AOA transcripts did not, nor with any other parameters (r = 0.515, P < 0.05;
Table 2.5). Tolar et al., (2016) suggested that AOA rates may not correlate with transcripts due to mRNA degradation since AOA mRNA have a shorter rate of decay than AOB (Bollmann et al., 2005; Nakagawa and Stahl, 2013; Tolar et al., 2016). Furthermore, PNR assays may favour AOB due to elevated ammonia concentrations used in the microcosm. However, the presence of AOA transcripts is a strong indication that they also are contributing to AO in intertidal sediments. In sediments of the Yangtze (China) and the Colne (UK) estuary, where amoA AOB were numerically more abundant than AOA (Li et al., 2015; Zheng et al., 2013), PNR assays amended with bacterial inhibitors (ampicillin and ATU) indicated a small contribution to AO from ATU/ampicillin sensitive AO. These may be AOA, however no genetic evidence to support this was presented in these papers. Nevertheless, these studies and the quantification of amoA AOA transcripts here indicate that AOA may be active in AOB dominant coastal sediments.

We also provide evidence of active ammonia oxidisers (mRNA) different to total (DNA) ammonia oxidisers, which adds new understanding and interpretation of active ammonia oxidisers in coastal ecosystems. Corresponding transcripts for the majority of AOB DNA OTUs (at 97% similarity) were not detected; however, the sequencing depth was low due to very low yield of PCR product (Table 2.6). The two AOB OTUs (amoAbacD5 and D4), found at DNA and/or mRNA level from all sites, were highly abundant in the cDNA clone libraries, indicating they are important contributors to AO in intertidal sediments. These two active OTUs grouped in a *Nitrosomonas urea* like cluster. *N. urea* like phylotypes have been reported as the dominant ammonia oxidiser in estuarine environments (Cébron et al., 2003; Stehr et al., 1995). These ecosystems are often characterised high ammonia concentrations. *N. ureae* and *N. oligotrophy*-like groups adapt to high initial ammonium concentrations (up to 10 mM) and due to their high affinity for ammonia they can grow at low ammonia concentrations (limiting concentration of 6 µM; Bollmann and Laanbroek, 2001). They are inhibited by ammonia above 10 mM typically only found in wastewater treatment plants (Bollmann and Laanbroek, 2001). This range is consistent with
ammonia concentrations in Rusheen bay where *in situ* concentrations never went above 1.2 mM NH$_4^+$.

We also report a large novel cluster of AOB, represented only by *amoA* transcripts, from the muddy sand and variable salinity sites. These were not detected in the DNA clone libraries, and are therefore not numerically dominant, albeit with low library coverage. Likely NGS sequencing of the *amoA* amplicons would detect them, but this less abundant but transcriptionally active group, even if detected by deeper sequencing effort, would not be regarded as a numerically dominant ammonia oxidiser in a gene only approach. Furthermore, this cluster is unique and unlike the DNA *amoA* gene profile and has not previously been detected in other environments. The most closely related AMO protein to these two clusters was between 89% and 99% similarity.

At DNA level, the community composition of AOB within Rusheen bay sediments was similar to other intertidal and coastal sediments globally (Bernhard et al., 2005b; Cébron et al., 2003; Stehr et al., 1995; Zheng et al., 2014). Clusters I and III were represented at DNA level only, indicating they may not be active or were transcribing at a low level below the detection limits and sensitivity of the approach. In addition clusters VI and VIII were composed of AO sequences recovered from transcripts only, without corresponding DNA sequences, indicating the presence of low abundance, but actively transcribing AOB. Therefore, a DNA and mRNA approach is required to reveal the diversity of, and differences between, total and active AO. When *amoA* transcripts are included, a different picture of rare, but active AO is revealed compared to a DNA only study, raising further questions in relation to presence of dominant but inactive (redundant) AO, in addition to contribution of less abundant but transcriptionally active AO (Wang et al., 2016). AOA diversity was lower, with gene and transcript *amoA* sequences primarily falling into group I.Ia, and similar to AOA retrieved from other coastal and marine sediments. AOA phylotypes from cluster I, represented by *amoA* genes and transcripts, clustered with *Can. Nitrosoarchaeum koreensis* MY1 from a rhizosphere soil enrichment (Kim et al., 2011; Fig. 2.7). *N. koreensis*, enrichment is inhibited by high salinity and grows
without inhibition up to 5 mM NH$_4^+$ and has a high affinity for ammonia 11 µM (Jung et al., 2011). While OTUs from this cluster were present at both the high and variable salinity sites, transcripts were only recovered from the variable salinity site at the estuary mouth (Rsed_6, 0 ppt), with a low ammonia concentration of 0.39 µM. Indeed, the majority of AOA amoA cDNA transcripts were recovered from this site. The most abundant amoA transcript (amoAArchR1, cluster V) was not present at DNA level. The DNA-only clusters (Cluster II, III, VI & VII) mostly comprised of clones from the muddy sediment site, from which the highly active mRNA AOB transcript cluster was recovered, but not AOA transcripts. While RT-Q-PCR quantified amoA transcripts from all sites, we were not able to amplify cDNA by end-point PCR from AOA amoA from this site. RT-Q-PCR is more sensitive and lower quantities of template can be detected. RT-Q-PCR Cq values for amoA transcripts were low, but always a log above the NTC (Smith and Osborn, 2009), and displayed the diagnostic melting curve for AOA amoA amplicons.

The final discussion point deals with understanding AOA and AOB community dynamics. What are the environmental drivers that are influencing the AO communities in intertidal coastal bay sediments? Substrate concentration is a major factor influencing AO; as such any environmental parameters that control substrate concentration are inevitably going to impact AO communities. Temperature, salinity and pH are three main environmental factors that directly regulate ammonia concentration and can place stress on cells. Temperature affects ammonia solubility and of course oxygen solubility in water; consequently, the lower the temperature the less ammonia and oxygen is available to AO (Bower and Bidwell, 1978; Table 1.3). In addition, AOA and AOB communities are made up of numerous different phylotypes that may have different optimal growth temperatures as shown by the cultivation of AOA where two *N. devanaterra* strains grew at a different optimal temperature (35 and 25°C) and *N. europea* an AOB isolate grew optimally at 30°C (Groeneweg et al., 1994; Lehtovirta-Morley et al., 2011). In this study, there are indications of temperature fluctuations influencing AOB and AOA communities. For example, spearman rank correlations show temperature negatively correlating with AOB gene
abundances and positively correlating with AOA gene abundances (Table 2.4), further suggesting that AOB and AOA communities may have different optimal growth temperatures and perhaps rely on different optimal substrate concentrations to grow to their full potential. Negative correlations of AOB and temperature were also shown in sediments from the Yangtze estuary (Zheng et al., 2014). Additionally, Salinity was shown to affect ammonia or benthic fluxes and act as an environmental stressor to cells through osmoadaptation, as discussed in section 1.4.1 and 1.4.5 (Bower and Bidwell, 1978; Roeßler and Müller, 2001; Weston et al., 2010). AOA gene abundance shows a negative correlation with high salinity over four time-points indicating AOA within intertidal sediments may prefer low salinity ecosystems (Table 2.4; Damashek et al., 2015). Moreover, salinity is a significant driver in the AOB community at transcript level (Fig. 2.5) signifying the AOB community may have specific high and low salinity phylotypes present in response to salinity. However, there was no specific trend in AOB transcript abundances. In contrast, in a study where coastal sediments were incubated with various salinities and AOA and AOB transcriptional response were measured, AOB were not sensitive to salinity changes but, they exhibited lower transcriptional activity as salinity increased (Zhang et al., 2015b). However, the AOB transcript community was not sequenced in this study (Zhang et al., 2015b). Lastly, pH influences ammonia oxidisers due to the role it plays in controlling the equilibrium of the NH$_4^+$: NH$_3$ ratio (Stein et al., 1997); the higher the pH the more NH$_3$ is available. Within Rusheen bay, there were some significant differences in AOB amoA gene abundance spatially. AOB gene copy numbers positively correlated with pH where the highest recorded pH was 8.27 and negatively correlated with NH$_4^+$ concentration. These correlations were also seen in the Yangtze estuary and Western English channel sediment (Table 2.4; Laverock et al., 2014; Zheng et al., 2014).

Another interesting point in this study is the difference in nitrification rates and gene abundances between bays. Rusheen and Clew bay were chosen from an urban and rural setting as described in the materials and methods section 2.3.1. AOA and AOB amoA gene abundances were similar in both bays (Fig. 2.3),
however PNR rates were significantly lower in Clew bay at all timepoints (Fig. 2.2). We see evidence of NH$_4^+$ concentrations dropping in Clew bay in November and February timepoints where the majority of sites had NH$_4^+$ at non-detectable levels and only one site (Csed_5) had high NH$_4^+$ concentrations in both November and February (95.15 - 182.44 µM NH$_4^+$; Table 2.3 B). From mid-September to mid-January there is a ban on fertilisers or manure spreading in Ireland according to the EU nitrates directive (91/676/EEC). This could be one reason why we see similar levels of NH$_4^+$ concentrations to the urban setting in Rusheen bay in the summer months (7.78 – 269.11 µM NH$_4^+$ in clew bay 12.44 – 304.99 µM NH$_4^+$ in Rusheen bay; Table 2.3 A, B). However, this does not explain low PNR rates throughout the entire year in Clew bay, perhaps the microbial community in Clew bay is significantly different to Rusheen bay and nitrifies ammonia at a much lower rate. A study carried out in soil microcosms tested the effect of different ammonia concentrations on soil AO communities, they looked at the influence of in situ ammonia concentration (no ammonia addition approximately < 0.5 µg NH$_4^+$ g$^{-1}$ soil), an intermediate ammonia concentration (20 µg NH$_4^+$ g$^{-1}$ soil) and high ammonia concentration (200 µg NH$_4^+$ g$^{-1}$ soil; Verhamme et al., 2011). They found that the communities shifted over time (from day 0 – to day 28) and were not the same as in situ communities, however, they also shifted due to changes in NH$_4^+$ concentrations. AOB communities were different from each other at each ammonia concentration while AOA were similar at low and intermediate ammonia concentrations but the community shifted at the high ammonia concentration. This shows community dynamics for both AOA and AOB change under varied ammonia concentrations. This shows that it is possible that Rusheen bay communities were more attuned to higher concentrations of ammonia therefore the PNR rate assay may have suited Rusheen bay sediment AO communities more than Clew bay AO communities (250 µM NH$_4^+$). Finally the natural geology of Clew bay contains large amounts of arsenopyrite (Gilligan et al., 2016). The oxidation of arsenopyrite can occur near the surface of the mineral and leads to arsenites and arsenates being formed which can leach into ground water thereby introducing the heavy metal arsenic into nearby waterways and
groundwater. Concentrations of arsenic have been measured in drinking water wells near Clew bay where concentrations ranged from 6.75 – 200 µg l⁻¹.

Within Rusheen Bay sediments, AOB and AOA transcripts are highly similar to known ammonia oxidisers’ N. ureae and N. koreensis MY1. AOA and AOB sequences closely related to N. ureae and N. koreensis were detected from the variable salinity site RSed_6. This prompts us to think about why both AOA and AOB are co-occurring. Niche differentiation strategy hypothesis facilitates this (Prosser and Nicol, 2012). We hypothesise that AOA and AOB are occupying separate niches within the same site, thus maintaining diversity within the ecosystem. The active amoA phylotypes recovered as transcripts were highly similar to AOB and AOA isolates N. ureae and N. koreensis MY1 as mentioned above. These isolates have different requirements for ammonia, and thus could occupy different niches when it comes to ammonia concentration. N. ureae can grow between 6 µM – 10 mM NH₄⁺ while N. koreensis grows without inhibition up to 5 mM and 10 mM with significant inhibition (Bollmann and Laanbroek, 2001; Jung et al., 2011). Therefore, both could persist in the same environment as they are not limited or inhibited by the ammonia concentrations present in situ (below 1.2 mM NH₄⁺). In any case, amoA transcripts highly similar to both of these AO were detected in situ providing strong evidence that both are potentially active at the same time (Fig. 2.6 and 2.7). Further work in this area using activity based analysis is required in order to test the niche differentiation strategy hypothesis, while incorporating different environmental drivers such as pH, temperature, salinity and varying concentration of ammonia and monitoring how the AO communities change over time in response to the environmental stimuli.

Our results demonstrated that AOB were the dominant group of ammonia oxidisers, they were more abundant at DNA and cDNA level than AOA and correlated with PNR rates. However, AOA were also active and likely contributing to nitrification as AOA gene abundances correlated with PNR rates and AOA transcripts were present in situ. Ammonia concentration in situ was suitable for AOB and AOA growth and showed a negative correlation with both
AOB and AOA gene abundances, indicating that ammonia concentration was a driver. Environmental conditions such as salinity, pH and temperature were discriminating factors driving the distribution of AOB and AOA communities within the sediments. AOB and AOA communities showed different responses to each parameter in terms of gene abundance correlations. Nitrification rates and AOA and AOB gene abundances positively correlated with pH which agrees with our hypothesis. Temperature also influenced AOB gene abundances more so than AOA gene abundances. Finally, salinity seemed to be the main driver of AOB community variability at transcript level demonstrating the separation of phylotypes at different salinities. Low and high salinity phylotypes were not clearly distinguished when looking at sequence data, possibly because the sequence depth was too low. However, sequence data did identify the most abundant active phylotypes (cDNA) and the most abundant phylotypes at DNA level which were closely related to *N. ureae* and *N. koreensis* for AOB and AOA, respectively. Salinity also had strong correlations with PNR rates and AOA and AOB gene abundances further prompting us to delve deeper and explore AOA and AOB community changes along a natural salinity gradient in the next chapter.
Coastal ecosystems are important to understand as they are heavily influenced by anthropogenic activity (Galloway and Cowling, 2002). Humans are constantly utilising coastal ecosystems and the resources they provide, such as food, power, transportation and waste disposal, making them an essential and valuable resource. Coastal environments are characterised by salinity, which is an important driver that separates terrestrial and marine systems. As mentioned in section 1.4.5 microorganisms must be specially adapted to saline conditions as it can cause osmotic stress and cell toxicity (Roeßler and Müller, 2001). To this end, Kinvarra bay was chosen as our study site based on its natural salinity gradient due to groundwater influences; no other source of freshwater enters the bay (Cave and Henry, 2011). Kinvarra bay is also heavily impacted by anthropogenic activity. Untreated sewage is currently entering Kinvarra bay due to poor wastewater treatment facilities. Irish water aims to upgrade and have a fully functional wastewater treatment plant running in Kinvarra by winter 2017. The population of Kinvarra is relatively small (620 people living there since 2011; Irish census 2011). However, untreated sewage entering Kinvarra bay may be contributing a significant source of fixed nitrogen (Smith and Cave, 2012). Bacterial, archaeal and nitrifier populations may be influenced by changes in salinity and high fixed nitrogen in the bay. Coastal sediments have diverse communities of microorganisms that vary across space and time due to influencing abiotic factors (Bernhard et al., 2005a; Bolhuis et al., 2014; Bolhuis and Stal, 2011; Webster et al., 2015; Xiong et al., 2012). The majority of these microorganisms are unculturable and so culture independent techniques are required to monitor changes in community composition in the environment (Ansorge, 2009). More specifically we can gain a better understanding of what external abiotic factors, like temperature, pH and salinity, influence microbial community change. Many studies look at bacterial community changes across
different environmental gradients such as nutrients, pH, temperature and salinity at DNA level only (Bernhard et al., 2005a; Bolhuis et al., 2014; Xiong et al., 2012). The problem with targeting DNA only is that it not only targets active populations but also targets cells that are dead, degrading and dormant (Cangelosi and Meschke, 2014). On the other hand, targeting RNA allows us to identify the active community, as the microorganisms can only actively transcribe when alive (Philippot et al., 2005); thus, RNA is an indicator of active or recently active cells (Blazewicz et al., 2013). A similar study on bacterial microbial community structure was carried out on soil focusing on community dynamics at both DNA level and RNA level (Mikkonen et al., 2014). Another study measured indexes of activity and potential growth rate using 16S rRNA relative abundances and the ratio of rRNA:rDNA in sediment down along the salinity gradient (Campbell and Kirchman, 2013). Moreover, few studies have looked at archaeal community change across an estuary (Bolhuis and Stal, 2011; Hugoni et al., 2013; Webster et al., 2015). The aim of this study is to understand total (DNA) and active (RNA) bacterial and archaeal community dynamics along a natural salinity gradient in Kinvarra bay and to elucidate the main environmental influences that drive these changes. Secondly, we aimed to understand how the nitrifying community responds to abiotic factors by targeting nitrifiers specifically during 16S rRNA analysis. Furthermore, we aimed to target ammonia oxidisers at functional gene level (amoA) in order to focus on how the ammonia oxidiser communities were influenced and varied across the bay.

3.2 AIMS

The objectives of the study were to identify total and active bacterial and archaeal communities along a natural salinity gradient at 16S rRNA level and functional amoA gene level to measure concentrations of environmental parameters, nitrification potential as well as AOA and AOB amoA gene abundances within Kinvarra bay. Moreover, we aimed to determine the influence environmental parameters have on total and active bacterial, archaeal and nitrifier microbial communities. In Chapter 2 we saw that intertidal bays are highly complex and dynamic ecosystems and are influenced by a range of
environmental parameters, including salinity. We therefore postulated that salinity would strongly influence AOA and AOB communities and drive potential nitrification rates. Furthermore, we hypothesised that total and active communities would also be impacted by various environmental factors, including salinity.

3.3 MATERIALS AND METHODS

3.3.1 SITE DESCRIPTION AND FIELD SAMPLING

This study was carried out in Kinvarra bay (53°08’30.4”N, 8°55’56.6”W) located in the west coast of Ireland in April 2015 (Fig. 3.1). Kinvarra bay was chosen as it is influenced by freshwater arising from ground water upwelling, which causes significant changes in salinity and nutrient input (Cave and Henry, 2011). Three biological replicates were sampled from the top 2 cm layer of sediment from three sampling sites. Each replicate was composed of 10 random samples taken from a 10 m² area. Sediment samples were sub-sampled from each replicate into 0.5 g aliquots and immediately stored at -80°C for subsequent molecular analysis. Samples for Potential Nitrification Rate (PNR) measurements and physicochemical analysis were stored at 4°C upon analysis.
3.3.2 DETERMINATION OF PHYSICOCHEMICAL PARAMETERS

Temperature, salinity, sediment classification, pH and inorganic nitrogen (NH$_4^+$, NO$_2^-$, NO$_3^-$) were all measured as described in section 2.3.2. DOC and TON were analysed from acidified samples on a Shimadzu TOC-L with TNM-L module according to Shimadzu manufacturer’s instructions. Chlorophyll A was extracted based on method by Castle et al., (2011). Briefly, chlorophyll A was extracted from 0.5 g sediment which was incubated with 5 ml 90% ethanol for 3 hours in darkness at 5 °C with intermittent shaking. Samples were centrifuged for 10 min at 3,000 r.p.m. Absorbance of samples was measured at 665 and 750 nm. Samples were acidified with one drop of 2 M HCl before measuring the absorbance at 665 nm and 750 nm again. Chlorophyll A was calculated using equation (2).
Chapter 3

Chlorophyll A (µg/g) = 29.6(665₀-665ₐ) v/m equation (2)

Where 665₀ is the absorbance at 665 nm subtracted from 750 nm before acidification and 665ₐ is the same as 665₀ except after acidification; V is the volume of ethanol (5 ml); m is the weight of the extracted sediment (0.5 g).

3.3.3 DNA AND RNA CO-EXTRACTION

DNA-RNA was co-extracted from all in situ samples in Kinvarra using the same method described in section 2.3.4.

