STUDIES ON NITRIFYING MICROORGANISMS IN COCHIN ESTUARY AND ADJACENT COASTAL WATERS

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Doctor of Philosophy in

MARINE MICROBIOLOGY

UNDER THE FACULTY OF MARINE SCIENCES

By

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March 2015

Certificate

This is to certify that the research work presented in this thesis entitled "**STUDIES ON NITRIFYING MICROORGANISMS IN COCHIN ESTUARY AND ADJACENT COASTAL WATERS**" is based on the original work done by Mr. Vipindas P V (Reg. No. 4012), under my supervision at National Institute of Oceanography, Regional Centre, Kochi, 682018, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in Faculty of **Marine Sciences,** Cochin University of Science and Technology, Kochi, 682018 and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition in any universities or institutes.

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March, 2015

Declaration

*The research work presented in this thesis entitled "**Studies on Nitrifying Microorganisms in Cochin Estuary and Adjacent Coastal Waters**" submitted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**, is a bonafide record of the research work done by me under the supervision of Dr. ShantaAchuthankutty, Chief Scientist, National Institute of Oceanography, Regional Centre, Kochi, 682018. No part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition in any universities or institutes*

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March, 2015 **Vipindas P V**

I am dedicating this work to my Family

Teachers and Friends

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Vipindas P V

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LIST OF ABBREVIATIONS

1. AMO	-	Ammonia Monooxygenase
2. ANOVA	-	Analysis of Variance
3. Anammox	-	Anaerobic Ammonia Oxidation
4. AOB	-	Ammonia Oxidizing Bacteria
5. AOA	-	Ammonia Oxidizing Archaea
6. BLAST	-	Basic Local Alignment Search Tool
7. CE	-	Cochin Estuary
8. DO	-	Dissolved Oxygen
9. DGGE	-	Denaturing Gradient Gel Electrophoresis
10. FISH	-	Fluorescent *In Stu* Hybridization
11. HAO	-	Hydroxylamine Oxidoreductase
12. NOB	-	Nitrite Oxidizing Bacteria
13. OTU	-	Operational Taxonomic Units
14. PCA	-	Principal Component Analysis
15. RNR	-	Recovery of Nitrification Rate
16. SPM	-	Suspended Particulate Matter

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Chapter - 1
INTRODUCTION

Contents

1.1 Estuary

Estuaries are dynamic aquatic systems that are transition zones linking fresh- and marine-water, greatly influenced by near shore activities, and experience large fluctuation in hydrological, morphological and chemical conditions. Estuaries are ecologically and economically important ecosystem, which function as transition zone between marine and freshwater systems. In this ecosystem, physical processes are the major determinants for the distribution and variability of chemical and biological variables. In particular, freshwater input and tidal currents structure the estuarine variables into spatial gradient and determine their characteristics scales by temporal variability. It is regarded as complex ecosystem with diverse habitats viz. sea grass beds, mangrove swamps, creeks, land bays. Estuarine areas are characterized by naturally derived organic matter that originates from auto-chthonous production, the open sea, surrounding salt marshes, river drainage and anthropogenic contribution. Among the various biogeochemical cycles of the estuarine system, nitrogen cycle is the most complex and important one. At the same time nitrogen also acts as a limiting nutrient for primary productivity in the marine environment (Howarth 1988).

1.2 Nitrogen Cycle

Nitrogen appears in Nature in multitude of compounds either in inorganic or organic forms. Nitrogen is fundamental to the structures and biochemical processes that define life and forms a critical component of nucleic acid and protein. Triple bonded N_2 gas is the largest reservoir of nitrogen in the Earth and it comprises about 78.08% of the total atmospheric gas. But most of the living forms are not able to directly utilize this largest reservoir of nitrogen and, so it must be fixed by certain group of microorganisms known as the nitrogen fixers to make it readily usable by other organisms. This fixed nitrogen and its different forms can be utilized by all other organisms including the autotrophs and heterotrophs as their source of nitrogen. Once utilized, nitrogen exists in its most reduced state within the organisms, and in most ecosystems nitrogen is primarily stored as living and dead organic matter. The reduced forms of nitrogen within the organism are released following cell death and are rapidly converted to ammonia. This ammonia is oxidized aerobically to nitrate via nitrite through nitrification. Nitrate is in turn denitrified to N_2 gas under suboxic to anoxic conditions and thus completes the cycle. A simple nitrogen cycle is illustrated in Figure 1.1.