3.3.4 CDNA SYNTHESIS

RNA was converted to cDNA using Superscript III (Life Science, USA). Two-step RT-PCR amplification was performed on RNA using random hexamers. The initial RT reaction mixtures contained 8 µl of a 1:4 dilution of environmental RNA, 5 µM of Random Hexamers (Life Science, USA) and 10 µM of each dNTP, making the total volume 10 µl. The mixture was denatured at 65°C for 5 min and transferred to ice for 1 min. 4 µl 5X first-strand buffer, 1 µl 0.1 mM DTT, and 200 units SuperScript III were added to the reaction mixture and incubated at 50°C for 50 min, followed by inactivation of the reaction at 72°C for 15 min. Samples were stored at -80°C until further analysis.

3.3.5 ILLUMINA MISEQ AMPlicon Sequencing of 16S rRNA gene from archaea and bacteria

DNA and cDNA samples were sent to Research and Testing Laboratory, Texas USA for PCR amplification and sequencing on the Illumina MiSeq platform. MacQiime 1.9.1 was used to analyse 16S RNA sequence data (Caporaso et al., 2010). Overlapping paired end reads were joined together and primers extracted from each sequence. Reads that were ≤ 75% of the original read length, were removed during quality filtering. Chimeric sequences were identified and removed using USEARCH 5.2236 (Edgar et al., 2011). An OTU table was built using the denovo OTU picking method and clustering OTUs
using uclust (Edgar, 2010) at 97% similarity to the latest greengenes database from August 2013. In order to reduce noise, singletons were filtered from the OTU table. This OTU table was then used to analyse the core diversity at the common depth of 27000 reads. Core diversity analysis included alpha and beta diversity which included distance boxplots, rarefaction curves, and weighted and unweighted unifrac. Principle coordinates analyses (PCoA) were performed on Bray-Curtis dissimilarity, weighted (abundance) and unweighted (presence-absence) UniFrac distances. Environmental vectors were introduced to the PCoA using RStudio version 3.3.1 with the calibrate v 1.7.2, vegan v 2.4 and ggplot2 packages in order to gain a better understanding of how the environmental parameters influence the community composition (Graffelman, 2012; Oksanen et al., 2012; The R Core Team, 2016; Wickham, 2009). A mean relative abundance graph and heatmap were created at phylum level using R-studio package ggplot2 to show the change across all sites (The R Core Team, 2016; Wickham, 2009). Venn diagrams were created using the amount of shared OTUs at 97% similarity at genus level using a presence/absence table with R-studio and limma package (Ritchie et al., 2015; The R Core Team, 2016).

3.3.6 POTENTIAL NITRIFICATION RATES (PNR)

PNR rates were measured on sediment of all three sites and incubated with artificial sea water instead of in situ sea water as described in section 2.3.3. Artificial sea water (ASW) contained 0.4 M NaCl, 0.1 M MgSO₄, 0.02 M KCl and 0.02 M CaCl₂ and was adjusted to pH 8. ASW salinity was also adjusted to in situ salinity concentrations. The mixture was then autoclaved at 121°C for 15 min and was used as seawater in PNR experiment (refer to section 2.3.3 for more detail).
3.3.7 QUANTITATIVE PCR (Q-PCR)

3.3.7.1 STANDARD CURVE CONSTRUCTION

Standard curves were constructed as described in section 2.3.6.1 for AOA and AOB Q-PCR. However, the standard gene copy number was re-calculated as the new primer lengths (Table 3.1) were shorter and within the older standards from section 2.3.6.1.
Table 3.1: Suite of primers used in this study

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer sequence (5′-3′)</th>
<th>Target gene</th>
<th>Thermal Profile</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cren-amoA23f</td>
<td>ATG GTC TGG CTW AGA CG</td>
<td>archaeal amoA gene (624 bp)</td>
<td>Q-PCR- 95°C, 3min; 40×(95°C, 30s; 47°C (AOB)/ 53°C (AOA) 30s/ 58°C, 40 s 72°C, 1 min, 80°C (AOA)/ 82°C (AOB) with plate read); Melt curve 65°C to 95°C, increment 0.5°C, 0:05+ plate read</td>
<td>(Tourna et al., 2008)</td>
</tr>
<tr>
<td>Cren-amoA616r</td>
<td>GCC ATC CAT CTG TAT GTC CA</td>
<td>bacterial amoA gene (435 bp)</td>
<td>Endpoint PCR - 94°C, 5 min; 35X (94°C, 30 s; 47°C (AOB)/53°C (AOA), 30 s; 72°C, 1 min) 72°C, 10 min</td>
<td>(Hornek et al., 2006)</td>
</tr>
<tr>
<td>BacamoA1 F*</td>
<td>GGG GHT TYT ACT GGT GGT</td>
<td>16S rRNA gene (291 bp) for bacteria and archaea</td>
<td>Endpoint PCR before sequencing-94°C, 3 min; 35X (94°C, 45 s; 50°C, 60 s; 72°C, 90 sec) 72°C, 10 min</td>
<td>(Parada et al., 2016)</td>
</tr>
<tr>
<td>BacamoA2 R</td>
<td>CCC CTC BGS AAA VCC TTC TTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>515F-Y</td>
<td>GTGYCAGC MGCCGCGG TAA</td>
<td>Universal 16S rRNA gene (291 bp) for bacteria and archaea</td>
<td></td>
<td>(Caporaso et al., 2011)</td>
</tr>
<tr>
<td>806R</td>
<td>GGACTACH VGGGTWTC TAAT</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Wobbles included: K: G/T; Y: C/T; V: A/G/C; B:G/C/T; W:A/T
Standard curve descriptors for all standard curves are reported in Table 3.2.

**Table 3.2: Q-PCR standard curve descriptors**

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>Genetic Target</th>
<th>Time point</th>
<th>Slope</th>
<th>% Efficiency</th>
<th>Y intercept</th>
<th>R²</th>
<th>NTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>BacamoA</td>
<td>in situ</td>
<td>3.596</td>
<td>90</td>
<td>42.06</td>
<td>0.98</td>
<td>33</td>
</tr>
<tr>
<td>DNA</td>
<td>ArchamoA</td>
<td>in situ</td>
<td>3.49</td>
<td>93</td>
<td>39.97</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

3.3.7.2 Q-PCR OF AMOA GENES OF ARCHAEAE AND BACTERIA

Archaeal and Bacterial *amoA* genes (Table 3.1) were amplified from triplicate sediments collected from each site in Kinvarra bay in April 2015. NTC and appropriate standard curve were included in each assay (Table 3.2). Q-PCR reaction mixture details are described in section 2.3.6.2.

3.3.8 CLONING AND SEQUENCE ANALYSIS OF BACTERIAL AND ARCHAEAL AMOA GENES IN SITU AND ACTIVE FRACTIONS

AOA and AOB *amoA* genes from replicate samples from *in situ* sites K1, K2 and K3 (Fig. 3.1) in Kinvarra Bay April 2015 were cloned and sequenced using Cren-amoaA23f and Cren-amoaA616r primers to target archaeal *amoA* and BacamoA1f* and BacamoA2R primers to target bacterial *amoA* (Table 3.1). More details on cloning and sequencing *amoA* gene can be found in section 2.3.8.
3.4 RESULTS:

3.4.1 SITE DESCRIPTION

Kinvarra bay was surveyed in April 2015 at low tide. The temperature of the sediment was 15°C, and salinity ranged from 0.38 to 28.65 psu (Table 3.3). Sediments were classified as muddy gravelly sand (mgS; 30-50% mud and gravel and at least 50% sand; Table 3.3). LOI ranged from 10.41 – 14.85 %. pH was stable in seawater with an average of 8.15 ± 0.08; however, sediment pH ranged between 7.89 - 8.27. NH$_4^+$ concentrations in sediment and porewater were highest in K3 934.5 and 227.95 µM$^{-1}$ g$^{-1}$ fs$^{-1}$ respectively, and the lowest NH$_4^+$ concentrations were found in K2 103.56 and 77.11 µM$^{-1}$ g$^{-1}$ fs$^{-1}$ in sediment and porewater, respectively (Table 3.3). NO$_3^-$ concentrations were undetected in porewater and detected when extracted from sediment, with concentrations ranging from 2.91 – 5.36 NO$_3^-$ µM$^{-1}$ g$^{-1}$ fs$^{-1}$ (Table 3.3). TN and DOC was measured in porewater, where the TN ranged from 1.92 – 3.82 mg l$^{-1}$ while DOC ranged from 11.14 – 13.75 mg l$^{-1}$ (Table 3.3)
Table 3.3: Physicochemical properties of sediments from three sites spanning Kinvarra bay salinity gradient.

<table>
<thead>
<tr>
<th>Sites</th>
<th>K1 (2-10 psu)</th>
<th>K2 (20-30 psu)</th>
<th>K3 (30psu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>53.142083, -</td>
<td>53.154105, -</td>
<td>53.163771, -</td>
</tr>
<tr>
<td>pH (seawater)</td>
<td>8.04</td>
<td>8.31</td>
<td>8.1</td>
</tr>
<tr>
<td>pH (sediment)</td>
<td>7.92</td>
<td>7.89</td>
<td>8.27</td>
</tr>
<tr>
<td>Salinity at time of sampling (psu)</td>
<td>0.38</td>
<td>20.12</td>
<td>28.65</td>
</tr>
<tr>
<td>NO$_3^-$ ($\mu$M$^{-1}$g$^{-1}$ sediment)</td>
<td>2.91 ± 5.04</td>
<td>5.36 ±2.08</td>
<td>3.84 ± 5.97</td>
</tr>
<tr>
<td>NH$_4^+$ ($\mu$M$^{-1}$g$^{-1}$ sediment)</td>
<td>243.67 ± 209.8</td>
<td>103.56 ± 175.4</td>
<td>934.5 ± 450.77</td>
</tr>
<tr>
<td>NO$_3^-$ ($\mu$M$^{-1}$ml$^{-1}$ PW)</td>
<td>Undetectable</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
<tr>
<td>NH$_4^+$ ($\mu$M$^{-1}$ml$^{-1}$ PW)</td>
<td>89.49 ± 5.69</td>
<td>77.11 ± 5.46</td>
<td>227.95 ± 10.22</td>
</tr>
<tr>
<td>TN (mg l$^{-1}$)</td>
<td>2.2 ± 0.92</td>
<td>1.92 ± 0.74</td>
<td>3.82 ± 1.16</td>
</tr>
<tr>
<td>DOC (mg l$^{-1}$)</td>
<td>11.14 ± 1.84</td>
<td>11.27 ± 1.84</td>
<td>13.75 ± 2.06</td>
</tr>
<tr>
<td>C:N ratio</td>
<td>5.13</td>
<td>5.96</td>
<td>3.65</td>
</tr>
<tr>
<td>Chlorophyll A ($\mu$g$^{-1}$ g$^{-1}$)</td>
<td>11.84 ± 0.99</td>
<td>13.62 ± 1.83</td>
<td>3.85 ± 0.77</td>
</tr>
<tr>
<td>LOI (%)</td>
<td>14.85 ± 2.35</td>
<td>10.41 ± 1.77</td>
<td>11.67 ± 1.93</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Particle size (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gravel (&gt;2 mm)</td>
<td>21.74 ± 2.24</td>
<td>25.47 ± 2.45</td>
<td>33.54 ± 3.38</td>
</tr>
<tr>
<td>Sand (63 µm-2 mm)</td>
<td>78.25 ± 5.32</td>
<td>74.53 ± 5.23</td>
<td>66.46 ± 4.68</td>
</tr>
<tr>
<td>Silt &amp; Clay (&lt; 60 µm)</td>
<td>7.85 ± 2.15</td>
<td>12.07 ± 1.84</td>
<td>10.88 ± 1.94</td>
</tr>
<tr>
<td>Eunis classification</td>
<td>mgS</td>
<td>mgS</td>
<td>mgS</td>
</tr>
</tbody>
</table>

All results include ± Standard Error (SE); porewater (PW); Total Nitrogen (TN); Dissolved organic carbon (DOC); Loss on ignition (LOI); muddy gravelly Sand (mgS)
3.4.2 16S rRNA BACTERIAL AND ARCHAEAAL DIVERSITY

Analysis of bacterial and archaeal 16S rRNA of DNA and RNA resulted in on average 52,815 sequences per site. Removing singletons, 32,725 OTUs (DNA and cDNA) at 97% similarity were retrieved in total from all sites at DNA and cDNA level, representing three archaeal phyla and 65 bacterial phyla. Alpha diversity was calculated at a rarefied depth of 27,000 from each sample at 97% similarity. From looking at the alpha diversity metrics chao 1 (Fig. 3.2 A) and observed OTUs (Fig. 3.2 B), it is clear that microbial diversity in coastal sediments is very high. 27000 reads was not enough to capture the alpha diversity as simpson diversity index is very close to 1 (Fig. 3.2 C; Table 3.4) and Shannon diversity index was an average of 9.8 indicating very high diversity in the communities in Kinvarra bay sediments (Table 3.4). The average richness was 45.5% coverage. No statistical difference was found between the sites for richness and evenness using both the Simpson and Shannon indexes (Fig. 3.2 C. and D). Of these sequences at 97% similarity, K1 was the most diverse with an average of 6531 OTUs (Std error ± 20.07), the high salinity site had the next highest number of OTUs with an average of 5717 OTUs (Std error ± 45.95), and lastly the intermediate site had an average of 5159 OTUs (Std error ± 111, Table 3.4, Fig. 3.2 B). From the cDNA data a similar pattern emerged, however, the numbers were lower than DNA. K1 was the most diverse with an average of 5885 OTUs (Std error ± 229.98) present, the high salinity site contained an average of 5595 OTUs (Std error ± 125.69) and finally the medium salinity site contained 4831 OTUs (Std error ± 83.7, Table 3.4, Fig. 3.2B).
Table 3.4: Species richness and diversity estimates based on 97% similarity

<table>
<thead>
<tr>
<th>Samples</th>
<th>Diversity Shannon</th>
<th>Diversity Simpson</th>
<th>Richness Observed OTUs</th>
<th>Chao 1</th>
<th>% Richness</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1a_DNA</td>
<td>10.61273</td>
<td>0.996564</td>
<td>6499</td>
<td>13413.55</td>
<td>48.45102</td>
</tr>
<tr>
<td>K1b_DNA</td>
<td>10.39423</td>
<td>0.995457</td>
<td>6568</td>
<td>14438.98</td>
<td>45.48797</td>
</tr>
<tr>
<td>K1c_DNA</td>
<td>10.53661</td>
<td>0.996105</td>
<td>6526</td>
<td>13933.45</td>
<td>46.83692</td>
</tr>
<tr>
<td>K2a_DNA</td>
<td>9.732195</td>
<td>0.994456</td>
<td>5215</td>
<td>11613.29</td>
<td>44.90544</td>
</tr>
<tr>
<td>K2b_DNA</td>
<td>10.05979</td>
<td>0.995792</td>
<td>5319</td>
<td>11030.78</td>
<td>48.21964</td>
</tr>
<tr>
<td>K2c_DNA</td>
<td>9.643642</td>
<td>0.994097</td>
<td>4945</td>
<td>10722.19</td>
<td>46.11931</td>
</tr>
<tr>
<td>K3a_DNA</td>
<td>10.1053</td>
<td>0.995691</td>
<td>5776</td>
<td>12704.61</td>
<td>45.46382</td>
</tr>
<tr>
<td>K3b_DNA</td>
<td>10.09928</td>
<td>0.995684</td>
<td>5627</td>
<td>11793.14</td>
<td>47.71416</td>
</tr>
<tr>
<td>K3c_DNA</td>
<td>10.14963</td>
<td>0.995899</td>
<td>5750</td>
<td>12648.5</td>
<td>45.45995</td>
</tr>
<tr>
<td>K1a_RNA</td>
<td>9.750622</td>
<td>0.986152</td>
<td>6048</td>
<td>13728.02</td>
<td>44.05588</td>
</tr>
<tr>
<td>K1b_RNA</td>
<td>9.142119</td>
<td>0.972669</td>
<td>5431</td>
<td>12222.14</td>
<td>44.43574</td>
</tr>
<tr>
<td>K1c_RNA</td>
<td>9.779685</td>
<td>0.985889</td>
<td>6176</td>
<td>13433.17</td>
<td>45.97574</td>
</tr>
<tr>
<td>K2a_RNA</td>
<td>8.973479</td>
<td>0.985667</td>
<td>4687</td>
<td>10658.71</td>
<td>43.97341</td>
</tr>
<tr>
<td>K2b_RNA</td>
<td>9.239582</td>
<td>0.981561</td>
<td>4830</td>
<td>10151.86</td>
<td>47.57747</td>
</tr>
<tr>
<td>K2c_RNA</td>
<td>9.186305</td>
<td>0.985976</td>
<td>4977</td>
<td>11782.6</td>
<td>42.24025</td>
</tr>
<tr>
<td>K3a_RNA</td>
<td>9.455112</td>
<td>0.98564</td>
<td>5638</td>
<td>12875.56</td>
<td>43.78838</td>
</tr>
<tr>
<td>K3b_RNA</td>
<td>9.432036</td>
<td>0.98174</td>
<td>5788</td>
<td>13196.55</td>
<td>43.85994</td>
</tr>
<tr>
<td>K3c_RNA</td>
<td>9.409444</td>
<td>0.985198</td>
<td>5359</td>
<td>11977.26</td>
<td>44.74313</td>
</tr>
</tbody>
</table>
Figure 3.2: Species richness and evenness of sites K1 to K3 DNA and RNA at 97% similarity. Rarefaction curves on the species richness (Chao1) and diversity (observed OTUs) are shown in A and B, respectively. Richness, diversity and evenness (Simpson and Shannon index) are shown in C and D, respectively. No statistical differences between sites were found when using group-wise averages of the diversity and species richness and evenness of the individual samples.
Salinity was used to organise the sites into categories. Low salinity site (LS) was 2 psu, intermediate salinity site (MS) was 20 psu and the high salinity site (HS) was at 30 psu. The most dominant phylum in all samples were the *Proteobacteria* (52.3%), with *Gammaproteobacteria* accounting for 32.6% of all sequences, followed by *Deltaproteobacteria* (14.7 %), *Alphaproteobacteria* (3.2%), *Epsilonproteobacteria* (1.5%) and finally *Betaproteobacteria* (0.3%; Fig. 3.3). All proteobacteria had a slight increase in numbers in the cDNA libraries compared to DNA level, indicating this phylum was quite active within the sediments. *Betaproteobacteria* were highest in the low salinity site (0.7% total community) compared to medium and high salinity sites (0.1% total community). The next highest phylum at all sites was *Bacteriodetes* accounting for 15.8% (Fig. 3.3). *Bacteriodetes* was in the range of approximately 20% of the community at DNA level but dropped to approximately 10% at RNA level. In addition, they were found to be higher in the high and medium salinity sites compared to the low salinity site (Fig. 3.3). Cyanobacteria also accounted for 3% of the community at DNA level but increased 7 fold at RNA level (22%).
Figure 3.3: Mean relative abundance of 16S rRNA Bacteria and Archaea at phylum level at different salinities and at RNA and DNA level. LSR, MSR and HSR stands for low medium and high salinity at RNA level respectively; LSD, MSD and HSD are low, medium and high salinity at DNA level, respectively. Below 0.01 indicates a combination of phyla that consisted of less than 0.01% of the community.
Archaea make up only 11.97% of the total community. Figure 3.4 depicts the Archaeal 16S rRNA community composition across the salinity gradient. *Crenarchaeota* were the most abundant phylum of Archaea making up 43.8% of archaeal community but only contributing 5.3% to the total community. *Parvarchaeota* and *Euryarchaeota* contributed 30.1% and 26% to the total archaeal community, respectively, and contributed 3.6% and 3.1% to the total community, respectively (Fig. 3.4). *Thaumarchaeota* are distinguished at class level and make up 1.6% of the total community. The relative abundance of this class is high in K1 and K2 but drops in K3 at DNA level. The same pattern is seen at RNA level (Fig. 3.4). 0.72% of the total community is attributed to *Crenarchaeota MBGB* at DNA level; this increases to 0.9% at RNA level (Fig. 3.4).
Figure 3.4: Diversity of archaeal 16S rRNA gene sequences at DNA and RNA level from Kinvarra bay sediments showing phylum, class and order level diversity.
The relative abundance of bacterial and archaeal 16S rRNA is quite stable across sites, however there are differences in the relative abundance of phylotypes between DNA and RNA (Fig. 3.5). The archaeal and bacteria community from the DNA libraries of K1 and K2 have a more similar community composition to each other than to K3, which separates on the heat map based on community composition (Fig. 3.5). A different picture is presented when examining the cDNA community. Here K2 and K3 communities are more similar to each other in terms of the active organisms present, the low salinity site K1 branches separately. Generally the differences in the 16S rRNA gene cDNA communities are attributed to higher abundances than observed in the 16S rRNA gene DNA communities in the majority of phyla, with the exception of Bacteriodetes, Planctomycetes, Verrucomicrobia, Chloroflexi, Chlorobi, Caldithrix, Actinobacteria, Gemmatimonadetes, WS3, Acidobacteria and a number of unassigned group of bacteria and archaeb.
Figure 3.5: Heatmap representing phylum level of 16S rRNA data of all sites at both DNA and RNA level. Heatmap is colour coded based on row Z scores. Analyses were performed on Bacterial and Archaeal 16S rRNA data to a rarefied depth of 27000 reads. Below 0.01 indicates a combination of phyla that consisted of less than 0.01% of the community.
DNA and RNA shared or unique OTUs are depicted in the venn diagram below (Fig. 3.6). 89% of OTUs are shared between DNA and RNA, while only 8% are unique to DNA and 4% are unique to RNA. This shows the majority of phylotypes are active, and represented in the RNA library, with only a small number of inactive 16S rRNA DNA genes (these could be extracellular DNA, dead or dormant cells). A small number of cDNA transcripts were not detected in the DNA library indicating these may represent rare but active phylotypes.

![Venn diagram](image.png)

**Figure 3.6: Venn diagram representing shared or unique OTUs** between DNA and RNA samples

Subsequently, we showed the unique/shared phylotypes in each site along the estuary. This is illustrated in Fig. 3.7 by venn diagrams showing the decimal fraction of OTUs unique or shared among sites at DNA (Fig. 3.7 a) and at RNA level (Fig. 3.7 b). 59% and 57% of OTUs are shared between all sites for DNA and RNA, respectively, showing that they are not affected by salinity (Fig. 3.6 b, c). The low salinity site had the most unique OTUs at 15% and 11% for DNA and RNA, respectively. While the medium and high salinity site had a smaller number of unique OTUs for DNA and RNA in the range of 3 - 6% (Fig. 3.6 b, c). 4% of sequences found outside the circle in figure 3.6 B represents the phylotypes that are not DNA and are found in RNA only; while similarly, 8% of
OTUs in the RNA venn diagram represent OTUs from DNA libraries that did not overlap with RNA.

Figure 3.7: Venn diagram representing shared or unique OTUs (A.) between sites at DNA level and (B.) between sites at cDNA level.
Next, we examined how the bacterial and archaeal communities changed along the bay by unweighted (presence-absence of OTUs) and weighted (relative abundance of OTUs) unifrac analysis on relative abundances of taxa at DNA and cDNA level along the salinity gradient (Fig. 3.8 & 3.9). A clear separation of the communities between sites is observed at both DNA and cDNA level (Fig. 3.8 and 3.9). At DNA level a range of environmental drivers influence the separation of communities between sites. K3 is influenced by high TN (total nitrogen), \( \text{NH}_4^+ \) concentration, pH and low carbon:nitrogen ratio. K1 is influenced by high organic carbon and low salinity. K2 is also influenced by salinity and carbon:nitrogen ratio (Fig. 3.8 a, b). Unweighted unifrac shows that replicates within a site cluster tightly together, indicating the same community composition is found between replicates. However, weighted unifrac shows the differences in relative abundances of the OTUs forcing the replicates to be slightly less similar to each other. Weighted unifrac had higher explanatory values than unweighted unifrac indicating that the relative abundance is very important in explaining the microbial communities along the bay (Fig. 3.8 a, b).

Similarly, at cDNA level, a range of environmental drivers influence the separation of communities between sites. Unweighted unifrac shows K3 is influenced by high salinity, TN, \( \text{NH}_4^+ \) concentration, pH, low carbon:nitrogen ratio and chlorophyll. The Y-axis explains 17.9% of variance. K1 is influenced by high LOI and low salinity. The X-axis explains 20.4% of variance. K2 is also influenced by salinity, chlorophyll, LOI and carbon:nitrogen ratio (Fig. 3.9 a). Unweighted unifrac shows that replicates within a site cluster tightly together, indicating the same community composition is found between replicates. However, weighted unifrac shows the differences in relative abundances of the OTUs, causing the replicates to shift slightly and become less similar to each other. Weighted unifrac is quite different to unweighted unifrac at cDNA level; the main environmental parameters driving separation of sites was DOC and salinity and again weighted unifrac shows higher explanatory values with the x-axis explaining 35.9% of variance and the Y-axis explaining 23.8% of the variance (Fig. 3.9 a, b).
Figure 3.8: Principle coordinate analysis plots showing differences in the phylogenetic diversities in DNA samples across a natural salinity gradient. Environmental parameters driving the communities are shown as vectors on each plot. Salinity, pH, NH$_4^+$ concentration, TN (total nitrogen), LOI (loss on ignition) and C:N ratio (carbon:nitrogen ratio). Low salinity DNA (LSD), medium salinity DNA (MSD), high salinity DNA (HSD) are shown in red, yellow and green, respectively. Unweighted unifrac is shown in graph A. and weighted unifrac is shown in graph B.
Figure 3.9: Principle coordinate analysis plots showing differences in the phylogenetic diversities in cDNA samples across a natural salinity gradient. Environmental parameters driving the communities are shown as vectors on each plot. Salinity, pH, NH$_4^+$ concentration, TN (total nitrogen), LOI (loss on ignition) and C:N ratio (carbon:nitrogen ratio). Low salinity DNA (LSD), medium salinity DNA (MSD), high salinity DNA (HSD) are shown in red, yellow and green, respectively. Unweighted unifrac is shown in graph A. and weighted unifrac is shown in graph B.
3.4.3 NITRIFIER COMMUNITY DYNAMICS IN KINVARRA BAY SEDIMENTS

After examining the total community diversity along the bay we next wanted to take a closer look at the Nitrifier community as revealed by 16S rRNA gene sequencing. Analysis of bacterial nitrifier community at DNA and RNA level showed that they represented on average 0.08% (Std error ± 0.02%) relative abundance of the total DNA community and 0.09% (Std error ± 0.01%) relative abundance in the cDNA data sets. The majority of bacterial nitrifiers were not identified at genus level (Fig. 3.10 a). *Nitrospirales* (phylum: *Nitrospirae*) and *Nitrosomonadales* (class: beta-proteobacteria) were identified at order level and represented 47.46%, 0%, 5.41% of the bacterial nitrifier community at DNA level at K1, K2 and K3, respectively. At RNA level there was a decrease at each site with 21.94% representing the RNA K1 community and none were detected at K2 and K3. *Nitrospiracea* (phylum: *Nitrospirae*) and *Nitrosomonadaceae* (class: beta-proteobacteria) were classified at family level and denoted 29.93%, 90.67% and 90.04% of the DNA bacterial nitrifier community for K1, K2 and K3, respectively. While at RNA level there was also a high level of activity with 66.58%, 90.85% and 89.2% for K1, K2 and K3, respectively. Finally *Nitrospina* (phylum: *Nitrospinae*), *Nitrospira* (phylum: *Nitrospirae*) and *Nitrosovibrio* (class: beta-proteobacteria) were classified at genus level and signified 22.62%, 9.33%, 4.55% of the bacterial nitrifier community at DNA level while at RNA level they made up 11.49%, 9.15% and 10.8% of the bacterial nitrifier active community in K1, K2 and K3, respectively (Fig. 3.10 a). *Nitrosovibrio* were observed at very low abundance appearing in some but not all of the replicates from K1, the high salinity sites (Fig 3.10 a).

Analysis of archaeal nitrifier community made up 0.23% of the K1 total archaeal community while at K2 and K3 nitrifiers were on average 0.06% (Std error ± 0.01%) of the total community at DNA level. At RNA level the percentage of the total community was more similar at all three sites, representing on average 0.05% (Std error ± 0.01%) of the total transcripts. Further analysis classified two archaeal nitrifiers at genus level *Nitrosopumilus*
and *Nitrospina*, both found in the phylum *Thaumarchaeota*. *Nitrospumilus* are the most abundant genus making up 74 – 100% of the total archaeal nitrifier community at all sites at both DNA and RNA level. While *Nitrospina* are lower in abundance and are sometimes absent from the higher salinity sites K2 and K3 (Fig. 3.10 b).

Figure 3.10: Nitrifier operational taxonomic units and their abundance within
each sampling site at DNA and cDNA level. (A) Bacterial nitrifier community
(B) Archaeal nitrifier community.

To examine the distribution of the nitrifier community along the bay, bray curts dissimilatory resemblances matricies were calculated on relative the taxonomic abundances of both archaeal and bacterial nitrifiers at DNA and cDNA level and visualised in PCoA plots (Fig. 3.11). Clear separation between K1 and the higher salinity sites of K2 and K3 was observed at DNA level. This separation was a little less clear at cDNA level (Fig. 3.11 a, b). For the nitrifier community at DNA level, salinity and LOI were drivers of separation of the communities between sites. K2 and K3 are influenced by high salinity and low LOI; in contrast, K1 was separated based on high LOI and low salinity. The explanatory values at DNA level were high with 53.9% of the variance explained on the x-axis and 25.2% explained on the y-axis (Fig. 3.11 a).

Similarly, at cDNA level pH salinity and DOC influenced the separation of communities between sites. K3 is influenced by high salinity, DOC and pH. While K1 and K2 are influenced by a lower salinity, DOC and pH. The explanatory variables are higher in cDNA where the x-axis explains 61.8 % of variance and the y-axis explains 25.7 % of variance (Fig. 3.11 b).
Figure 3.11: Principle coordinate analysis plots of bray curtis distances showing differences in the phylogenetic diversities samples across a natural salinity gradient. Environmental parameters driving the communities are shown as vectors on each plot. Salinity, pH, DOC (dissolved organic carbon) and LOI (loss on ignition). Low salinity DNA (LSD), medium salinity DNA (MSD), high salinity DNA (HSD) are shown in red, yellow and green, respectively. DNA samples are shown in graph A. and cDNA samples are shown in graph B.
Spearmann rank correlations were performed by correlating nitrifier phylotypes with environmental parameters measured in situ (significant correlations are shown in Table 3.6). Salinity negatively correlated with all archaeal phylotypes (P < 0.001) and all bacterial phylotypes, except for *Nitrosomonadaceae*, *Nitrosovibrio*, *Nitrospina* (P < 0.001; P < 0.05; Table 3.6). Genus *Nitrosopumilus* is a known AOA and positively correlated with sand, PNR and LOI (r = 0.517, P < 0.05; r = 0.838, P < 0.001; r = 0.571 P < 0.05 respectively; Table 3.6). Order *Nitrosomonadales* includes ammonia oxidisers and they positively correlated with PNR (r = 0.519, P < 0.05) but negatively correlated with salinity (r = -0.545, P < 0.05). *Nitrospirales*, *Nitrospiracea*, *Nitrospira* are all nitrite oxidisers and all positively correlate with PNR. This makes sense as PNR is a measure of ammonia oxidation which provides the nitrite substrate for nitrite oxidisers. *Nitrospiracea* also negatively correlate with ammonium concentrations in sediment and porewater pH and TN (r = -0.498, P < 0.05; r = -0.659, P < 0.05; r = -0.734 P < 0.001; r = -0.722, P < 0.001 respectively; Table 3.6).

**Table 3.6: Significant Spearman Rank Correlations on nitrifiers in archaeal and bacterial 16S rRNA community at DNA and RNA level**

<table>
<thead>
<tr>
<th>Domain</th>
<th>Archaea</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Nitrosopumilus</em></td>
<td><em>Nitrospira</em></td>
</tr>
<tr>
<td></td>
<td><em>Nitrososphaera</em></td>
<td><em>Nitrospiracea</em></td>
</tr>
<tr>
<td></td>
<td><em>Nitrospirales</em></td>
<td><em>Nitrospira</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Nitrosomonadales</em></td>
</tr>
<tr>
<td>Gravel</td>
<td>-.0517</td>
<td>* -.54</td>
</tr>
<tr>
<td>Sand</td>
<td>* 0.517</td>
<td>*0.54</td>
</tr>
<tr>
<td>PNR</td>
<td>** 0.838</td>
<td>* 0.667</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td></td>
<td>** 0.73</td>
</tr>
<tr>
<td>NH₄⁺ PW</td>
<td></td>
<td>** 0.829</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>* 0.519</td>
</tr>
<tr>
<td>LOI</td>
<td>* 0.571</td>
<td>* 0.66</td>
</tr>
<tr>
<td>Salinity SW</td>
<td>**-.864</td>
<td>**-.691</td>
</tr>
<tr>
<td>TN</td>
<td></td>
<td>**-.761</td>
</tr>
<tr>
<td>CN ratio</td>
<td>**-.545</td>
<td>**-.864</td>
</tr>
</tbody>
</table>

*P < 0.05 **P < 0.001; N= 18
Subsequently, we used PNR to estimate the activity of the nitrifying communities observed in the sediments along the salinity gradient of the bay. PNR reflects potential changes in \textit{in situ} nitrification rates. PNR in Kinvarra bay ranged from 0 - 1596 $\mu$M NO$_2^-$ g$^{-1}$ FS d$^{-1}$ ± 21.87. PNR was highest in the low salinity site and intermediate salinity site (K1 and K2); both were significantly higher than K3 where a PNR rate was undetectable (high salinity site; Fig. 3.12; One way ANOVA P > 0.05).

\textbf{Figure 3.12: Potential nitrification rates (PNR) \textit{in situ} in Kinvarra bay.} PNR ($\mu$M$^{-1}$ NO$_2^-$ g$^{-1}$ d$^{-1}$) amended with 250 $\mu$M NH$_4^+$ is represented by the bars (mean n=3, standard error indicated by error bars). Blue bars indicate sediments were incubated with 2 psu artificial seawater; green bars indicate sediments were incubated with 20 psu artificial seawater and red bars indicate sediments were incubated with 30 psu artificial seawater. Letters denote significant differences across sites within a bay (One-way ANOVA P < 0.05).
The ammonia oxidising community in the bay was then quantified via the *amoA* gene of AOB and AOA. AOB *amoA* gene abundances ranged from $1.20 \times 10^6$ to $1.18 \times 10^9$ (total average $3.88 \times 10^8 \pm SE 1.88 \times 10^8$) and AOA from $5.28 \times 10^6$ to $4.66 \times 10^8$ (average $9.87 \times 10^7 \pm SE 5.14 \times 10^7$) copies per gram of sediment (Fig. 3.12). There were no significant differences in gene copy numbers across sites for *amoA* from AOA, with gene abundances similar among all sites. In contrast, AOB gene abundances were significantly higher at K1 (fresh water) than K2 and K3 (One-way ANOVA P < 0.05). There were no significant differences in AOB *amoA* gene abundances between K2 and K3, medium and high salinity sites respectively (Fig. 3.3).

![Figure 3.12: AOB and AOA gene abundances from Kinvarra site K1, K2 and K3. Black and red bars indicate log_{10} gene copy numbers of *amoA* gene in bacteria and *Thaumarchaeota*, respectively. The asterisk symbol represents significant differences between AOA and AOB gene copy numbers at each site within the bay (Paired t-test P < 0.05). The black letters indicate significant differences across AOB gene copy numbers (One-way ANOVA P < 0.001).](image)

AOB gene abundances correlated positively with LOI ($r = 0.780$, P < 0.05) and negatively with salinity ($r = -0.922$, P < 0.001). AOA gene abundances positively correlated with PNR and LOI ($r = 0.712$, P < 0.05; $r = 0.751$, P < 0.05) and negatively with salinity ($r = -0.913$, P < 0.05). Finally, PNR positively correlated with Chlorophyll A, porewater NH$_4^+$ and pH ($r = 0.683$, P < 0.05; $r = 0.828$, P < 0.05; $r = 0.904$, P < 0.05) and negatively with salinity, TN and C:N ratio ($r = -0.699$, P < 0.05; $r = -0.804$, P < 0.05; $r = -0.714$, P < 0.05; Table 3.7)
Table 3.7: Significant Pearson Correlations carried out on Kinvarra April 2015 dataset

<table>
<thead>
<tr>
<th></th>
<th>PNR</th>
<th>Salinity</th>
<th>Chlorophyll A</th>
<th>NH$_4^+$ Porewater</th>
<th>pH</th>
<th>TN</th>
<th>C:N ratio</th>
<th>LOI</th>
</tr>
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<tr>
<td><strong>PNR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pearson r</strong></td>
<td>-</td>
<td>-0.699</td>
<td>0.683</td>
<td>0.828</td>
<td>0.904</td>
<td>-0.804</td>
<td>-0.714</td>
<td></td>
</tr>
<tr>
<td><strong>P (2-tailed)</strong></td>
<td>-</td>
<td>0.036</td>
<td>0.042</td>
<td>0.006</td>
<td>0.001</td>
<td>0.009</td>
<td>0.031</td>
<td></td>
</tr>
<tr>
<td><strong>N</strong></td>
<td>-</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td><strong>AOB gene abundance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pearson r</strong></td>
<td>-0.922</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.780</td>
</tr>
<tr>
<td><strong>P (2-tailed)</strong></td>
<td>0.000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.013</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9</td>
</tr>
<tr>
<td><strong>AOA gene abundance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pearson r</strong></td>
<td>0.712</td>
<td>-0.913</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.751</td>
</tr>
<tr>
<td><strong>P (2-tailed)</strong></td>
<td>0.031</td>
<td>0.001</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td>9</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9</td>
</tr>
</tbody>
</table>
Next we explored the diversity and identified ammonia oxidisers along the bay. Duplicate AOB *amo*A clone libraries were created from each site. *amo*A AOB clone libraries were retrieved from all sites, but *amo*A AOA sequences were only recovered from the freshwater site K1. From these, 157 *amo*A AOB DNA and 53 *amo*A AOA DNA sequences correctly translating to protein were recovered in total. From these, eleven AOB and 25 AOA OTUs at 97% similarity were identified (Table 3.8). The AOA community is quite diverse in comparison to AOB at site K1 *in situ*. AOB diversity decreases slightly as salinity increases (Table 3.8).

**Table 3.8: Number of AO clones sequenced per site in Kinvarra, and identity at OTU level**

<table>
<thead>
<tr>
<th>Samples</th>
<th>K1</th>
<th>K2</th>
<th>K3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of clones</td>
<td>97% identity</td>
<td>No. of clones</td>
</tr>
<tr>
<td>AOA</td>
<td>53</td>
<td>25</td>
<td>*</td>
</tr>
<tr>
<td>AOB</td>
<td>65</td>
<td>5</td>
<td>45</td>
</tr>
</tbody>
</table>

* PCR product was too weak to clone and sequence

For AOB, all of the OTUs fell into the *Nitrosomonas*-like group (Fig. 3.14) alongside *amo*A sequences from coastal and estuarine sediments found in the Baltic Sea and Eastern China marginal sea. The majority of OTUs fall into a cluster relating to *N. ureae*. It contains 10 OTUs representing 98.5% of all sequences. It also contains the most abundant OTU (*amo*ABacK1a_9) recovered from all sites. One OTU from K1 was highly similar to *Nitrosospira briensis*. 