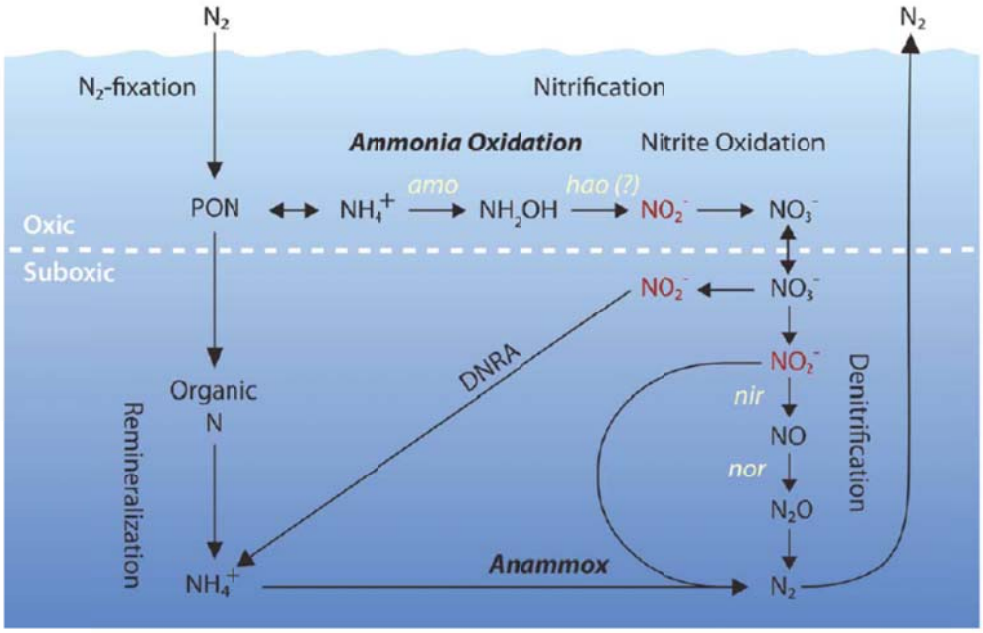


Figure 1.1 Microbial nitrogen transformations above, below and across an oxic/anoxic interface in the marine environment (based on Arrigo, 2005).

1.3 Nitrification

Nitrification occupies a central position within the nitrogen cycle by linking nitrogen mineralization to potential nitrogen loss from the system through denitrification. Nitrification is the major oxidative reaction in the nitrogen cycle in which organic nitrogen is sequentially converted to its highest oxidation state, i.e. nitrate by a microbial mediated process. The two steps involved in nitrification are "ammonia oxidation" and "nitrite oxidation". Aerobic ammonia oxidation is the first and rate-limiting step in nitrification (Purkhold et al. 2000) through which ammonia is oxidized to nitrite. Oxidation of nitrite to nitrate is the second step and both processes usually involve oxygen as the terminal electron acceptor. Nitrification was traditionally considered to be restricted to aerobic environments (Froelich et al. 1979), but recent study (Mortimer et al. 2004) has shown that nitrification also happens in anoxic environments at the expense of elements like manganese and/or iron.

Nitrification does not alter the quantity of nitrogen in any ecosystem but affects the composition, distribution, and fate of dissolved inorganic nitrogen (DIN) compounds. The products of nitrification serve as the substrates for denitrification and/or anaerobic ammonium oxidation (anammox), which remove bioavailable N from the system in the form of N_2 gas (Jenkins and Kemp 1984, Codispoti and Christensen 1985, Mulder et al. 1995, Hellinga et al. 1998). It is also linked with the enhanced production of the potent greenhouse gases nitric oxide (NO) and nitrous oxide (N_2O). These processes determine the concentrations and distribution of bio-available nitrogen in aquatic systems. Nitrification can also influence the ecosystem oxygen distribution by consuming 1.5 M of O_2 for every mole of ammonia oxidized. The O_2 consumed by nitrification is ultimately converted to H_2O, rather than back to O_2 (Mayer et al. 2001), and so the removal may have significant consequences in nutrient rich, high productivity regions which are susceptible to hypoxia (Lohrenz et al. 2008). It is an important oxygen consuming process in nutrient rich estuarine environment (Garnier et al. 2001, Gazeau et al. 2005). Nitrification invited the research interest of both chemical and biological oceanographers due to various reasons, extending from its intricacies of chemical transformations, environmental

importance in eutrophication and nitrous oxide emission and also due to the involvement of diverse groups of microorganisms in this process. Nitrification is particularly important in estuarine and coastal environments where the anthropogenic nitrogen eutrophication is removed by this process and it is estimated that around 30 % of globally fixed nitrogen loss occurs in estuarine and coastal environment (Voss et al. 2011). At the same time nitrogen also acts as a limiting nutrient for the primary productivity in the marine environment.

1.4 Nitrifiers- Diversity and Abundance

The involvement of bacteria in ammonia oxidation and the existence of ammonia oxidizing bacteria (AOB) were identified in 1890 by Winogradsky (1890). To date, all known AOB fall into two distinct phylogenetic microbial groups within the β- and γ-proteobacteria as reviewed by (Kowalchuk and Stephen 2001). A persistently increasing gene sequence database of AOB 16S ribosomal DNA (rDNA) has produced descriptions of distinct lineages and clusters within the group. Pure culture representatives are available for most of the groups and lineages. The more recent revision in our understanding of the nitrogen cycle has come after hundred years of AOB discovery, followed by the analysis of sequence libraries from seawater (Venter et al. 2004) and soil (Treusch et al. 2005). These studies have revealed the presence of putative genes involved in ammonia oxidation in genomic fragments extracted from uncultivated Crenarchaeota. The confirmation of this process was obtained by the isolation and cultivation of *Candidatus Nitrosopumilus maritimus* (Könneke et al. 2005). After the discovery of ammonia oxidizing archaea (AOA), numerous studies have focused on this group of organisms and provided persuasive data to support their wide existence and dominance over AOB from most of the natural environments where ammonia is present in low concentrations (Mincer et al. 2007, Shen et al. 2008). High affinity of AOA for ammonia than AOB supports their dominant existence in oligotrophic environment (Martens-Habbena et al. 2009). The actual chemical mechanism of archaeal ammonia oxidation is distinctive and as yet unresolved, but it shares genes distantly related to ammonia monoxygenase gene of bacterial ammonia oxidizers (Treusch et al. 2005). The habitat range of AOA, which is significantly broader than that of AOB, includes

extreme environments like hot springs, acidic soils, Arctic and Antarctic regions (Murray et al.1998).

The processes and the players involved in the second step of nitrification process i.e. oxidation of nitrite into nitrate, have been less studied. The genus *Nitrobacter* was previously considered to be the major nitrite oxidizer (Bock and Koops 1992). However, the application of recent molecular techniques in the field of microbial ecology made it possible to explore the composition of the nitrite-oxidizing community in the environment and to improve our understanding of its functioning (Daims et al. 2001, Dionisi et al. 2002). At present four phylogenetically distinct genera have been reported, and these are *Nitrobacter* (alpha subclass of Proteobacteria), *Nitrococcus* (gamma subclass of Proteobacteria), *Nitrospina* (delta subclass of Proteobacteria) and *Nitrospira* (distinct phylum). Among the four genera *Nitrobacter* strains are omnipresent in natural environment and other three genera are generally assumed as the players in marine environments (Bock and Koops 1992). Most of the studies on nitrifiers are limited to the temperate and subtropical areas, and further most of the recent published studies on nitrifying organisms are pertaining to sediment (Bernhard and Bollmann 2010). Therefore, there is a general lacuna on these organisms from the tropical waters particularly from estuaries.