102
Figure 3.14: Neighbour-joining phylogenetic tree of AOB amoA genes at protein level (≥ 97% similarity). Bootstrap values shown are greater than 70% of 1000 which are shown near nodes. Accession numbers from GenBank are shown from other studies and isolates. Circles indicate K1 freshwater site, triangles indicate intermediate salinity site and squares indicate full salinity site. The numbers in brackets indicate the number of sequences recovered from that site.
AOA were more diverse than AOB; however, sequences were only retrieved from the freshwater site due to insufficient amplification of PCR products from K2 and K3 (Fig. 3.15). The majority of AOA fell into group I.Ia, with two OTUs falling into group I.Ib. In group I.Ib, OTU amoAArchK1a_3 was similar to Can. Nitrososphaera evergladensis and gargensis while amoAArchK1a_21 branched off and wasn’t closely related to any isolated representative but was closely related to a Hong Kong bay sediment clone. Group I.Ia, was composed of Nitrosopumilus-like clusters containing 22 OTUs and 84.9% of sequences. Only one OTU amoAArchK1a_1 was found in the cluster relating to Nitrosoarchaeum. The most abundant OTUs were found in the Nitrosopumilus-like clusters and they were the most closely related to clones found in estuarine sediments from the Yangtze estuary and Erhai lake basin.
Figure 3.15: Neighbour-joining phylogenetic tree of AOA amoA genes at protein level (≥ 97% similarity). Bootstrap values shown are greater than 70% of 1000 which are shown near nodes. Accession numbers from GenBank are shown from other studies and isolates. Circles indicate K1 freshwater site. The numbers in brackets indicate the number of sequences recovered from that site.

CCA revealed two different groupings of AO amoA sequences (Fig. 3.16; r = 0.754, P < 0.001). Phylotypes at the freshwater site, K1, separated primarily due to salinity explaining 32.2% of variance (pseudo f = 1.9, P < 0.05). While the higher salinity marine sites, K2 and K3, were influenced by high salinity but also mud grain size which explained 26.3% of the variance (pseudo f= 1.9; p < 0.05). CCA X-axis explained 34.57% variance while the Y-axis explains 23.97% variance.

Figure 3.16: CCA illustrates the separation of AO community amoA gene clones for AOA and AOB based on physicochemical parameters measured. The most significant parameters are represented by the labelled vectors.
3.5 DISCUSSION

Kinvarra bay has a natural salinity gradient due to fresh groundwater inputs from the surrounding catchment at the top of the bay, and seawater entering from Galway bay through a narrow channel. The bay is also strongly influenced by anthropogenic pollution caused by an improper wastewater treatment plant which overloads the bay with nitrogen. To this end, Kinvarra bay was chosen as an ideal ecosystem to explore the effects of salinity on benthic microbial communities, and in particular nitrifiers due to the steep environmental gradients of nitrogen, carbon and salinity along the bay (Cave and Henry, 2011). The aim of this study was to determine the microbial community in situ along Kinvarra bay. We revealed a diverse community of microorganisms (section 3.4.2), the true diversity of which was not fully accessed with the current sequencing depth, as on average only 45.5% of the community was uncovered through ‘illumina’ sequencing of the DNA and RNA out of a huge number of sequence reads retrieved per site (mean = 52,815 reads per site; Table 3.4; Fig. 3.2). This is not unusual in coastal benthic sediments. A number of other studies carried out in coastal sediments also reported that they did not reach the asymptote of the rarefaction curves, illustrating the high diversity of microbial communities within sediments (Bolhuis et al., 2014; Bolhuis and Stal, 2011; Zhang et al., 2008). The daily fluctuations of key nutrients and other environmental parameters mean that intertidal sediments are highly productive ecosystems that support very diverse microbial communities. This may be achieved by microorganisms occupying a range of separate niches within a single ecosystem. For examples, Ley et al., (2006) provided evidence of this in coastal microbial mats, environments that also support highly diverse microbial communities. They showed this by mapping microbial diversity onto chemically defined zones, for example the oxic zone, low-H₂S zone and the high-H₂S zone, and observed clustering of communities specific to the chemical zones (Ley et al., 2006). Another potential factor that contributes to highly productive and diverse microbial communities in mud sediments is epipelic diatoms, who live in close association with heterotrophic bacteria and can be found together in biofilms (Agogué et al., 2014). They contribute to primary productivity and provide
carbon to heterotrophs (Amin et al., 2012). Their interactions with the bacterial community in intertidal coastal ecosystems is an important one, a wide range of bacteria are known to interact with diatoms, such as species from the phyla *Cyanobacteria, Bacteriodetes, α-proteobacteria, γ-proteobacteria* and to a lesser extent *β-proteobacteria* (Schäfer et al., 2002). However, the interactions between them are very difficult to study *in situ*. A study carried out investigating the interactions of diatoms and bacteria in a biofilm from intertidal mudflats tried to simulate summertime environmental conditions *in situ* by using tidal mesocosms. This study successfully showed both positive and negative influences EPS has on bacteria and diatom interactions in a controlled setting (Agogué et al., 2014).

While amplification and sequencing of the 16S rRNA gene DNA detects living, dead, dormant and even extracellular DNA, targeting 16S rRNA gene transcripts is a way of distinguishing between viable and inactive cells that may be an issue when examining DNA alone, as discussed in section 2.5. RNA analysis is a step closer to identifying the active members within a community. However, there are some caveats that must be borne in mind when accessing 16S rRNA gene transcript data (Blazewicz et al., 2013). 16S rRNA does not represent real time growth or activity in a sample, due to 16S rRNA gene production under an array of different scenarios such as life strategies of the phylotypes, the history of the cell and the cell may remain active without growing (Blazewicz et al., 2013). What it does tell us is the phylogenetic, taxonomic and functional composition of the community and potential activity in the environment (Blazewicz et al., 2013), cells that are currently active or have the potential to become active or have been active in the recent past. In Kinvarra bay we compared 16S rRNA genes and transcripts from each site along the salinity gradient. 8% of OTUs at genus level in the total community are not found at RNA level (Fig. 3.6). This indicated that 8% could represent dormant OTUs or OTUs that have very low expression levels (Fig. 3.6), or that the transcripts were expressed at a low level that was not detected by the sequencing depth. Alternatively, targeting RNA may aid and recover low abundant yet actively transcribing OTUs not detected at DNA level due to being rare (Wang et al., 2016). For example, *Nitrosovibrio* was detected at RNA level in the high salinity
sites but not at DNA level (Fig. 3.9 b). We also showed an example of this (chapter 2) using the amoA gene as a functional marker where a new cluster was identified containing only RNA transcripts that were not closely related to current AOB isolates or gene sequences from other studies (Fig. 2.6). Analysis of 16S rRNA transcripts support stronger correlations of community response to certain environmental parameters measured \textit{in situ}, thereby, adding further understanding to the potential active organisms present \textit{in situ} at the time of sampling. We see this first hand in figure 3.6, 3.7 and 3.10 where the explanatory values on the PCoA axes are always higher in the RNA plots. Nevertheless, caution must be taken when analysing 16S rRNA gene and transcript data. Bacteria and Archaea can have multiple copies of the 16S rRNA gene in their genome and may also transcribe numerous copies, therefore we cannot equate 16S rRNA gene abundances and transcripts to cell numbers (Klappenbach et al., 2000). 16S rRNA gene copy number per cell may reflect the cells response to resources (Klappenbach et al., 2000). Although large numbers of 16S rRNA gene copies are advantageous to microorganisms (it gives them the ability to transcribe and translate the gene more rapidly), the disadvantage is revealed in low nutrient environments; having numerous copies has adverse effects as it consumes and costs energy. Therefore phylotypes with fewer copies of 16S rRNA gene may be more attuned to low energy environments and out compete organisms with more copies. Both DNA and RNA amplicon analysis can be affected by PCR due to PCR biases from selective amplification of some templates over others (Suzuki and Giovannoni, 1996). In addition, generation of cDNA involves a reverse transcriptase step, the efficiency of which may also impact the final results.

The dominant phyla within the sediments were the \textit{Proteobacteria}, \textit{Bacteriodetes} and \textit{Cyanobacteria}. This is consistent with previous studies in similar ecosystems such as the mudflats of the Bornean estuary in southeast Asia and coastal microbial mats in the North sea (Bolhuis et al., 2014; Bolhuis and Stal, 2011). In contrast, in Baltic sea sediments \textit{Cyanobacteria} only represented 1% (Vetterli et al., 2015) relative abundance of the total community. \textit{Betaproteobacteria} were highest in the low salinity site (0.7% total community)
compared to medium and high salinity sites (0.1% total community). In a study exploring all freshwater sequences in the ribosomal database (RDP), they found five phyla were frequently recovered and are listed in order of most abundant *Actinobacteria, Proteobacteria* (especially *Betaproteobacteria*), *Bacteriodetes, Cyanobacteria*, and *Verrucomicrobia* (Newton et al., 2011). Despite the differing environmental pressures along the bay, over half of the community at genus level was shared among all three sites at both DNA and RNA level. This indicates the presence of a core microbial community dispersed along the bay gradient that is unaffected by the regular tidal driven changes in salinity. The other 50% of the community seemed to respond to the varying environmental parameters. It is clear from figures 3.8 and 3.9 that it is not only salinity driving community change but key nutrients like carbon, nitrogen and pH. In the Venn diagram we also see that the low salinity site had the highest number of unique OTUs while the highest salinity site had the lowest number of unique OTUs (Fig. 3.7). Studies carried out along natural salinity gradients along an estuary and a bay also found that the freshwater sites had more unique OTUs (Campbell and Kirchman, 2013; Francis et al., 2003). This shows us that salinity may put a lot of stress on microorganisms and is likely a strong environmental selector for microorganisms that are adapted to withstand this stress. In contrast, in the freshwater environments, greater niche separation is occurring and the community can adapt faster as there is less selective pressure. Evidence of selective pressure was seen in a large dataset of the biodiversity of AOA in a range of different habitats where AOA *amoA* gene sequences clustered by habitat showed that salinity was among the significant drivers of niche differentiation (Biller et al., 2012).

The nitrifier community along the estuary was explored using both the 16S *rRNA* and *amoA* gene. Archaeal ammonia oxidisers were classified at genus level *Nitrosopumilus* and *Nitrosphaera* (Fig. 3.9 a). These were also found in the freshwater site when amplifying the *amoA* gene (Fig. 3.14). AOB communities were also targeted at both 16S *rRNA* level and *amoA* gene level. *Nitrosovibrio* is an ammonia oxidiser that is potentially active in Kinvarra bay. This ammonia oxidiser was detected in medium and high salinity sites as a 16S *rRNA* transcript
but was not detected at DNA level and was not targeted by the functional *amoA* gene primers (Fig. 3.9 b). *Nitrosovibrio* are from the *Beta-proteobacteria* class (Koops et al., 2015) and should have been detected by the *amoA* primers; however, the 16S *rRNA* transcripts of *Nitrosovibrio* were detected at very low levels and only in some replicates at the higher salinity sites K2 and K3 (Fig. 3.9 b). This shows that *Nitrosovibrio* are a very low abundant species in coastal bays but may be transcribing at a high level and are potentially important phylotypes in high salinity AO communities. This is an example of where RNA based community analysis has helped us uncover a less abundant ammonia oxidiser not recovered in the DNA libraries with the depth of sequencing we were able to achieve in this study. *Nitrosovibrio* were detected in a coastal microbial mat in the North sea at high salinity using the Rotthauwe primers also used in this study (Table 3.1; Fan et al., 2015). *Nitrosomonas* were detected with the *amoA* primers at all sites at DNA level, which was not seen at genus level using 16S *rRNA* primers but is part of the *Nitrosomonadaceae* family which was detected at 16S *rRNA* gene and transcript level. A number of coastal sediment studies have found *Nitrosomonas* like sequences present and abundant in the AOB community (Cébron et al., 2003; Stehr et al., 1995; Zheng et al., 2014). Finally, NOBs *Nitrospina* and *Nitrospira* were detected in 16S *rRNA* gene and transcript datasets and both have previously been detected in other marine environments such as the open ocean and coastal waters (Haaijer et al., 2013; Mincer et al., 2007).

Salinity, pH and carbon seem to be the most significant drivers separating nitrifier communities (Fig. 3.10). Salinity drives separation of nitrifiers because of the stress it inflicts on microorganisms but does not drive the separation completely (Bollmann and Laanbroek, 2002; Francis et al., 2003; Roeßler and Müller, 2001; Zheng et al., 2014). Other factors such as carbon and nitrogen play a role in nitrifier separation (Table 3.6 & Fig. 3.10). *Nitrosopumulis*, *Nitrospirales* and *Nitrospira* were all positively correlated with high carbon sites these organisms may be present due to higher rates of mineralisation providing a source of ammonia and thus an increase in nitrifiers, that may in turn provide nitrate for primary production (Table 3.6; Herbert, 1999). Furthermore, we see
evidence of the nitrifier community separating based on the carbon content of the sediments. In figure 3.10 AOA and AOB gene abundances positively correlate with LOI (Table 3.6). In the previous study in Chapter 2, AOB gene and transcript numbers correlated with TOC and was a significant parameter driving the active AOB community change across Rusheen bay. Similarly, in Yangtze estuarine sediments in China, both AOB and AOA gene abundances significantly correlated with organic carbon (Zheng et al., 2014). Finally, Nitrospiraceae and Nitrospira negatively correlated with pH, perhaps because of the indirect influence it has on the AO substrate ratio \( \text{NH}_4^+ : \text{NH}_3 \), i.e. AOA and AOB oxidise \( \text{NH}_3 \) which is reduced in low pH environments, subsequently, AO supply NOB with \( \text{NO}_2^- \) as their substrate which consequently would be reduced due to AO converting less substrate to \( \text{NO}_2^- \) (see section 1.4.2 for more information on pH; Stein et al., 1997). In chapter two, AOB gene abundances in Rusheen bay positively correlated with pH and negatively with \( \text{NH}_4^+ \) concentration (Table 2.4). These correlations were also seen in the Western English channel sediment and the Yangtze estuary sediment (Laverock et al., 2014; Zheng et al., 2014).

Finally, ammonia oxidisers were sequenced to identify the OTUs within the bay and to determine if the changes in the AO community were observed across the bay and examine their response to salinity. First, PNR decreased significantly as salinity increased (Fig. 3.11). AOB gene copy numbers were significantly higher at the low salinity site than the high salinity sites while AOA showed no significant difference in gene copy numbers across sites (Fig. 3.12). However, AOA gene abundances positively correlated with PNR rates, possibly suggesting that they may have been driving nitrification rates in Kinvarra bay (Table 3.7). Still, both AOB and AOA gene copy numbers significantly negatively correlated with salinity (Table 3.7). AOA sequences were only retrieved from the freshwater site as endpoint PCR amplicons and sufficient product did not amplify from K2 and K3. The AOA community in situ was composed of Nitrosopumilus like sequences which are usually part of the marine AOA community whereas Nitrosoarchaeum clusters are usually associated with low-salinity sites (Damashek et al., 2015; Mosier and Francis, 2008); yet there is only one sequence from our study closely related to the Nitrosoarchaeum cluster.
Despite AOA sequences coming from a low salinity site, AOB clustered in *Nitrosomonas* like clusters relating to *N. ureae* in all sites, regardless of salinity concentration. Only one OTU from the low salinity site was related to *Nitrospira birensis*. The CCA analysis of AOB did not show any significant environmental parameters influencing the community (data not shown). However, when paired with the AOA community, both mud and salinity were found to be important parameters driving community separation (Fig. 3.15). For AOA it is clear that most of the evidence presented above points to high salinity impacting AOA community change and even shows that high salinity may inhibit their growth due to the lack of sequences retrieved from the medium and high salinity sites. This is not the only study where AOA communities are seen to prefer lower salinity environments (Damashek et al., 2015; Mosier and Francis, 2008). Conversely, contrasting evidence is observed for AOB communities as they correlate negatively with salinity while common coastal phylotypes (*Nitrosomonas* like sequences) are found along the estuary regardless of salinity concentration. AOB may be influenced more strongly by substrate concentration than salinity as *Nitrosomonas* like sequences that are closely related to *N. ureae* and *N. oligotropha* have a high affinity for NH$_4^+$ but can also withstand NH$_4^+$ concentrations up to 10 mM and so can be found in both high and low NH$_4^+$ ecosystems; additionally, *Nitrosospira* are more active in low NH$_4^+$ environments (Bollmann and Laanbroek, 2001; Taylor and Bottomley, 2006). Perhaps because of *N. ureae* versatility when it comes to NH$_4^+$ concentration means they do well in Kinvarra bay, which is impacted by pollution. In contrast, *Nitrosomonas* like sequences that are closely related to *N. eutropha* are associated with NH$_4^+$ rich environments which were not found in our bay (Dang et al., 2010; Martens-Habbena et al., 2009). A similar study in the Plum Island Sound estuary had a comparable AOB community structure to this study in two out of three of their sites. The AOB community in the Sound estuary was dominated by *N. ureae* like sequences at the low and medium salinity sites. In contrast, the high salinity site (25 psu) contained *Nitrosospira* like sequences and so they found salinity impacted the AOB community (Bernhard et al., 2005b). A study in the Sacramento-San Joaquin Delta also
reported *N. ureae* like sequences as the dominant AOB cluster in the estuary (Damashek et al., 2015). Due to the lack of correlation of AOA and AOB communities with salinity and ammonium concentration, perhaps the AOA and AOB community structure at DNA level is not representative of the active community response to environmental parameters. Further study on the active AO community is required to elucidate how they respond to salinity and other environmental parameters along the natural salinity gradient. Additionally, further investigation of numerous different coastal sediments impacted by different salinity concentrations is required to elucidate whether salinity is a driver in niche differentiation and creates separate ecotypes. For example, a paper surveying AOA sequences from nine different marine ecosystems ranging from sediments to water column elucidated two ecotypes (Francis et al., 2005). They found AOA communities unique to each geographic location in sediments. This included a cluster of only San Francisco bay sequences (0.5 psu) which they classified as a low salinity cluster as the other sites were influenced by high salinity. They found coastal sediments were very diverse and so more sites were required to elucidate specific ecotypes in sediments. However, two distinct ecotypes were recognised in the water column, named water column A (WCA) and water column B (WCB) (Francis et al., 2005). These clusters are taxonomically distinct and so were further investigated to determine whether they also had distinct biogeochemical and ecological niches. WCA were classified as quite active and consistent in the water column, while WCB responded to environmental parameters such as temperature, chlorophyll and nutrients, and were less consistent (Smith et al., 2014a). Ecotypes are difficult to characterise and assign, however, it is worth investigating. Another study also carried out a geographic study covering 51 stations of water column samples. They found two distinct ecotypes that had differences at protein level where glycine in position 65 and 77 were exchanged with valine and alanine, respectively, contributing to a change in the 3D structure of the enzyme (Sintes et al., 2016). This shows us that in order to elucidate specific AOA and AOB ecotypes that have occupied a specific niche and respond to environmental
parameters such as salinity, surveys of a large number of coastal sediment sites are required.

Overall from this study we show that coastal sediments are diverse communities. We showed by studying the active communities in situ that there are low abundant but active OTUs not observed at DNA level. Analysis of 16S rRNA transcripts supported stronger correlations of community response to in situ environmental parameters. Additionally, salinity was shown to separate the community structure and diversity, with decreasing diversity as salinity increased along the bay. This was also observed for nitrifiers within the bay, with salinity driving the separation of nitrifier communities. However, it is not the only parameter that influences the nitrifier community; other factors such as carbon and nitrogen also play a role, in addition to parameters that were not measured in this study. AOB and AOA respond to salinity through PNR and gene abundances, however, the effect salinity has on the community of AOA and AOB along a salinity gradient requires further investigation.
4) Chapter 4: Growth and Activity Response of Ammonia Oxidisers Along an Estuarine Salinity Gradient in Sediment Microcosms

4.1 Introduction

Ammonia concentration is a primary environmental parameter influencing niche differentiation between AOA and AOB due to differences in ammonia affinities of the organisms (Martens-Habbena et al., 2009; for more information refer to section 1.4.1).