1.5 Factors Affecting Nitrification

Nitrification in a system is regulated by the distribution, abundance and activity of the nitrifiers community in that system, which is in-turn influenced by the changing environmental conditions. Influence of environmental factors, such as salinity, temperature, pH and nutrients on nitrification in estuarine and marine environments has been elaborated by various studies (Jones and Hood 1980, Allison and Prosser 1993, Rysgaard et al. 1999, Cébron et al. 2003, Caffrey et al. 2007) and all these studies suggest different contributing factors or different optimum environmental conditions at different geographical locations. So the influence of these factors on the distribution and dynamics of nitrifiers is complex to be studied separately. The role of environmental and biological factors on diversity, distribution and activity of nitrifiers is not well established (Bernhard et al. 2005, Erguder et al. 2009). Studies on distribution of nitrifying microorganisms and the

nitrification rates in estuaries are very limited as compared to the total number of estuaries (ca 1200) and these are mostly from temperate and subtropical estuaries (Bernhard and Bollmann 2010) while the tropical estuaries especially those along the southeast Arabian Sea have not received enough attention.

Despite the obvious ecological importance of nitrification, the microbial population that mediate the process and the environmental factors which regulate the population are poorly understood. High abundance of AOA in natural system especially in oligotrophic environment have been reported (Leininger et al. 2006, Erguder et al. 2009), however high AOB abundance in nutrient rich estuaries (exceptions also there) have also been reported (Wankel et al. 2011), but still there are many questions that are unanswered by scientific community. The important questions include, what is the actual contribution of AOA and AOB to the actual process? Do AOB and AOA co-exist and compete each other as these organisms share the same substrate and niche? Do AOA have unique biogeochemical links to the carbon and nitrogen cycles?

1.6 Hypothesis and Objective

Estuaries are dynamic aquatic systems that are transition zones linking fresh- and marine-water, greatly influenced by near shore activities, and experience large fluctuation in hydrological, morphological and chemical conditions. From the previous sections it is obvious that nitrification, though a key reaction in the environment, is influenced by an array of environmental factors. Some of these have positive influence while some others have negative influence. Hence, the health and sustainability of the estuary and coastal environments need to be studied from the perspective of nitrogen cycling and nitrification in particular. A well balanced functioning of nitrogen biogeochemical cycle is a prerequisite for sustainable maintenance of the estuary. In the estuary, nitrifiers might experience large changes as well, some species may be limited to narrow variations in the habitat, while others may tolerate wide range of environmental conditions. Paradoxically not much study on the microbiological aspects of tropical estuaries has been carried out. Thus, it is imperative to understand the influence of environmental factors on the distribution and community structure of nitrifiers and their activities in the water

column across the salinity gradient in a tropical estuary. The Cochin estuary (CE) was selected for this study which is the second largest wetlands ecosystem along the southwest coast of India. A considerable amount of freshwater (20000 mm^3) is added to the CE during the Southwest monsoon season from precipitation and runoff from terrestrial sources (Srinivas et al. 2003). A continuous supply of fresh water keeps the estuary turbid for a longer period compared to the saline conditions (Madhu et al. 2007). The seawater influx into the estuary is from the two openings into the Arabian Sea (Menon et al. 2000). The reported dissolved nutrient concentration in the CE is high and has increased substantially in the past decades (Martin et al. 2010). A recent study classified the CE as a sink of nutrients as only 50 % of the 2.69 kg day^{-1} of inorganic nitrogen are exported to coastal waters (Martin et al. 2011). It can be assumed that a considerable fraction of the remaining 50 % nitrogen could be processed by microorganisms in this estuary. Despite high nutrient load in the CE, there is only one study on nitrification rate (Miranda et al. 2008).