However, salinity seems to be a secondary factor that shapes ammonia oxidiser communities. In the literature there is contrasting evidence on the response of AOA and AOB to salinity (see section 1.4.5). Studies mentioned in section 1.4.5 mainly focus on AOA and AOB communities as a whole. When analysing the communities down to ecotype level a clearer picture may be obtained. For example in an ecophysiology study by Mosier et al (2012b) showed AOA activity to be influenced by salinity. Nitrosoarchaeum limnia was incubated with 75 ppt salinity resulting in a slow growth rate along with incomplete ammonia oxidation because they prefer low salinity environments (Mosier et al., 2012b). While N. maritimus was shown to grow optimally between 32 – 40 ppt and was inhibited by 20 ppt salinity (Qin et al., 2014). In addition, the genomes of both N. koreensis and N. limnia lack genes involved in the ectoine biosynthesis pathway. These genes are involved in tolerance to salinity, further suggesting that this group prefer low salinity (Kim et al., 2011; Mosier et al., 2012a). To my knowledge, no physiology studies on how salinity affects AOB have been carried out. But, a transcriptional microcosm study over a range of salinities revealed low AOB transcriptional activity in high salinity environments compared to an AOA community dominated by Nitrosopumilus maritimus like sequence clusters (Zhang et al., 2015b).

Ammonia oxidisers are not readily cultivated; another way of studying their activity under certain conditions is to use stable isotope probing (SIP). SIP allows identification of specific microorganisms that can assimilate a labelled
Chapter 4

substance such as carbon dioxide (CO$_2$; refer to section 1.4.5 part II). This works for identifying autotrophic ammonia oxidisers, as carbon fixation and ammonia oxidation are coupled i.e. any labelled CO$_2$ incorporated into an ammonia oxidiser’s genome is most certainly active as it cannot incorporate the heavy labelled carbon if it is not growing. Still, caution must be taken when analysing SIP data as any $^{13}$C incorporated into a microorganism’s genome can be from heterotrophs consuming autotrophs or other organic molecules produced by autotrophs that have incorporated the heavy labelled carbon. Hence, a control for autotrophic ammonia oxidation must be employed. Acetylene is a suicide inhibitor that competes with NH$_3$ to bind to the active site of AMO (Keener and Arp, 1993). Acetylene is used as an inhibitor of autotrophic ammonia oxidation, whilst heterotrophic ammonia oxidation can’t be abolished by acetylene (Berg et al., 1982; Hynes and Knowles, 1982). Hence, significantly higher growth found in the $^{12}$C and $^{13}$C microcosms than the acetylene treatment would confirm autotrophy. From the literature, a single study by Freitag et al (2006) was conducted along an estuarine salinity gradient with the aim of identifying the active AOB in nitrifying SIP microcosm (also mentioned in section 1.4.5). Nitrosospira cluster I dominated the AOB community in situ in marine sites ranging from 16 – 28 ppt salinity while Nitrosomonas oligotropha dominated freshwater site at 0 ppt in situ. Both Nitrosospira- and Nitrosomonas-like sequences were found in stations with 10 and 11 ppt salinity. The aim of the study was to link the community structure to ecosystem function. However, they could only confirm that the active ammonia oxidiser present in the marine salinity station (16 ppt) was actually Nitrosomonas Nm 143 not the dominant organism in situ Nitrosospira cluster I (Freitag et al. 2006). To this end, a lot remains to be elucidated when it comes to the influence of salinity on ammonia oxidiser communities.

In this study, microcosm incubations combined with SIP were set up to carry out hypothesis-based experiments keeping environmental factors consistent and changing only one variable between microcosms. The presence of ammonia oxidisers in sediments does not always mean they are functionally active. We aim to get one step closer to confirming their activity in intertidal coastal
sediments. In this study sediment microcosm incubations and SIP were performed to demonstrate whether AOA and AOB are capable of autotrophic nitrification under varying salinities. We investigated the autotrophic activity of AOA and AOB and explored the niche separation of AOB and AOA along the *in situ* salinity gradient. In addition, we applied different salinities that deviate from *in situ* salinity to nitrifier communities to test how they would respond to surges and drops in salinities (2 to 30 PSU). Finally, SIP was used to identify the active AO in the most suitable microcosm that demonstrated the occurrence of autotrophic nitrification and was due to at least one group of AO (AOA/AOB).

### 4.2 AIMS

The specific aims were firstly to identify whether nitrification occurs under a natural salinity gradient and if both AOA and AOB communities contributed to autotrophic nitrification at each salinity concentration. In chapter 3 we saw AOB were more abundant at all sites except the intermediate salinity site. We expect that AOA and AOB gene copy numbers will increase in numbers within our nitrifying microcosms at all sites; however, AOA sequences were only retrieved from freshwater sites in both chapter 2 and chapter 3 indicating there is a possibility that AOA in coastal ecosystems prefer freshwater.

Secondly, we aim to implement a forced salinity gradient i.e. varying salinities to the extremes in the microcosm, thereby testing salinity as a significant driving factor. In chapter’s 2 & 3 we see the nitrifier communities are influenced by salinity. We envisage a significant difference in community response to different salinities that deviate from *in situ* salinity.

Finally, we will choose the most suitable microcosm in order to perform SIP and identify who the active ammonia oxidisers are under a certain set of microcosm conditions.
Chapter 4

4.3 MATERIALS AND METHODS

4.3.1 SITE DESCRIPTION AND FIELD SAMPLING

Site description and field sampling of Kinvarra are described in section 3.3.1

4.3.2 SEDIMENT MICROCOSM INCUBATION

Two sediment microcosm experiments were conducted. The first incubated sediments from each site at the salinity recorded from the site at low tide. This is referred to as the natural salinity gradient microcosms. For the second sediment microcosm sediments from each of the sites were incubated at higher and lower salinities than naturally occur. This is referred to as the forced salinity gradient. A schematic of sediment microcosms is shown in Figure 4.1 a. Sediments were incubated for up to 29 days. For each experiment, natural and forced, 12 g of sediment and 12 ml ASW (pH 8.0), were added into 120-ml serum bottles. One mM ammonium in the form of (NH₄)₂SO₄ was amended on the first day of incubation and again on day 14 for the longer incubations. Acetylene (0.2%) was added to the headspace every 2 - 3 days. The headspaces were flushed with CO₂-free air, and ¹²C or ¹³C bicarbonate (Sigma Aldrich, Ireland) was added to a final concentration of 1 mg ml⁻¹ and refreshed weekly. Sodium bicarbonate salt (NaHCO₃) consists of an ion Na⁺ and an anion HCO₃⁻ which separate in water and react to form CO₂ (eqn 3), providing light and heavy labelled CO₂ in the microcosm.

\[ \text{HCO}_3^- \rightarrow \text{CO}_2 + \text{OH}^- \quad \text{equation (3)} \]

Bottles were capped with butyl stoppers and incubated at 15°C in the dark with shaking. Triplicate bottles were destructively sampled at day 0, 14 and 29. Sediment samples were spun down and water was removed. 0.5 g sediment was transferred to 2-ml tubes and were immediately frozen in liquid nitrogen and stored at -80°C until nucleic acid extraction. The remaining sediment and water mixture was extracted with 2 M KCl and used to determine the inorganic
nitrogen concentrations of NH$_4^+$, NO$_2^-$ and NO$_3^-$ in the sediment (refer to section 2.3.2 for analysis method used).

Figure 4.1: Flow diagram of sediment microcosm experimental setup for i) natural salinity gradient and ii) forced salinity gradient. Schematic of experimental design (the small circles represent microcosms and large circles represent sample site. The small blue circle indicates in situ salinity of that site.
and green circle indicates the ‘forced’ salinity the sediment was incubated at.

Flow diagram of sediment microcosm protocol (b.) Three sets of treatments were set up for each site and salinity incubation consisting of microcosms amended with $^{13}$C, $^{12}$C bicarbonate ($H^{13}CO_3^-$, $H^{12}CO_3^-$; 1 mg$^{-1}$ml$^{-1}$) and $^{13}$C bicarbonate + C$_2$H$_2$ (0.2% C$_2$H$_2$). In total, 147 microcosms were set up for destructive sampling at days 0, 14 and 29 (d0, d14, d29). A pairwise comparison between the $H^{13}CO_3^-$-labelled and the $H^{12}CO_3^-$ control treatment was used to assess whether the nitrifying populations assimilated $^{13}CO_2$ for autotrophic growth, whereas the $H^{13}CO_3^-$ + C$_2$H$_2$ treatment was used as a control to block autotrophic ammonia oxidation.

### 4.3.3 POTENTIAL NITRIFICATION RATES (PNR)

PNR measurements were carried out as described in section 3.3.2. ASW salinity was adjusted to in situ and forced experimental salinity concentrations to test how AO communities would respond to changes in salinity.

### 4.3.4 DNA AND RNA CO-EXTRACTION

DNA-RNA was co-extracted from in situ samples and sediment microcosms using the method described in section 2.3.4.

### 4.3.5 QUANTITATIVE PCR (Q-PCR)

#### 4.3.5.1 STANDARD CURVE CONSTRUCTION

Standard curves constructed in section 2.3.6.1 were used for AOA and AOB Q-PCR. However, the standard gene copy number was re-calculated as the new AOA amoA primers (Table 4.1) amplified a shorter amplicon from within the previous standard from section 2.3.6.1
Table 4.1: Suite of primers used in this study

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer sequence (5’-3’)</th>
<th>Target gene</th>
<th>Thermal Profile</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arch-amoWAF</td>
<td>CTGAYTGG GCYTGGAC ATC</td>
<td>archaeal amoA gene (256 bp)</td>
<td>Q-PCR - 95°C, 3min; 40×(95°C, 30s; 47°C (AOB)/ 53°C (Tourna AOA) 30s/ 58°C, 40 s (Wuchter AOA) 72°C, 1 min, 80°C (Both AOA)/ 82°C (AOB) with plate read); Melt curve 65°C to 95°C, increment 0.5°C, 0:05+ plate read</td>
<td>(Wuchter et al., 2006)</td>
</tr>
<tr>
<td>Arch-amoWAR</td>
<td>TTCTTCTTT GTTGCCCAG TA</td>
<td>bacterial amoA gene (435 bp)</td>
<td>Endpoint PCR - 94°C, 5 min; 35X (94°C, 30 s; 47°C (AOB)/53°C (Tourna AOA), 30 s; 72°C, 1 min) 72°C, 10 min</td>
<td>(Hornek et al., 2006)</td>
</tr>
<tr>
<td>BacamoA1 F*</td>
<td>GGG GHT TYT ACT GGT GGT</td>
<td>archaeal amoA gene (624 bp)</td>
<td>DGGE - 94°C, 5 min; 35X (94°C, 30 s; 53°C (Tourna AOA), 30 s; 72°C, 1 min) 72°C, 10 min</td>
<td>(Tourna et al., 2008)</td>
</tr>
<tr>
<td>BacamoA2 R</td>
<td>CCC CTC BGS AAA VCC TTC TTC</td>
<td>bacterial amoA gene (435 bp)</td>
<td>Endpoint PCR - 94°C, 5 min; 35X (94°C, 30 s; 47°C (AOB)/53°C (Tourna AOA), 30 s; 72°C, 1 min) 72°C, 10 min</td>
<td>(Hornek et al., 2006)</td>
</tr>
<tr>
<td>Cren-amoA23f</td>
<td>ATG GTC TGG CTW AGA CG</td>
<td>archaeal amoA gene (624 bp)</td>
<td>DGGE - 94°C, 5 min; 35X (94°C, 30 s; 53°C (Tourna AOA), 30 s; 72°C, 1 min) 72°C, 10 min</td>
<td>(Tourna et al., 2008)</td>
</tr>
<tr>
<td>Cren-amoA616r</td>
<td>GCC ATC CAT CTG TAT GTC CA</td>
<td>bacterial amoA gene (435 bp)</td>
<td>Endpoint PCR - 94°C, 5 min; 35X (94°C, 30 s; 47°C (AOB)/53°C (Tourna AOA), 30 s; 72°C, 1 min) 72°C, 10 min</td>
<td>(Hornek et al., 2006)</td>
</tr>
</tbody>
</table>

Wobbles included: K: G/T; Y: C/T; V: A/G/C; B:G/C/T; W:A/T
Standard curve descriptors for all Q-PCR assays are reported in Table 4.2.

Table 4.2: Q-PCR standard curve descriptors

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>Genetic Target</th>
<th>Time point</th>
<th>Slope</th>
<th>% Efficiency</th>
<th>Y intercept</th>
<th>r²</th>
<th>NTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Wuchter ArchamoA C¹² Fraction</td>
<td>3.324</td>
<td>99.9</td>
<td>41.51</td>
<td>1</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Wuchter ArchamoA C¹³ Fraction</td>
<td>3.314</td>
<td>100.3</td>
<td>38.36</td>
<td>1</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Wuchter ArchamoA C₂H₂ Fraction</td>
<td>3.307</td>
<td>100.6</td>
<td>39.24</td>
<td>1</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>BacamoA C¹² Fraction</td>
<td>3.912</td>
<td>81</td>
<td>37.5</td>
<td>0.99</td>
<td>35</td>
<td></td>
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<tr>
<td>DNA</td>
<td>BacamoA C¹³ Fraction</td>
<td>3.8</td>
<td>83</td>
<td>35.4</td>
<td>0.99</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>BacamoA C₂H₂ Fraction</td>
<td>3.43</td>
<td>93</td>
<td>33.18</td>
<td>0.99</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Tourna ArchamoA Natural gradient</td>
<td>3.372</td>
<td>98</td>
<td>33.91</td>
<td>1</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Tourna ArchamoA K1 and K3 forced gradient</td>
<td>3.498</td>
<td>93</td>
<td>35.13</td>
<td>0.99</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Tourna ArchamoA K2 forced gradient</td>
<td>3.432</td>
<td>95.6</td>
<td>33.93</td>
<td>0.99</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>BacamoA Natural gradient</td>
<td>3.365</td>
<td>98.2</td>
<td>38.25</td>
<td>0.99</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>BacamoA K1 and K3 forced gradient</td>
<td>3.517</td>
<td>92.4</td>
<td>39.84</td>
<td>1</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>BacamoA K2 forced gradient</td>
<td>3.532</td>
<td>92</td>
<td>39.66</td>
<td>0.99</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>
4.3.5.2 Q-PCR OF AMOA GENES OF ARCHAEA AND BACTERIA

AOA and AOB *amoA* genes were amplified from each sediment microcosm sample in results part I and each fraction fractionated in results part II. Triplicate no-template controls (NTC) and appropriate standard curve were included in each assay (Table 4.2). Q-PCR reaction mixture details are described in section 2.3.6.2.

**Troubleshooting:**

Q-PCR AOA primers were changed from Cren-amoA23f and Cren-amoA616r to ArchamoWAF and ArchamoWAR (Table 4.1) when amplifying fractions in results part II because of a smear found in the negative fractions when using Cren-*amoA* primers. This smear impacted the fractionation assays only and gave false positives. Therefore these primers could no longer be used and a shorter gene target of 256 bp was used (Figure 4.2).

![Figure 4.2: Schematic of amoA gene](image)

**Figure 4.2: Schematic of amoA gene** and where AOA *amoA* primers used in this study fall. Cren-amoA23f starts at 7 bp on the *amoA* gene and ends at 631 bp of the *amoA* gene while ArchamoWAF starts at 111 bp and ends at 367 bp.

4.3.6 FRACTIONATION

DNA and RNA were co-extracted from 0.5 g sediment samples, aliquoted from microcosms by spinning down overlying water in a centrifuge and pouring off excess water until 0.5 g sediment was reached. The Griffith method previously described in section 2.3.4 was used to extract DNA and RNA from the sediment sample. The concentration of DNA was determined photometrically.
using a Nanodrop 2000 UV-Vis spectrophotometer (Thermo Scientific, Ireland). Density gradient centrifugation was performed in 3.9-ml Quick-Seal®, Polypropylene tubes (BeckmanCoulter, USA) in a TLN-100 near vertical rotor (Beckman Coulter), subject to centrifugation at 127000 xg (65000 r.p.m.) for 72 h at 20°C (Hungate et al., 2015). Extracted DNA (5 µg) was added into CsCl gradients with an initial density of 1.696 g ml⁻¹, prepared by adjusting the refractive index to 1.399 with an DR201-95 digital hand-held refractometer (Kruss Optronic, Germany; Zhang et al., 2012). Centrifuged gradients were fractionated into 25 equal volumes (approximately 150 µl) by displacing CsCl solution with mineral oil at the top of the tube 0.3 ml min⁻¹, using a fraction recovery system (Beckman Coulter, USA) and a Masterflex L/S peristaltic pump (Cole-Parmer, Ireland). Buoyant density of each collected fraction was measured by determining the refractive index of 15 µl aliquots. Nucleic acids were precipitated from CsCl overnight by adding two volumes of PEGG 6000 in 1.6 M NaCl and 70% ethanol washing (Neufeld et al., 2007b), then dissolved in 20 µl sterile water.

### 4.3.7 DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

DGGE analysis was carried out on AOA active fractions and acetylene fraction in K1 day 29 2 psu. AOA *amoA* genes were amplified for DGGE with CrenamoA23f and CrenamoA616r primers (Table 3.1). DGGE analysis was performed using a DCode Universal Mutation Detection System (Bio-Rad Laboratories). PCR products were loaded onto a 6% polyacrylamide gel containing a 15% - 45% denaturant. The gel was run at 70 V for 15 h with a constant temperature of 60°C. The gel was removed from the electrophoresis tank and stained with GelStar™ Nucleic Acid Gel Stain 10,000X (Fisher Scientific, Ireland) for 30 min and washed in 1X TAE buffer for 5 min before taking the final picture under 300 nm UV light.

Bands were excised and placed in a sterile 1.5 ml Eppendorf tube and frozen at -20°C until further amplification. Bands were amplified using primers
just inside the original forward primer ArchamoWAF and CrenamoA616r Wuchter conditions were used as seen in Table 4.1. PCR products were gel purified using Gel Purification Kit (Qiagen, Ireland) according to manufacturer’s instructions and sent for Sanger sequencing (Macrogen, Netherlands).

### 4.3.8 CLONING AND SEQUENCE ANALYSIS OF BACTERIAL AND ARCHAEAL AMOA GENES IN SITU AND ACTIVE FRACTIONS

Active fractions chosen from DGGE analysis were cloned and sequenced using Cren-amoA23f and Cren-amoA616r primers to target archaeal amoA (Table 4.1). More details on cloning and sequencing amoA gene can be found in section 2.3.8. AOA and AOB amoA genes from replicate samples from in situ sites K1, K2 and K3 (Figure 3.1) in Kinvarra Bay April 2015 were cloned and sequenced using Cren-amoA23f and Cren-amoA616r primers to target archaeal amoA and BacamoA1F* and BacamoA2R primers to target bacterial amoA (Table 4.1).

### 4.3.9 STATISTICAL ANALYSIS

Statistical analysis was carried out on PNR and microcosm data, including nutrient measurements and amoA gene abundances for AOA and AOB. All data was tested for normality using Kolmogorov-Smirnov. When data was not normal, it was log-transformed (Bartlett, 1947). Variation in potential nitrification rates or gene abundances were analysed using a one-way ANOVA followed by a post-hoc Bonferroni test (Dunn, 1961) in SPSS v21 (IBM, USA). A one-way ANOVA was used on microcosm data to explain significant differences over time (days 0, 14 and 29) and also between salinities or sites. A paired t-test was also carried out comparing differences between $^{12}\text{C}$ and $^{13}\text{C}$ microcosms to the acetylene treatment microcosm in SPSS v21. Differences were considered significant when $P < 0.05$. 

126
4.4 RESULTS

PART I: SEDIMENT MICRO COSM EXPERIMENT

4.4.1 SEDIMENT NITRIFICATION ACTIVITY AND GROWTH OF AOA AND AOB COMMUNITY ALONG A NATURAL SALINITY GRADIENT

Sediments sampled from Kinvarra bay were incubated with *in situ* salinity and amended with 1 mM NH$_4^+$, differentially labelled bicarbonate (H$^{12}$CO$_3^-$ or H$^{13}$CO$_3^-$) and acetylene (C$_2$H$_2$) was added to the control microcosm only. CO$_2$-free air was also included in each microcosm. Microcosms were incubated at 15°C with shaking and were destructively sampled at day 0, day 14 and day 29 (Fig. 4.6). To determine if nitrification occurred with the microcosms over the 28 day incubation, ammonia and nitrite/nitrate concentrations were measured in all treatments. A decrease in ammonia accompanied by an increase in nitrate in both $^{12}$C and $^{13}$C microcosms compared to the acetylene treated microcosms indicated active nitrification. Nitrification occurred at all sites in the natural salinity gradient microcosms (Fig. 4.3 a, b). Nitrification was highest at the intermediate salinity site K2 after 14 days (paired t-test P < 0.05). There was no statistical difference between sites in terms of ammonia and nitrate concentration (One-way ANOVA P = 0.334, P = 0.951, respectively). However, there was a significant increase in nitrate concentrations from day 0 to day 14 and a decrease again after 29 days at all sites (One-way ANOVA P < 0.001; Fig. 4.3 a, b).

*amoA* gene abundances in AOA and AOB were quantified in order to gain a better understanding of the growth of the ammonia oxidising communities in the microcosms over time in sediments from along the natural salinity gradient. Significant increases in AOA and AOB *amoA* gene copy numbers compared to the acetylene treatment signify strong evidence of growth due to ammonia oxidation. In the microcosms, incubation from the K1 site (freshwater) by day 29 in both the $^{12}$C and $^{13}$C microcosms AOA *amoA* gene abundances were significantly higher than the acetylene treatment (Paired t-test; P < 0.001; Fig. 4.3 a, b).
4.3 d). This result indicates that AOA gene abundances increased within the microcosm and thus that they may be the dominant drivers of nitrification at the low salinity site. On the other hand, at the higher salinity sites (K2 and K3) AOA amoA gene numbers dropped significantly (One-way ANOVA; P < 0.001; Fig. 4.3 d). Over the incubation period there was not a significant increase in AOB amoA gene abundances across sites (One way ANOVA; P = 0.449). In fact, on day 0 the initial AOB amoA gene abundance was significantly higher than AOB amoA gene copy numbers after day 14 and 29 (One way ANOVA; P < 0.05). As we know nitrification occurred within the microcosm based the disappearance of ammonia and production of nitrate compared to the acetylene microcosms, the decrease in AOA and AOB indicates that the microcosm conditions selected for a smaller number of phylotypes in the community and there may have been a die off of certain phylotypes and/or a change in community structure during the incubation (Fig. 4.3 c). Taken together, these results indicate that AOA were active nitrifiers at low salinity. Further investigation is required to understand AOB response to salinity.
Figure 4.3: Change in nitrification activity and amoA gene abundance over a natural salinity gradient in sediment microcosms over a period of 29 days. Sediment ammonia depletion (A) and nitrate production (B) were measured to assess nitrification activity within sediment microcosm’s overtime. amoA gene
copy numbers of AOB (C) and AOA (D) were determined using Q-PCR. SIP microcosms were incubated with $\text{H}^{12}\text{CO}_3^-$ (Blue), $\text{H}^{13}\text{CO}_3^-$ (Purple), or $\text{H}^{13}\text{CO}_3^- \& \text{C}_2\text{H}_2$ (Orange) for 29 days. $\text{H}^{13}\text{CO}_3^- \& \text{C}_2\text{H}_2$ represent sediment microcosm incubated with $\text{H}^{13}\text{CO}_3^-$ in the presence of the nitrification inhibitor acetylene ($\text{C}_2\text{H}_2$). All treatments were conducted in triplicate microcosms. The error bars represent the standard errors of the mean of the triplicate microcosms. The asterisk above the columns indicates a significant difference between that and the acetylene treated microcosm (Paired t-test $P < 0.05$). $^{12}\text{C}$ and $^{13}\text{C}$ microcosms had the same trend over time and across sites (One-way ANOVA). The letters above represent significant differences with time within a site (One-way ANOVA $P < 0.05$). The Greek letters indicate significant differences between sites and were carried out on both $^{12}\text{C}$ and $^{13}\text{C}$ microcosms (One-way ANOVA $P < 0.001$).
### 4.4.2 SEDIMENT NITRIFICATION ACTIVITY AND GROWTH OF AOA AND AOB COMMUNITY WHEN SALINITY IS VARIED TO A LOWER OR HIGHER SALINITY THAN IN SITU MEASUREMENTS

Potential nitrification rates carried out using a range of salinities (2, 20 and 30 psu) showed no statistical differences in rates among the adjusted salinities after 24 hour incubation (One-way ANOVA; P = 0.668; Fig. 4.4). PNR at K1 and K2 were not significantly different from each other but were significantly higher than K3, the high salinity site (One-way ANOVA; P < 0.05; Fig. 4.4). PNR from K1, the low salinity site ranged from 1596 – 2007 µM NO₂⁻ g⁻¹ FS d⁻¹ ± 21. While the intermediate salinity site (K2) PNR ranged from 480 – 666 µM NO₂⁻ g⁻¹ FS d⁻¹ ± 10. Finally, the high salinity site (K3) PNR ranged from 0– 39 µM NO₂⁻ g⁻¹ FS d⁻¹ ± 0 (Fig. 4.4). Interestingly, the highest rate of PNR at K3 occurred at 20 psu, lower than the natural salinity from that site (30 psu).

**Figure 4.4: Potential nitrification rates (PNR) in Kinvarra bay.** PNR (µM⁻¹ NO₂⁻ g⁻¹ d⁻¹) amended with 250 µM NH₄⁺ is represented by the bars (mean n=3, standard error indicated by error bars). Blue bars indicate sediments were incubated with 2 psu artificial seawater; green bars indicate sediments were incubated with 20 psu artificial seawater and red bars indicate sediments were...
incubated with 30 psu artificial seawater. Arrows signify in situ salinity PNR. Letters denote significant differences across sites within a bay (One-way ANOVA P < 0.05). There was no significant difference between salinities within a site.

Sediments from site K1 with an in situ salinity of 2 psu at low tide were also incubated with a higher (30 psu) salinity and destructively sampled at day 0, day 14 and day 29 (Fig. 4.6). Microcosm results from the natural (2 psu) and forced (30 psu) salinity were compared over the 29 day incubation. Nitrification increased significantly when K1, an in situ low salinity site, was incubated at 30 psu (One-way ANOVA; P < 0.05; figure 4.5 a, b). However, AOA amoA gene copy numbers dropped in the 30 psu treatment incubation compared to K1 sediment incubated with 2 psu although it was not a significant drop (One-way ANOVA; P = 0.766). In addition, we observed AOA gene copy numbers in \(^{12}\)C and \(^{13}\)C microcosms were significantly higher than the acetylene treated microcosm at day 14 in the high salinity incubation. After 29 days the AOA numbers were no longer significantly higher, indicating the AOA may be tolerant of high salinity fluxes but not for long periods of exposure (Paired t-test P < 0.05 and P = 0.133, respectively; Fig. 4.5 d). AOB amoA gene copy numbers showed no significant change between salinity incubations and there was no significant increase in \(^{12}\)C and \(^{13}\)C microcosms compared to the acetylene treatment (Paired t-test; P = 0.428; Fig. 4.5 c). AOB gene copy numbers showed no growth at either salinity, further strengthening the hypothesis that AOA may be the main drivers of nitrification at the low salinity site.
Figure 4.5: Change in nitrification activity in freshwater site K1 and amoA gene abundance in sediment microcosms incubated with different salinities over a period of 29 days. Sediment ammonia depletion (a) and nitrate production (b) was analysed to assess nitrification activity in sediment microcosms. amoA gene copy numbers of AOB (c) and AOA (d) were
determined using Q-PCR. SIP microcosms were incubated with H$^{12}$CO$_3^-$ (Blue), H$^{13}$CO$_3^-$ (Purple), or H$^{13}$CO$_3^-$ & C$_2$H$_2$ (Orange) for 29 days. H$^{13}$CO$_3^-$ & C$_2$H$_2$ represent sediment microcosms incubated with H$^{13}$CO$_3^-$ in the presence of the nitrification inhibitor acetylene (C$_2$H$_2$). All treatments were conducted in triplicate microcosms. The error bars represent the standard errors of the mean of the triplicate microcosms. The asterisk above the columns indicates a significant difference between that and the acetylene treated microcosm (Paired t-test P < 0.05). The letters above represent a one way ANOVA across time within a site (One-way ANOVA P < 0.05). The Greek letters denote significant differences across salinities (One-way ANOVA P < 0.05).

The intermediate salinity site K2 (20 psu) was incubated with a higher (30 psu) and lower (2 psu) salinity, and again destructively sampled at day 0, day 14 and day 29 (Fig. 4.6). Nitrification was significantly higher at the *in situ* salinity site compared to the forced salinity microcosms (One-way ANOVA; P < 0.05; Fig. 4.6 a,b). AOA *amoA* gene copy numbers dropped significantly after day 0 (One-way ANOVA; P < 0.05) indicating microcosm condition did not suit the AOA community present *in situ*. There were no significant differences in AOA *amoA* gene copy numbers between salinity incubations (One-way ANOVA; P = 0.401; Fig. 4.6 d). On the other hand, AOB *amoA* gene copy numbers remained high and increased significantly from day 0 to day 29 (One-way ANOVA; P < 0.05). There was no significant differences between salinities (One-way ANOVA; P = 0.988). However, there were significant increases in $^{12}$C and $^{13}$C microcosms compared to the acetylene treatment for AOB gene copy numbers after incubating with 2 and 30 psu (Paired t-test; P < 0.05; Fig. 4.6 c). This may have stimulated AOB growth. Further analysis is required to see if AOB were still oxidising ammonia at 20 psu even though there was no evidence of growth after 14 and 29 days.
Figure 4.6: Change in nitrification activity in intermediate salinity site K2 and amoA gene abundance in sediment microcosms incubated with different salinities over a period of 29 days. Sediment ammonia depletion (a) and nitrate production (b) was analyzed to assess nitrification activity in sediment.
microcosms. The *amoA* gene copy numbers of AOB (c) and AOA (d) were determined using Q-PCR. SIP microcosms were incubated with H^{12}CO\textsubscript{3}\textsuperscript{-} (Blue), H^{13}CO\textsubscript{3}\textsuperscript{-} (Purple), or H^{13}CO\textsubscript{3}\textsuperscript{-} & C\textsubscript{2}H\textsubscript{2} (Orange) for 29 days. H^{13}CO\textsubscript{3}\textsuperscript{-} & C\textsubscript{2}H\textsubscript{2} represent sediment microcosms incubated with H^{13}CO\textsubscript{3}\textsuperscript{-} in the presence of the nitrification inhibitor acetylene (C\textsubscript{2}H\textsubscript{2}). All treatments were conducted in triplicate microcosms. The error bars represent the standard errors of the mean of the triplicate microcosms. The asterisk above the columns indicates a significant difference between that and the acetylene treated microcosm (Paired t-test \( P < 0.05 \)). The letters above represent a one-way ANOVA across time within a site (One-way ANOVA \( P < 0.05 \)). The Greek letters indicate significant differences across sites (One-way ANOVA \( P < 0.05 \)).

Finally, the high salinity site K3 (30 psu) was incubated with a lower (2 psu) salinity, with destructive sampling at day 0, day 14 and day 29 (Fig. 4.7). Nitrification was similar at the in situ salinity site compared to the forced salinity microcosms (One-way ANOVA; \( P = 0.944 \); Fig. 4.7 a,b).

AOA *amoA* gene copy numbers showed no significant growth with time (One-way ANOVA; \( P = 0.12 \); Fig. 4.7 d). There was no significant differences in AOA *amoA* gene copy numbers between salinity incubations (One-way ANOVA; \( P = 0.561 \)). AOB *amoA* gene copy numbers decreased slightly over time (One-way ANOVA; \( P < 0.05 \); Fig. 4.7 c). There was no significant differences between salinities (One-way ANOVA; \( P = 0.272 \)). However, there were significant increases in \(^{12}\)C and \(^{13}\)C microcosms compared to the acetylene treatment for AOB gene copy numbers at both salinities and time-points (Paired t-test; \( P < 0.001 \); Fig. 4.7 c). This data provides further evidence that AOB communities may be the dominant nitrifiers at the high salinity sites and that salinity variations over time did not affect the abundance of the high salinity AOB communities.
Figure 4.7: Change in nitrification activity in full salinity site K3 and amoA gene abundance in sediment microcosms incubated with different salinities over a period of 29 days. Sediment ammonia depletion (a) and nitrate production (b) was analyzed to assess nitrification activity in sediment
microcosms. The *amoA* gene copy numbers of AOB (c) and AOA (d) were determined using Q-PCR. SIP microcosms were incubated with H$^{12}$CO$_3^-$ (Blue), H$^{13}$CO$_3^-$ (Purple), or H$^{13}$CO$_3^-$ & C$_2$H$_2$ (Orange) for 29 days. H$^{13}$CO$_3^-$ & C$_2$H$_2$ represent sediment microcosms incubated with H$^{13}$CO$_3^-$ in the presence of the nitrification inhibitor acetylene (C$_2$H$_2$). All treatments were conducted in triplicate microcosms. The error bars represent the standard errors of the mean of the triplicate microcosms. The asterisk above the columns indicates a significant difference between that and the acetylene treated microcosm (Paired t-test P < 0.05).

**PART II: WHO ARE THE ACTIVE AMMONIA OXIDISERS?**

4.4.3 ACTIVE NITRIFYING COMMUNITY WITHIN FRESHWATER SEDIMENT SITE K1 AS REVEALED BY SIP

Due to the significant growth of AOA (Fig. 4.3 d) in freshwater salinity microcosms K1 at 2 psu by day 2, it was selected for further analysis to identify active community present via the incorporation of $^{13}$C by active AO and subsequent fractionation. DNA from triplicate day 29 $^{13}$C, $^{12}$C and acetylene treatment microcosms were subjected to density gradient fractionation (Fig. 4.2). Q-PCR was performed on the *amoA* genes of both AOA and AOB in the fractionated DNA; the relative abundance of DNA was calculated for each fraction (Fig. 4.8 a,b). The relative abundance of AOB populations showed the DNA in the experimental microcosm ($^{13}$C) and control microcosms ($^{12}$C and $^{13}$C & C$_2$H$_2$) were situated in the lighter fractions, typical of unlabelled DNA (Fig. 4.8 a). However, the relative abundance of AOA shows higher *amoA* gene abundances in the heavy fraction with a buoyant density of 1.66 g ml$^{-1}$ in the experimental microcosm ($^{13}$C). Importantly, in comparison, in the $^{12}$C and C$_2$H$_2$$^{12}$C unlabelled microcosms, all AOA amoA genes were quantified in the lighter buoyant density 1.64 g ml$^{-1}$. Labelling of *amoA* genes was not observed in the $^{13}$C & C$_2$H$_2$ microcosm suggesting that any labelling of *amoA* genes was due
to autotrophic nitrification growth. The active fractions are labelled 4 – 7 in Fig. 4.8 b.

**Figure 4.8: K1 2 psu day 29 DNA fractions quantified from light to heavy buoyant density.** The relative abundance of the (A) bacterial *amoA* and (B) archaeal *amoA* genes across the buoyant density gradient of the DNA fractions from sediment microcosms incubated with H$^{12}$CO$_3^-$ (Blue), H$^{13}$CO$_3^-$ (Purple), C$_2$H$_2$ (Red) for 29 days. The normalized data are the ratios of the gene copy number in each DNA fraction to the sum of the *amoA* genes across the entire gradient of DNA fractions for each treatment. The blue shaded area indicates the lighter fraction (12C labelled). The grey shaded area indicates where the active
fractions should be ($^{13}$C labelled) in the labelled microcosms. The error bars represent the standard errors of the triplicate microcosms.

DGGE analysis of archaeal *amoA* gene was carried out across the four heavy labelled fractions 4 - 7 from each of the triplicate (A, B, C) $^{13}$C day 29 2 psu and compared to fraction 2, from the control microcosm K1 A, B, C $^{13}$C + C$_2$H$_2$ in order to determine if there was a community shift across the heavy labelled fractions and to compare this to the control microcosm (Fig. 4.8). Fractions 5 and 6 of the heavy labelled fractions had the highest diversity (Fig. 4.9). Fractions 4 and 7 showed reduced diversity, but had some bands in common with fractions 5 and 6. No new bands were present in fraction 4 and 7 that were not already present in fractions 5 and 6. In addition, the acetylene treatment also contained one strong band which was also present in all of the $^{13}$C fractions (Fig. 4.9).
Figure 4.9 Dendrogram based on presence absence of DGGE bands from an archaeal amoA gene banding pattern of the active fractions 4 - 7 from K1 A, B, C $^{13}$C day 29 2 psi and fraction 2 from the unlabelled control K1 A, B, C $^{13}$C + $^{2}$H$_2$. Asterisks indicate the samples that were sequenced further.
Eleven bands were excised from the DGGE gel and cloned and sequenced (Fig. 4.10).

![Figure 4.10: DGGE archaeal amoA gene banding pattern of K1C day 29 2 psu fractions 4 -7. The numbers indicate bands extracted for sequencing.](image)

In addition, a single replicate from fractions 4 (C), 5 (C) and two replicates from fraction 6 (A and C) were chosen for cloning and sequencing (Fig. 4.9). In total, 215 AOA DNA, correctly translating to proteins were recovered (Table 4.3). From these, 22 AOA OTUs at 97% similarity were identified (Wang et al., 2015a; Table 4.3).
Phylogenetic analyses of archaeal amoA genes retrieved from $^{13}$C-labelled heavy fractions in K1 day 29 2 psu DNA-SIP microcosm revealed that active AOA belonged to group I.Ia (Fig. 4.11). Group I.Ia, was composed of two main sub-clusters, where all of the active sequences were found in the marine estuarine sediment cluster while the other cluster represented acidic soil isolates. The marine sediment cluster was further broken into two clusters one of which was a *Nitrosopelagicus* like cluster and the other broken into three clusters of which contained the *Nitrosopumilus* like cluster, an estuarine sediment cluster with no isolated representative and finally the *Nitrosoarchaeum* like cluster (Fig. 4.11). The majority of active AOAs 96.7% of total active sequences were found in the *Nitrosoarchaeum* like cluster. The most highly abundant OTU was closely related to *Nitrosoarchaeum koreensis* and DGGE band 8 was also closely related and reflected the most abundant OTU on the DGGE gel (Fig. 4.9 and Fig. 4.11). The second most abundant OTU was found in the *Nitrosoarchaeum* cluster and was most closely related to *Nitrosoarchaeum limnia*. Two active OTUs were found in *Nitrosopumilus* like cluster indicating that cluster also had active members but not as abundant as the *Nitrosoarchaeum* cluster. In addition, three active OTUs were found in the *Nitrosopelagicus* like cluster (Fig. 4.11). A different story is revealed when looking at day 0 OTUs, the *Nitrosopelagicus* like cluster is the most abundant cluster containing 66% of total Day 0 sequences, while *Nitrosoarchaeum* like cluster only contains 23% of total sequences (Fig. 4.11).
All DGGE bands extracted and sequenced were found in the *Nitrosoarchaeum* like cluster.

**Figure 4.11:** Neighbour-joining phylogenetic tree of AOA amoA genes at nucleotide level (≥ 97% similarity). Bootstrap values shown are greater than 70% of 1000 which are shown near nodes. Accession numbers from GenBank are shown from other studies and isolates. Blue open circles indicate K1 freshwater site from *in situ* data; Purple circle indicates sequences from K1 d29 2 psu active fractions; Green squares are sequences from DGGE bands seen in figure 4.9. The numbers in brackets indicate the number of sequences recovered from that site. Coloured circles on branches indicate different clusters. The main
branches are labelled group I.Ia, and group I.Ib, represented by the blue and green circle respectively. Sub-clusters are labelled *Nitrosopeplicus* like cluster, *Nitrosopumilus* like cluster, estuarine sediment cluster and *Nitrosoarchaeum* like cluster, represented by the orange, pink, yellow and beige circles respectively.