The hypothesis put forward for the nutrient rich CE is that 1) nitrification process is mediated by the interplay among nitrifiers, which in turn is influenced by environmental factors; 2) bacteria may dominate over archaea in nitrification in this nutrient rich tropical estuary; and 3) being a biogeochemically active zone significant amount of estuarine inputs will be transferred to the coastal waters.

The present study was carried out for a period of one year (2011) covering three seasons with following objectives:

1. Distribution pattern of nitrifying bacteria and their response to environmental changes in the CE and adjacent coastal waters.
2. Molecular investigation on the community structure of ammonia oxidizers and its spatio-temporal variation.
3. Nitrification activity in the CE and adjacent coastal waters and the differential contribution of ammonia oxidizers towards the process.

1.7 Significance

Very limited research has been carried out on nitrifying microorganisms from the marine environment of India, especially from the estuaries. This study is probably the first of its kind addressing the nitrification process in conjunction with the bacterial and archaeal contribution to this process. This study also addresses the spatial and temporal variations of nitrifiers abundance and community structure along with its activities. This research will provide a new dimension in the management of our estuaries as it focuses on the functioning and influence of environmental variables on the nitrification process.

Chapter - 2
REVIEW OF LITERATURE

Contents

2.1. General Introduction

2.1.1 Nitrogen and nitrogen cycle

Nitrogen forms the seventh element in the modern periodic table and has an atomic mass of 14. Nitrogen, the fourth most abundant element (after hydrogen, oxygen, and carbon) in organic matter is an important nutrient for all living forms. Nitrogen exits either in organic or inorganic form. Organic nitrogen exists in compounds like amino acids, amines, proteins and humic compounds with low nitrogen content. Inorganic nitrogen consists of ammonium nitrogen, nitrate and nitrite nitrogen. The gaseous form of nitrogen includes ammonia, dinitrogen and nitrous oxide. Nitrogen appears in both oxidized and reduced states and a single nitrogen atom can serve as a terminal electron acceptor for eight electrons, from N (+5) of nitrate ions to N (-3) of ammonium ions. In most compounds, nitrogen is either bonded to carbon and hydrogen, where the oxidation state of the nitrogen is negative (such as amines, amides, proteins and urea), or bonded to oxygen (such as nitrate, nitrite and nitrous oxide), where the oxidation state is positive. Number of chemical reactions are involved in the transformation of atmospheric nitrogen to organic nitrogen and again back to environment as atmospheric nitrogen. The major chemical transformations in this biogeochemical cycle of nitrogen are nitrogen fixation, ammonia assimilation, assimilatory ammonia and nitrate reduction, ammonification, nitrification and denitrification. Recently a novel microbial

pathway Anammox was introduced in the nitrogen cycle. It is anaerobic oxidation of ammonia using nitrite as the electron accepter and nitrogen gas as the end product (Van de Graaf et al. 1995). It was first postulated by Richards (1965) in oxygen minimum marine environment and was first observed in wastewater treatment plants by Mulder et al. (1995). Recently, the importance of anammox process in the global nitrogen cycle and its geographical distribution gained considerable attention (Thamdrup and Dalsgaard 2002, Kuypers et al. 2003, Rich et al. 2008). In closed coastal seas eutrophication leads to the occurrence of oxygen-deficient water during particular seasons (Conley et al. 2009). Anammox may have significant contribution to the nitrogen loss in such environment. The latest calculations suggest that anammox may be responsible for the removal of up to 50% of the globally fixed nitrogen from the oceans (Arrigo 2004).

Nitrogen cycle is of great concern because, together with carbon, hydrogen and oxygen, it is intimately associated with reactions carried out by all living organisms. Nitrogen cycle normally occurs in close association with carbon cycle and these two associated cycles regulate the entire productivity of an ecosystem. The cycling of other essential nutrients, especially phosphorous and sulphur are also closely linked with biochemical nitrogen transformations (Vitousek et al. 1997, Gruber 2004, Arrigo 2005, Galloway et al. 2008).

2.1.2 Nitrification

Nitrification occupies a central position within the global nitrogen cycle. It is an autotrophic microbial process by which ammonium is sequentially oxidized to nitrite and nitrate. Most of the above chemical reactions are exclusively mediated by microorganisms. Ammonia oxidization is chemolitho-autotrophic process and it consumes oxygen to generate energy which can be then used to fix inorganic carbon (Kowalchuk and Stephen 2001). It is first and rate limiting step in nitrification (Prosser 1989). Oxidation of ammonium to nitrite is a two-step process catalysed by ammonia monooxygenase (AMO) and hydroxylamine oxido-reductase (HAO). AMO catalyses the oxidation of ammonium to hydroxylamine in an energetically unfavorable reaction (Hollocher et al. 1981). It is a membrane bound enzyme found in all ammonia oxidizers, with three subunits, amoA, amoB, and amoC respectively.

In the second process HAO catalyses the oxidation of hydroxylamine to nitrite (Figure 1). HAO is located in the periplasm and is a homotrimer with each subunit containing eight C-type hemes (Arp et al. 2002). The overall reaction of ammonia oxidation is $NH_3 + 1.5O_2 \leftrightarrow lNO_2 + H_2O + H^+ + 84$ kcal M^{-1}. The second step of nitrification is the conversion of nitrite to nitrate. It gains energy from the conversion of nitrite to nitrate: by using enzyme nitrite oxidoreductase and the process is reversible $NO_2 + 0.5\ O_2 \leftrightarrow NO_3$. Detailed mechanisms of the coupling of nitrite oxidation to a proton motive force have yet to be elucidated. Nitrification is more than just a nutrient transformation process. Oxygen consumption due to nitrification can be a major consumer of oxygen in natural system (Heip et al. 1995, Soetaert and Herman 1995). Energy yield of nitrification is comparatively small and it consumes 2 moles of oxygen per mole of ammonium and produces nitrate. About 80% of the energy generated by chemolithotrophic nitrification is utilized for CO_2 fixation by Calvin cycle (Hagopian and Riley 1998). Thirty five molecules of ammonia are needed for fixing a single carbon. Though nitrification is an autotrophic process, heterotrophic nitrification is also reported, though at a slow rate (Verstraete and Alexander 1973, Watson et al. 1981, Yool et al. 2007).