### 4.5 DISCUSSION

#### 4.5.1 NITRIFIER ACTIVITY AT VARIOUS SALINITIES

AOB gene copy numbers were higher in all sites along the inlet with no significant change among sites while AOA amoA gene copy numbers decreased from low to high salinity (One-way ANOVA P < 0.05; Fig. 4.3 c, d). AOB gene copy numbers ranged from $2.18 \times 10^8$ – $6.08 \times 10^8$ gene copy g$^{-1}$ sediment while AOA gene copy numbers ranged from $6.22 \times 10^5$ - $1.72 \times 10^6$. AOB gene copy numbers are quite high in comparison to other studies which are usually in the range of $10^4$ – $10^7$ (Smith et al., 2014b; Zhang et al., 2015b). However, AOA gene copy numbers are comparable as most AOA amoA gene copy numbers in sediment studies are in the range of $10^4$ - $10^6$ (Santoro et al., 2008; Smith et al., 2014b).

The majority of ammonia present in the sediment was consumed in all sites after 14 and 29 days; however, nitrate accumulation did not correspond to the amount of ammonia consumed. Nitrate accumulation decreased from 14 days to 29 days indicating that perhaps conditions for denitrification and anammox improved with time and they may have been consuming nitrate as it was produced (Bodelier et al., 1996; Lohse et al., 1993; Smith et al., 2015). A possible explanation for anaerobic processes taking place in the microcosms is that dissolved oxygen decreases with increased salinity (Bank et al., 1967). However, the main driver of decreased oxygen solubility is high organic carbon content in sediments (Wang et al., 2015; refer to section 1.4.4). Sediments used in this study had high organic carbon content (Table 3.3). The highest organic carbon content was in K1 with the next highest found in K3 ($14.85\% \pm 2.35$ and $11.67\% \pm 1.93$, respectively). There was evidence of less nitrate accumulation in K1 salinity incubations with the next lowest found in K3 salinity incubations.
Lack of nitrate accumulation may be as a result of the combination of high organic carbon content and salinity contributing to a decrease in dissolved oxygen which in turn contributes to denitrification and anammox in the microcosms. Additionally, there has been evidence in oxic and anoxic microenvironments in sediments where nitrification and denitrification can be coupled in close proximity (Gilbert et al., 1998; Laverock et al., 2014). Nevertheless ammonia oxidation was demonstrated by the significant decrease in ammonia compared to the acetylene treatments, ensuring the decrease was due to autotrophic nitrification.

**4.5.2 EFFECT OF SALINITY ON AOA**

Significant increases in AOA and AOB amoA gene copy numbers compared to the acetylene treatment signified strong evidence of growth due to ammonia oxidation. Figure 4.3 c and d show AOA and AOB amoA gene abundances across a natural salinity gradient after 29 days of incubation. AOA amoA gene abundances significantly decrease in the higher salinity sites suggesting AOA prefer low salinity habitats (One-way ANOVA; P < 0.05). In addition, when looking at the influence of high salinity fluxes impacting a low salinity site, we observed AOA gene copy numbers in $^{12}$C and $^{13}$C microcosms were significantly higher than the acetylene treated microcosm at day 14 in the high salinity incubation, but after 29 days the AOA numbers were no longer significantly higher. These results suggest AOA may be tolerant of high salinity fluxes for a short period of exposure (Paired t-test P< 0.05 and P = 0.133, respectively; Fig. 4.5 d). Together these results demonstrate that AOA may be the major contributor to AO in the low salinity site K1. Conversely, a study by Qin et al., (2014) showed AOA prefer high salinity. They grew three *N. maritimus* related strains of AOA from 15 to 40 ppt salinity concentration, strain SCM1 was isolated previously (Könneke et al., 2005) and strains HCA1 and PS0 were enriched in this study. They found each strain grew optimally at different salinities, for example SCM1 showed no growth at 15 ppt and was severely inhibited at 20 ppt but grew well between 32 - 40 ppt. While strain HCA1 was inhibited by 15 ppt and 40 ppt but grew optimally between 20 to 35 ppt. Finally,
strain PS0 was inhibited by 15 ppt but grew optimally between 20 and 40 ppt. Hence, this study shows the importance of delving further into AOA community analysis and deducing the phylogeny contained within the active communities as different strains and ecotypes occupy different niches.

Therefore, we went a step further and performed SIP followed by cloning and sequencing on active fractions of AOA to confirm the observations from microcosm experiments that indicated that AOA were the active nitrifiers in low salinity sediments as shown in the heavy labelling of AOA DNA (Fig. 4.8 b). Phylogenetic analysis of AOA in the heavy fractions revealed a different picture that you wouldn’t normally see when looking at the community in situ (Fig. 4.11). The *Nitrosopelagicus* like cluster seemed to be the most abundant cluster at day 0; however, when looking at the active fractions the *Nitrosoarchaeum* like cluster is clearly the most active. This study is the first study to demonstrate growth and activity of *Nitrosoarchaeum* like AOAs at low salinity (Fig. 4.11). The most highly abundant active OTUs were closely related to *N. koreensis* and *N. limnia*. These AOA do not possess genes involved in the ectoine biosynthesis pathway which enables tolerance of salinity (Blainey et al., 2011; Kim et al., 2011; Mosier et al., 2012a). *N. koreensis* was shown to be inhibited by salinity above 0.4 ppt when grown in an enrichment culture (Jung et al., 2011). In this study the *Nitrosoarchaeum* like cluster were found to be active at 2 ppt and could tolerate salinities up to 30 ppt for 14 days, perhaps different strains of *Nitrosoarchaeum* can grow optimally at different salinities in a low salinity range in the same way *N. maritimus* strains grew at different salinity ranges as shown earlier (Qin et al., 2014). In addition, a survey of 500 environmental clones from GenBank carried out by Blainey et al., (2011) fell into the *Nitrosoarchaeum* cluster with the majority from freshwater habitats indicating these AOAs are particularly attuned to low salinity environments. Moreover, approximately 10% of sequences were found in salt marsh sediments, further sanctioning their ability to tolerate high salinities.

*N. koreensis* has a high affinity (Km) for ammonia. The Km of *N. koreensis* is 0.69 µM and they have a Vmax of 11 µM NH₃. Nevertheless, *N.
Chapter 4

*maritimus* still holds the lowest recorded Km for ammonia 0.13 µM and has a Vmax of 24 µM (Jung et al., 2011; Martens-Habbena et al., 2009). Despite *N. koreensis* high affinity for ammonia they can also tolerate ammonia up to 10 mM NH₃ (Jung et al., 2011).

This particular community of AOA have occupied a niche in low salinity intertidal bay sediments, as they thrive well in low salinity environments and can withstand a wide range of ammonia concentrations. They also have the ability to withstand salinity fluxes for short periods of time which occurs daily in K1 at low (0 - 2 ppt) and high (15 – 20 ppt) tide.

4.5.3 AUTOTROPHIC ACTIVITY OF AOB

AOB gene copy numbers showed no change in overall gene abundances between sites and salinities (Fig. 4.3 – 4.7). However, sediments sampled from site K3 were incubated with low and high salinity and AOB amoA gene copy numbers showed a significant increase in ¹²C and ¹³C AOB numbers compared to the acetylene treatments at both salinities (Fig. 4.7), supporting the hypothesis that AOB are potentially contributing to ammonia oxidation at high salinity sites. In similar studies, AOB diversity was reduced as salinity increased along a natural salinity gradient, showing that few AOB phylotypes are suited to a high salinity niche, making AOB high salinity phylotypes more specialised (Bernhard et al., 2005b).

Although, in this study, AOB gene copy numbers are as high as 10⁸ cells per g of sediment. They are lower than the day 0 initial AOB gene copy numbers and in most cases the experimental microcosm ¹³C are not significantly higher than the acetylene treatment meaning firstly, AOB may be growing at all, or secondly, that AOB may not be growing due to autotrophic nitrification (Fig. 4.3, 4.5, 4.6 c). In addition, they have not incorporated the heavy labelled ¹³C into their genome at the low salinity site (Fig. 4.8 a). Why is there such high numbers present and no autotrophic nitrification occurring? Firstly, we may not see clear evidence of growth in AOB due to slow doubling times which can be in the range of 12 hours to several days (Prosser, 1990). For example, if a community grew
Chapter 4

exponentially with a doubling time of two days from a starting community of $10^3$ cells per gram of sediment, they may take up to 20 days to reach $10^6$ cells per gram of sediment. Secondly, we may not see clear evidence of growth for AOB as some species may be dying off in response to microcosm conditions while other phylotypes may be actively growing and replacing the dying species. For example, changes in AOB and AOA community dynamics over time in soil plots with a pH range of 4.9 to 7.5 were investigated where it was found that AOB amoA gene fingerprints were significantly different when pH was increased from 4.9 to 7.5, certain bands decreased in intensity while others increased in intensity (Nicol et al., 2008). Another reason could be that AOB are active but not multiplying. Methods like RNA-SIP could potentially resolve this issue in the future as it does not solely rely on the replication of cells as is needed for DNA-SIP (Pratscher et al., 2011). From looking at the in situ community structure of AOB community in Kinvarra (Fig. 3.10; section 3.4.4) we see that dominant species in the environment is *Nitrosomonas ureae*. *N. ureae* has the ability to break down urea inside the cell to NH$_3$ and CO$_2$ (Fig. 1.4). This is an avenue that has not been explored in this study as we did not account for some AOB’s ability to utilise urea within the sediments and incorporate CO$_2$ produced from the breakdown of urea rather than utilising the heavy labelled CO$_2$ supplied in the microcosm by the addition of $^{13}$C- bicarbonate. Interestingly, AOA *N. limnia* do not possess urease genes and so may not be able to use urea directly as a source of ammonia. Another avenue to explore would be alternative primers. At the high salinity site in Kinvarra bay autotrophic nitrification is still occurring as we see from the nutrient dynamics of ammonia and nitrate compared to the acetylene treatment (Fig. 4.3). However, our primers do not pick up which community is more responsible for this activity. Perhaps the primers in this study are not picking up an important group of AOB or AOA that is potentially contributing to autotrophic nitrification. For example, in Chapter 3 we saw evidence of *Nitrosovibrio* appearing in Kinvarra high salinity sites at RNA level which was not picked up the Rotthauwe AOB amoA primers in this study at DNA level (Fig. 3.9).
Future fractionation of high salinity microcosms will be carried out to further elucidate if there has been heavy labelling of the AOB community and the AOA community.

4.5.4 CONCLUSIONS

This study determined how AOA communities in Kinvarra intertidal bay sediment responded to salinity. AOA thrived in low salinity environments and could withstand high salinity fluxes for short periods of time. However, AOA amoA gene copy numbers dropped significantly at high salinity and growth of AOA also stopped. This is the first study to link *Nitrosoarchaeum* like AOAs to ammonia oxidation growth and activity in a low salinity habitat through the use of experimental sediment microcosm incubations combined with SIP. The response of AOA and AOB to salinity has been ambiguous; however we now know for certain that within Kinvarra bay regardless of AOB gene copy numbers being numerically dominant, AOA were shown to be the active nitrifiers within sediment microcosm from this low salinity site. More specifically, the *Nitrosoarchaeum* like cluster are the dominant active AO in low salinity habitat in Kinvarra; whereas AOB activity remains ambiguous and further investigation is required to elucidate whether AOB actively nitrify at high salinity and whether the abundant *N. ureae* like sequences found in situ utilise urea rather than inorganic ammonia substrate provided in the microcosms.
CHAPTER 5: GENERAL CONCLUSIONS & FUTURE DIRECTIONS

Environmental parameters such as ammonia, salinity, pH and temperature all play a role in influencing ammonia oxidiser activity. Ecological response of ammonia oxidisers to abiotic variability is important to understand in terms of the ecological implications anthropogenic activities can cause. Each experiment in this thesis firstly dealt with surveying the coastal bays of interest and understanding how the natural communities respond to varying environmental parameters in situ. Secondly, we linked the active nitrifier community response to environmental variables in situ to bring current understanding a step closer to linking function to community structure. Thirdly, we applied what we learned from the in situ studies to experimentally explore AO community response to significant environmental parameters (in this case, salinity). This was achieved via laboratory time-series microcosms where experimental conditions are more easily controlled. Finally, we used SIP to test hypotheses derived from our in situ studies to identify the active growing AO under a certain set of conditions, thereby linking identity of AO organisms to autotrophic ammonia oxidation function. To this end, the research presented in this thesis contributes to the understanding of AO response to a range of environmental parameters in intertidal coastal bays, especially salinity. In addition, we showed the importance of understanding the ecotypes involved in the active community and how they differ from ecotypes found when targeting the total AO community.

5.1 AOB VERSUS AOA: WHO IS THE DOMINANT DRIVER OF AMMONIA OXIDATION IN INTERTIDAL SEDIMENTS?

The first aim of the thesis was to determine if AOA or AOB were more abundant in intertidal sediments, and thus indicate the primary drivers of AO. We hypothesise that AOB would be more abundant than AOA due to their preference for high ammonia concentrations which are usually typical of coastal bays. Gene abundances are measured in numerous studies as they are considered proxies for cell abundance (Li et al., 2014; Lisa et al., 2015; Yu et al., 2016; Zheng et al., 2014; Damashek et al., 2015; Lipsewers et al., 2014a; Mincer et al.,
Chapter 5

2007; Santoro et al., 2010; Zhang et al., 2015a; Zheng et al., 2013); the rationale being that numerically abundant AO are likely the primary drivers of ammonia oxidation. In our studies, AOB were found to be more abundant AO within three geographically separated coastal bays (Rusheen, Clew and Kinvarra) on the west Atlantic coast of Ireland (chapters 2, 3 and 4; Fig. 2.3, 3.12, 4.3). Within each bay, at DNA level, AOB *amoA* gene abundances were 1 to 2 orders of magnitude greater than AOA *amoA* except for the intermediate site K2 in Kinvarra where AOA was slightly higher than AOB but not even by a magnitude of one (Fig. 3.12). As discussed in chapter 2, one to three copies of the *amoA* gene are potentially present in AOB (Norton et al., 2002), whereas only a single copy is present in AOA. However, to date there are very few enrichments or isolates of AOA so this number is based on the only AOA isolated in pure culture to-date, *N. maritimus* (Berg et al., 2015; Walker et al., 2010; Zhalnina et al., 2014). Due to the variation in copy number AOA *amoA* gene abundances cannot be used directly to represent cell numbers in the environment. In a review on niche specialisation in AO it was proposed that AOA must be 10-fold higher than AOB gene abundances to be considered the main contributor of ammonia oxidation (Prosser and Nicol, 2012). In our studies AOA never outnumber AOB ten-fold indicating that AOB should be the main contributor of ammonia oxidation in all of the coastal bay intertidal sites. Another issue to be considered when quantifying DNA is that this targets dormant, dead and active cells. Thus, to detect functionally active AO, *amoA* gene transcripts can be targeted as a stronger indicator of AO activity (Philippot et al., 2005). In chapter 2 both AOB and AOA gene abundances and transcripts were measured in Rusheen bay, both AOB gene abundances and transcript abundances were higher than AOA. In this case gene abundances and transcript data aligned to support the hypothesis that AOB are the dominant driver of ammonia oxidation in these sediments. Conversely, in Kinvarra at the low salinity site K1, AOB were more abundant than AOA (chapter 3 and chapter 4), yet when looking at the active autotrophic ammonia oxidiser community in the microcosms we found AOA were the active community regardless of higher AOB gene abundances (Fig. 4.3 and 4.8). Further, in the microcosms, AOB did not grow. From the evidence presented in
this thesis, it is clear that gene abundances alone are not sufficient to determine the active AO. For this a combination of techniques are required, such as in situ transcript analysis, as conducted in chapter two, combined with laboratory testing of the observation using controlled microcosm experiments, such as the inclusion of acetylene to block nitrification. We went a step further and combined these experiments with \(^{13}\)CO\(_2\) to exploit the autotrophic nature of AO to confirm activity and growth via the incorporation of \(^{13}\)C into active cells. Using this approach we showed that AOA were driving ammonia oxidation in the low salinity site in Kinvarra bay. Further evidence points to AOA preferring low salinity sites in intertidal sediments in chapter 2 where AOA gene abundance negatively correlated with salinity and *amoA* sequences were only recovered from the low salinity sites (Table 2.4; Fig. 3.14; Damashek et al., 2015). The more difficult part to untangle is the specific reasons behind this and to determine the environmental drivers of AO communities spatially, temporally and between bays.

### 5.2 TEMPORAL AND SPATIAL CHANGES IN AO DYNAMICS WITHIN INTERTIDAL BAYS

The second aim of the thesis was to understand AOA and AOB dynamics temporally and spatially within intertidal bays. We hypothesised temporal increases in PNR activity and AOB gene abundances due to previous studies, using laboratory based experiments, showing that AOB responded to temperature fluctuations more than AOA (Urakawa et al., 2014). To this end we undertook an extensive temporal field campaign in two bays to monitor nitrifier activity via PNR, and AOB and AOA gene abundances, while simultaneously recording a range of environmental parameters from each sampling time point and site in Rusheen and Clew bay. Spearman rank correlations showed that AOB gene abundances negatively correlated with temperature while AOA gene abundances positively correlated with temperature (Table 2.4). AOB correlations were stronger than AOA which disagrees with Urakawa et al., (2014) who showed the contribution of AOB was minor compared to AOA in the water column along the Hood Canal, except at the shallow site (15 m depth) where the AOB single cell
ammonia oxidation rate dropped significantly when temperature changed from 11.5 (October) to 8.7°C (December). In contrast, the AOA single cell ammonia rates stayed consistent. This shows that AOB responded to temperature more than AOA and became a more competitive player in this environment. Our data indicates that AOB and AOA are responding to temperature in situ and that there may be a temporal pattern. While the effect of temperature on the AO community within our sediments requires further investigation, the fact that AOB and AOA have opposite responses to temperature further suggests niche partitioning and that they may have different optimal temperatures for growth. Negative correlations of AOB and temperature were also shown in intertidal sediments from the Yangtze estuary and Laizhou Bay (Yang et al., 2015; Zheng et al., 2014). Other fluctuating parameters that significantly correlated with AOB and AOA gene abundances were pH and NH₄⁺. AO substrate NH₄⁺ correlated negatively with both AOB and AOA gene abundances for obvious reasons as both AOB and AOA oxidise ammonia to nitrate in order to grow. pH directly affects ammonia availability (Stein et al., 1997); the higher the pH the more NH₃ is available. AOB and AOA both positively correlated with an increase in pH which agrees with correlations also seen in the Yangtze estuary and Western English channel sediment (Table 2.4; Laverock et al., 2014; Zheng et al., 2014). Lastly, AOA gene abundance negatively correlated with salinity indicating AOA within intertidal sediments may prefer low salinity ecosystems (Table 2.4; Damashek et al., 2015). The data shows that fluctuations in environmental parameters influence AOA and AOB dynamics in intertidal sediments.