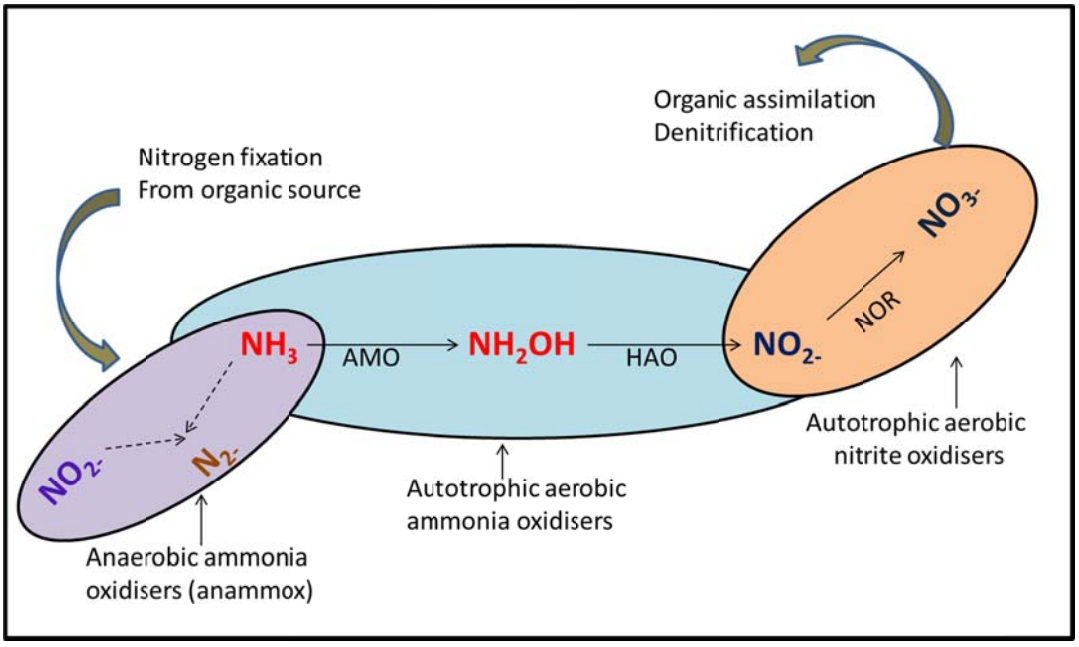


Figure 2.1 Schematic representations of all the reactions involved with nitrification.

2.1.3 Nitrifying microorganisms

Nitrifying organisms are the only organisms which are capable of converting the most reduced form of nitrogen (ammonium), to the most oxidised form (nitrate) and also can carry out a range of other processes within the nitrogen cycle. Two predominant groups of microorganisms are involved in the nitrification process viz. Bacteria and Archaea.

2.1.3.1 Bacteria

Nitrifying bacteria was discovered at the end of the 19th century and are ubiquitous in marine, freshwater and soil environments (Winogradsky 1890). They have been reported from even in extreme habitats such as alkaline soda biotopes (Sorokin et al. 1998), Antarctic ice (Wilson et al. 1997, Arrigo 2005), hot springs (Golovacheva 1975) or in association with marine sponges (Diaz and Ward 1997). No bacteria have been found which can convert NH_3 to NO_3 directly (Hooper et al. 1997). Two groups of bacteria are involved in this process 1) ammonia oxidizing bacteria (AOB) and 2) nitrite oxidizing bacteria (NOB). AOB come under the family *Nitrobacteriaceae* based on their physiology and morphology. AOB have a unique metabolism with many enzymes that have been found only in this group of organisms. Physiological diversity among cultivated isolates of AOB has been well-documented. Growth rate and yield is low with a minimum doubling time of one week. It has five different genera: *Nitrosomonas, Nitrosospira, Nitrosococcus, Nitrosovibrio* and *Nitrosolobus.* Among this *Nitrosomonas, Nitrosococcus, and Nitrosospira* are the most frequently observed genera. AOB were initially thought to be associated with β and γ proteobacteria. Based on the sequence similarity of 16S rRNA, recently *Nitrosospira* sp.*, Nitrosovibrio* sp. and *Nitrosolobus* sp. were suggested to be combined into one common genus *Nitrosospira*. Molecular studies of ammonia-oxidizing populations in their natural habitat have led to a more extensive classification into further sub groupings within the *Nitrosomonas*/ *Nitrosospira* clades (Wagner et al. 1995). With the exception of *Nitrosococcus*, all genera represent closely related organisms with in the β subclass of Proteobacteria. The genus *Nitrosococcus* is phylogenetically not homogeneous. *N. mobilis* is a β subclass organism, while two other species, *N. oceani*, and *N. halophilus*, are

affiliated to γ-Proteobacteria (Holmes et al. 1995) and is restricted in marine and saline environments (Purkhold et al. 2000). Members of the *Nitrosomonas/ Nitrosospira* group appear to dominate in most terrestrial and aquatic environments, including marine sediments. Several species (*Nitrosomonas marina, Nitrosomonas aestuarii, and Nitrosomonas cryotolerans*) are obligatory halophilic, while the remaining species in the *Nitrosomonas* and *Nitrosospira* genera either have no salt requirement, are often halotolerant or moderately halophilic (Koops and Pommerening-Röser 2001). The first AOB to be cultivated from the marine environment was *Nitrosocystis oceanus*, now it renamed as *Nitrosococcus oceani*. About 14 sp of AOB are now available in culture. Other than autotrophic nitrifiers, heterotrophic nitrifying bacteria like *P. denitrifcans and Peudomonas strain PB16* have been reported (Jetten et al. 1997) from natural environment. Currently, in Planctomycetales with anammox have four genera *Brocadia*, *Kuenenia*, *Scalindua*, and *Anammoxoglobus* recorded (Francis et al. 2007).