Nitrification was very low in Clew bay and so spatial variation was not clearly seen within the bay. There may be a number of reasons why nitrification rates are low, but an unmeasured variable in this study that may negatively impact upon nitrification is heavy metal concentration. There are high arsenic concentrations in Clew bay (pers. comm. Dr. Liam Morrison) that are a result of the natural geology in the surrounding area, Clew bay contains large amounts of arsenopyrite (Gilligan et al., 2016). The oxidation of arsenopyrite can occur near the surface of the mineral and leads to arsenites and arsenates being formed which can leach into ground water thereby introducing the heavy metal arsenic.
into nearby waterways and groundwater. This heavy metal input could potentially lead to the low nitrification rates found in Clew bay. Concentrations of arsenic have been measured in drinking water wells near Clew bay and concentrations ranged from (6 - 200 µg l\(^{-1}\); Gilligan et al., 2016). Studies carried out in Laizhou Bay show that AOB gene abundance were negatively correlated to arsenic however concentrations were much higher than measured in Clew bay and ranged from 840 – 3910 µg l\(^{-1}\) (Yang et al., 2015; Zhang et al., 2014). Further work is required to explore concentrations of arsenic found in sediments in Clew bay and whether the concentrations in situ impact nitrification. Due to the higher rates of nitrification, spatial dynamics of AO and diversity, Rusheen and Kinvarra bay were studied in more detail. Rusheen and Kinvarra showed significant spatial variability in PNR (Fig. 2.2 and 3.11) and AO gene abundances (Fig. 2.3 and 3.12). AOB and AOA gene abundances in Kinvarra showed a strong negative correlation with salinity indicating that salinity affects both AOB and AOA in Kinvarra bay (Table 3.7), where salinity ranged from 0.38 to 28.65 psu in situ at low tide. AOB and AOA gene abundances in Rusheen bay correlated positively with salinity (Table 2.5); however, the highest salinity at low tide recorded in Rusheen bay during the month of February was 24 psu which is classified as a medium salinity. The intermediate salinity site showed high activity in the Plum Island Sound estuary (Bernhard et al., 2007). Contrasting evidence of how salinity impacts AOA and AOB gene abundances in Kinvarra and Rusheen bay may indicate that the community composition of AOA and AOB are quite different between bays. However, caution must be taken when analysing correlations as salinity may co-correlate with other variables that were not measured in this study. Low salinity ecotypes of AOA have been documented in the North San Francisco bay (0.5 psu; Francis et al., 2005), sequences falling into this cluster were further seen in the Sacramento and San Joaquin rivers (Damashek et al., 2015). The sequences in the low salinity cluster are closely related to isolates *Nitrosoarchaeum limnia* and *Nitrosoarchaeum koreensis*. Ecophysiology studies have demonstrated that these two microorganisms prefer low salinity habitats (Jung et al., 2011; Mosier et al., 2012b). Sequences from both Kinvarra and Rusheen bay found in cluster I, II and
III seem to correspond to this low salinity cluster also found in the North San Francisco bay, Sacramento and San Joaquin rivers (Fig. 5.2). Six distinct ecotypes were observed after analysis of archaeal amoA sequences from the Genbank database ranging over 13 different habitats. The six ecotypes had strong influences from both salinity and habitat type. One of the ecotypes was dominated by high salinity estuarine sequences while another ecotype was dominated by low salinity estuarine sequences (Biller et al., 2012). For AOB large surveys have not been carried out to elucidate specific ecotypes, however smaller studies have found some distinct clustering based on salinity. In Chesapeake bay sequences from medium and high salinity sites fell into a Nitrosospira like cluster (Francis et al., 2003). In the Plum Island Sound estuary AOB amoA gene from a low intermediate and high salinity site were sequenced, the intermediate and high salinity site also fell in the Nitrosospira like cluster first recognised in Chesapeake bay, which seems to represent a medium to high salinity cluster (Bernhard et al., 2005b; Francis et al., 2003). Bernhard et al., 2005b also found that the low salinity site clustered more with Nitrosomonaes like sequences especially with N. ureae and N. oligotropha which are known to have no salt requirement (Bernhard et al., 2005b; Koops et al., 2006). This prompts us to think about the significance of AOA and AOB community composition across the bays and why AOA and AOB are co-occurring.

### 5.3 WHAT IS DRIVING AMMONIA OXIDATION DYNAMICS IN INTERTIDAL SEDIMENTS?

Diversity has been associated with functional redundancy, the more diverse a community the better it is able to withstand changes in environmental parameters (Konopka, 2009). For example, if a stress such as salinity is applied to a community, a community that is more diverse is more likely to have the ability to withstand that stress as the stress selects for a different set of taxa that may not have been active beforehand; this ensures the function of the community as a whole is not lost in an ecosystem. The data generated in this thesis indicates that AOA and AOB are occupying separate niches within the same site, thus maintaining diversity within the ecosystem. In particular, the evidence from the RT-Q-PCR of AOA and AOB across Rusheen bay showed that both groups were
transcriptionally active. In contrast, we also showed in the Kinvarra microcosm study (Chapter 4) that AOA were active despite high AOB gene numbers, however the diversity was still seen in the AO community regardless of activity as dormant and inactive cells also contribute to diversity of an ecosystem.

Ammonia concentration is one of the main factors influencing AOA and AOB dynamics and niche differentiation in situ. In general, AOB prefer higher ammonia concentrations to AOA (Martens-Habbena et al., 2009). We see from community analysis that *Nitrosomonas* like sequences were the most abundant in both Rusheen and Kinvarra bay (Fig. 2.6 and 3.13). *N. ureae, N. oligotropha* like sequences made up the largest cluster of AOB sequences retrieved. *N. ureae, N. oligotropha* isolates have a high affinity for ammonia (1.9 – 4.2 µM). Within that same cluster there were also sequences closely related to *N. marina* and *N. aestuarii* which also have a high affinity for ammonia (50 – 52 µM; Koops and Pommerening-Roser, 2001). Based on the physiology of the closest known isolates, it indicates that the AOB within this cluster may have the ability to withstand very high concentrations of ammonia, ranging from 50 to 400 mM, which wouldn’t normally be found in coastal ecosystems (Koops et al., 1991). This *Nitrosomonas* like cluster is found in numerous coastal sediment studies and is potentially a cluster that is typical of coastal sediment environment (Bernhard et al., 2005b; Cébron et al., 2003; Damashek et al., 2015; Zheng et al., 2014). High affinity for ammonia may be a physiological trait that is shared among all OTUs in this cluster as they are found in coastal ecosystems that experience high inputs of nitrogen from anthropogenic sources but can also experience periods of low ammonia due to ammonia benthic fluxes (section 1.4.5). But, we cannot assume that the AOB identified only by a fragment of a gene have the same physiology as that of closely related organisms. Indeed, Lehtovirta-Morley et al., 2014 showed that closely related strains of *Nitrosotalea devanaterra* had different physiological responses to pH. Environmentally relevant isolates of AOB and AOA are urgently required in order to explore this further.

The *Nitrosomonas* isolates have shown previously in the section above that they have high affinity for ammonia; however they can also be separated by
salinity. *N. marina, N. aestuarii* both require salt to grow, while *N. oligotropha* and *N. ureae* do not (Koops et al., 1991; Koops and Pommerening-Roser, 2001). We see this ecotype separation within our bays in the *Nitrosomonas* cluster, with *Nitrosomonas* like OTUs that were ubiquitous at all sites in Rusheen and Kinvarra in addition to the occurrence of freshwater or high salinity OTUs only (Fig. 2.6 and 3.13). This further indicates salinity as a significant driver of niche separation for AOB in both Rusheen bay and the active nitrifier community in Kinvarra bay (Fig. 2.5 and 3.10 b). However the sequencing depth in Rusheen and Kinvarra bay for both DNA and RNA may have been too low to capture the full extent of the AOA and AOB total and active communities. Further analysis is required to test this, perhaps using next generation sequencing methods as opposed to clone libraries. In addition to this, sequencing methods still rely on primers and so the depth of sequencing will only be good enough to capture the various clusters and groups of AO that primers target. Therefore metagenomics could help alleviate the biases and limitations primers and PCR based methods incur this will be discussed in more detail in section 5.5.

AOA in Rusheen bay clustered with *Nitrosoarchaeum* in clusters I, II and III. In addition, AOA in clusters IV, V, VI and VII were unrelated to any known isolate, albeit the limited number currently in isolation, but did cluster with AOA *amoA* sequences from other similar intertidal sediments (Fig. 2.7). In Kinvarra bay there were four unknown clusters relating to other intertidal sediments and only one OTU was related to *Nitrosoarchaeum* (Fig. 3.14). *N. koreensis* is found mostly in freshwater systems. *N. koreensis* has an even higher affinity for ammonia (0.69 µM) than *N. ureae* and *N. oligotropha*. These AOA in the freshwater *Nitrosoarchaeum*-like clusters could have the ability to outcompete AOB if they had a similar affinity for ammonia as the known isolate, which can withstand ammonia concentrations up to 5 mM (Jung et al., 2011). However, to test these hypothesis isolates are required.

Ammonia concentrations in Rusheen bay February ranged from 0 to 56.5 µM, while in April in Kinvarra bay ammonia concentrations were higher and ranged from 77 to 227 µM (Fig. 5.1 and 5.2; Table 2.3 a and 3.3). In Kinvarra
bay, transcripts for AOA and AOB were not sequenced, instead, the low salinity site sediment was enriched for 29 days under low salinity conditions and the active community was targeted through SIP. AOB showed no growth after 29 days and their overall numbers dropped (Fig. 4). On the other hand, sequencing of the heavy labelled SIP fractions, identified *N. koreensis* and *N. limnia*-like AOA. In both bays, the ammonia concentration did not go above the inhibitory concentrations for either *Nitrosomonas* like clusters in AOB and *Nitrosoarchaeum* like clusters in AOA. Perhaps a possible explanation for AOB inactivity is that AOA were able to out-compete AOB in K1 freshwater site due to their higher ammonia affinity and ability to grow well in freshwater. These observations suggest ammonia and salinity are primary drivers of AO community composition.

5.4 THE IMPORTANCE OF ECOTYPES IN AO COMMUNITIES

Community composition can inform on how individual taxa respond to environmental perturbation; however it is important to confirm the communities’ activity as some of the community may be inactive at a given time. Combining Rusheen and Kinvarra bay total and active communities in one tree allows us to observe any changes between the active and total community and if there are any clusters specific to certain environmental parameters such as high or low salinity concentrations. Here we will compare all sequences retrieved from both Kinvarra and Rusheen bay studies, some of which were amoA genes, transcripts or genes from heavy labelled SIP fractions.

Rusheen bay and Kinvarra bay AOB OTUs overlap and are found within the same clusters (Fig. 5.1). In Rusheen bay mRNA amoA amplicons, indicating active AOB, are from the sites with low ammonia (cluster VII and VIII in Fig. 5.1). Furthermore, the two clusters contained sequences generated from mRNA and were from medium and low salinity sites with ammonia concentrations much less than 50 µM. This cluster could be representative of AOBs that have the ability to scavenge ammonia environments in addition to being halotolerant, withstanding salinities up to 24 psu. A Kinvarra freshwater sequence at DNA
level also fell within this cluster but was not observed as active, as defined by retrieval of a corresponding transcript or heavy labelling in the SIP microcosm study from that site (Fig. 4.1). Clusters IV, V and VI contain the majority of AOB sequences from both Kinvarra and Rusheen bay (Fig 5.1). They contain DNA and RNA sequences from all salinity ranges and ammonia concentrations. These clusters seem to represent versatile and resilient AOB phylotypes that may be typical of coastal ecosystems, as they are also found globally in a range of other studies of coastal ecosystems (Bernhard et al., 2005b; Cébron et al., 2003; Damashek et al., 2015; Zheng et al., 2014). Evidence of an AOA coastal-marine sediment cluster was seen in a study analysing a relatively large dataset containing 6203 unique OTUs from 13 different habitats. They showed four major clusters representing coastal and marine sediments, one representing soil and two from the water column in the open ocean (Biller et al., 2012). However, AOB coastal marine sediment clusters have not been identified yet, as further analysis is required to confirm specific clusters associated with coastal marine sediments. Finally clusters I and II are more closely related to *Nitrosospira briensis* and contain DNA sequences from both Rusheen and Kinvarra bay (Fig. 5.1). These clusters seem to be inactive, based on this current study, and are usually found in soils (Rice et al., 2016; Watson, 1971). However, further analysis is required to confirm this due to low sequencing depth within our clone libraries. So why are dormant AO present?
Figure 5.1: Neighbour-joining phylogenetic tree of AOB amoA genes at protein level (≥ 97% similarity). Bootstrap values shown are greater than 70% of 1000 which are shown near nodes. Accession numbers from GenBank are shown from other studies and isolates. Circles indicate Rusheen bay DNA sequences, open circles specify Rusheen bay cDNA sequences. Squares indicate Kinvarra DNA sequences. The coloured symbols represent different salinities blue, maroon and green indicate low, medium and high salinities, respectively. Ammonia concentration of Rusheen and Kinvarra bay are represented by the highlighted clones, low medium and high ammonia concentrations are in white, pink or green respectively. The numbers in brackets indicate the number of sequences recovered from that site.
Dormant cells are cells that are considered inactive; these cells will not grow unless resuscitated with conditions that they require to grow (Roszak and Colwell, 1987). Bacteria that are exposed to stress that is unfavourable to them may reduce their metabolism to very low levels for a while, if conditions become favourable again they can grow and replicate as normal. However, if conditions continue to be stressful for the cell, complete metabolic shutdown may arise leading to dormancy (Mukamolova et al., 2003). Stresses such as changes in temperature, pH (Shleeva et al., 2011), salinity and nutrients can all trigger dormancy (Mukamolova et al., 2003) which has been shown to allow bacteria to survive in extremely difficult conditions for long periods of time, such as in the permafrost layer in the Arctic (Vorobyova et al., 1997). Perhaps dormancy is an adaptive mechanism for microorganisms to survive in environments with constant fluctuations in environmental parameters. We hypothesise that dormant cells persist in a community as a survival mechanism when conditions become unfavourable and their presence contributes to the diversity of the ecosystem, thereby maintaining ecosystem function when conditions become favourable again and select for a different set of taxa. Nonetheless, more studies are required to elucidate whether dormancy occurs in AOA and AOB and if they use this as a survival mechanism in situ. This hypothesis requires isolates in order to demonstrate the possibility of dormancy.

The majority of AOA sequences in Rusheen and Kinvarra bay are found in group I.Ia. AOA OTUs from both bays are found within the same clusters (Fig. 5.2). In cluster I, active transcripts from both Rusheen and Kinvarra bay are found from freshwater sites, these sequences are closely related to *N. koreensis*, a freshwater AOA (Kim et al., 2011; Mosier et al., 2012a). We didn’t find evidence of activity from the medium salinity site in Rusheen bay based on the methods we used in this study suggesting that they may be transcribing at undetectable levels or are dormant in the community and will become active in the event conditions become favourable again as mentioned above. Cluster II has no isolated representative but was actively transcribing in freshwater and was present in the Rusheen bay medium salinity site at DNA level. Cluster III contains active sequences from freshwater and is closely related to *N. limnia* and
\textit{N. maritimus} which occupy opposite niches when it comes to salinity, \textit{N. limnia} prefers freshwater while \textit{N. maritimus} prefers high salinity habitats (Blainey et al., 2011; Qin et al., 2014); only one DNA sequence from Rusheen bay medium salinity site was found in this cluster. \textit{Nitrosopelagicus brevis} usually found in the open ocean is found in cluster IV and is closely related to one of the active sequences from Kinvarra (Santoro et al., 2015). Cluster V has no isolated representative but consists of DNA sequences from both Rusheen and Kinvarra and active sequences from Rusheen bay at medium and low salinity sites indicating this cluster is robust because it can grow in a wide range of salinities from 0 to 24 psu. Lastly very few sequences were found in group I.Ib, usually found in soils (Bates et al., 2011; Pester et al., 2012), however there was a representative from both Kinvarra and Rusheen bay (Fig. 5.2).
Figure 5.2: Neighbour-joining phylogenetic tree of AOA amoA genes at protein level (≥ 97% similarity). Bootstrap values shown are greater than 70% of 1000 which are shown near nodes. Accession numbers from GenBank are shown from other studies and isolates. Circles indicate Rusheen bay DNA sequences, open circles specify Rusheen bay cDNA sequences. Squares indicate Kinvarra DNA sequences, open squares specify Kinvarra bay active DNA sequences from the microcosm experiment. The coloured symbols represent different salinities; blue and maroon indicate low and medium salinities, respectively. Ammonia concentration of Rusheen and Kinvarra bay are represented by the highlighted clones, low medium and high ammonia.
concentrations are in white, pink or green respectively. The numbers in brackets indicate the number of sequences recovered from that site.

Active and potentially inactive clusters found in coastal intertidal bays suggest first of all that sequencing efforts may not have deep enough to capture the diversity at DNA and cDNA. However, amoA amplicons were in general very weak and not suitable for high throughput sequencing. Secondly, potentially inactive clusters are present contributing to the diversity of a community and may enable the community to maintain ecosystem function when there are unexpected changes in the environment that the current active community cannot deal with (Konopka, 2009). To this end, *in situ* and *ex situ* total and active community analysis combined with environmental parameter measurements can bring us closer to elucidating ecotype response to environmental perturbations.

### 5.5 FUTURE WORK AND CONCLUSIONS

Our results demonstrated that coastal sediments are diverse communities driven by a range of environmental parameters. More specifically we demonstrated that AOB were the dominant group of ammonia oxidisers in Rusheen and Kinvarra bay, however AOA were confirmed to be more active in Kinvarra low salinity site. Both AOA and AOB were shown to actively co-occur in Rusheen bay, indicating that both are likely contributing to nitrification. Environmental conditions such as nutrient concentrations, salinity, pH and temperature influenced the distribution of AOB and AOA communities within intertidal sediments. Salinity was a major driver of AOA and AOB community variability. Sequence data identified the most abundant total and active phylotypes which were closely related to *N. ureae* and *N. koreensis* for AOB and AOA, respectively, in both Rusheen and Kinvarra bay. AOA thrived in low salinity environments and could withstand high salinity fluxes for short periods of time. A further significant contribution to current understanding of nitrification conducted in this thesis was that we, for the first time confirmed the activity of autotrophic AOA in freshwater using DNA-SIP.
To learn more about this community in the future, we will combine DNA-SIP with metagenomics to target the uncultured active AOA community that we have enriched in freshwater microcosms. DNA-SIP combined with metagenomics is a powerful tool that enables us to target active microorganisms from the rare biosphere combined with the ability to recover the entire genome of the organisms, enabling us to discover potential novel enzymes without prior knowledge of gene targets (Coyotzi et al., 2016). In addition, the information we discern could also be used to reconstruct metabolic pathways which would allow us to predict some of the uncultivated AOA functions. This information would facilitate greater understanding of AOA that would be exploited to enable enrichments and eventually isolate AOA from the environment using a more informed approach. The information contained within the genomes of yet to be cultured AO may reveal key processes the organism requires for growth, for example, pathways requiring trace elements. A study using this approach has recently been carried out in soil and lake sediment, with the aim of understanding the functional aspects of dimethylsulphide degraders (Eyice et al., 2015). The authors confirmed that a previous known group of *Thiobacillus* were actively degrading dimethylsulphide and also discovered, for the first time, that *Methylophilaceae* were dimethylsulphide degraders (Eyice et al., 2015). A similar metagenomic study, this time using a targeted DNA-SIP approach recovered an almost complete genome of *Methylotenera mobilis* from lake water sediment (Kalyuzhnaya et al., 2008).

In conclusion, the research presented in this thesis has shown that firstly ammonia and salinity are major environmental drivers in the AO community in intertidal coastal bay ecosystems. Other drivers include carbon, pH and temperature. Secondly, AOA and AOB occupy separate niches within the same site, thus preserving diversity within the ecosystem and maintaining ecosystem function. Thirdly, both transcript analysis and acetylene controlled experimental microcosms combined with SIP revealed the differences in active communities compared to total communities. This highlights the importance of delving deeper than DNA studies alone to gain a better understanding of active ecotypes and their response to environmental parameters. Combinations of ‘omics approaches,
with experimental hypothesis testing will lead to a greater understanding, and ultimately aid us in bringing more environmentally relevant nitrifiers into pure culture and provide us with the ability to test various hypotheses put forward in this thesis. Overall this is an exciting time to study microbial ecology as more and more methodologies are allowing us to link microbial identity to function and using an experimental approach we can construct connections between the microbial diversity of a community and ecosystem function.


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