NOB are chemolithoautotrophs that catalyses the oxidation of nitrite to nitrate, the final product of nitrification. Phylogeny and functionality of both AOB and NOB appear to be well interrelated. Nitrite oxidizers can also survive with alternative substrate like nitrous oxide. *Nitrobacter* sp., *Nitrospina* sp., *Nitrococcus* sp., *Nitrospira* sp. etc. are involved in nitrite oxidation (Watson et al. 1981). Traditionally, *Nitrobacter* sp. *and Nitrospira* sp. were considered as the most important nitrite-oxidizers in natural environment (Cebron and Garnier 2005, Santoro 2010). *Nitrobacter* sp. are fast-growing organisms with low affinities to nitrite and oxygen and can also grow heterotrophically (Bock 1976, Steinmüller and Bock 1976, Bock et al. 1990, Vadivelu et al. 2006). *Nitrospira* sp. possess a low maximum specific growth rate, but are well-adapted to low nitrite and oxygen concentrations (Maixner et al. 2006, Blackburne et al. 2007). These organisms have evolved strategies that enable them to successfully survive and maintain themselves within the ecosystem. These life cycles indicate the possible complicities of electron-transport system in nitrite oxidizers. Nitrite oxidizing organisms are least studied group of organisms in nitrification (Cébron et al. 2003). Till date, only bacterial nitrite oxidation was identified. Involvement of heterotrophic bacteria in nitrification was also proved by various studies, but their contribution toward the

process is minor when compared to chemoautotrophic nitrification and is not covered in this review.

2.1.3.2 Archaea

Archaea were previously considered as extremophilic organisms present in the extreme environmental conditions like hot springs and volcanic areas. The existence of mesophilic Archaea has been proved and its ubiquitous existence in aquatic environment confirmed by various studies (DeLong 1992, Fuhrman 1992, Karner et al. 2001). Later reports showed the wide presence of Archaea, Crenarchaeota in marine environments and which alone comprised over 20-40% of bacterioplankton in the world ocean (Karner et al. 2001). However, the biogeochemical significance of marine Archaea was an enigma till data from marine metagenome sequencing reported an archaeal open reading frame codes for a protein distantly related to monooxygenases of ammonia-oxidizing bacteria (AOB) and methanotrophs (Venter et al. 2004). At the same time (Treusch et al. 2005) proved the existence of ammonia monooxygenase (amoA) genes in soil DNA sample also. The perfect link between these novel amoA genes and archaeal ammonia oxidation was recently and believably established by cultivation of an ammonia-oxidizing Crenarchaeon – designated *Nitrosopumilus maritimus* – from a saltwater aquarium (Könneke et al. 2005). This organism is placed in the group 1.1a Crenarchaeota based on 16S phylogeny analysis. Their physiology also established these chemo lithoautotrophic organism convert ammonia in to nitrite and utilize bicarbonate as sole source of carbon.

In Archaea, all essential genes of the bacterial pathway other than genes coding for the presumptive AMO are lacking. The completion of the genome sequence 1.64 Mbp circular chromosome of the first isolated AOA, *N. maritimus*, described three major deviations from the typical bacterial system of ammonia oxidation and carbon fixation: (1) The involvement of copper (instead of iron) as the major redox active metal in electron transport chain reactions, (2) The lack of any homolog to the bacterial oxidoreductase (hydroxylamine oxidoreductase, HAO) responsible for the oxidation of hydroxylamine to nitrite, and (3) A variant of the 3-hydroxypropionate/4-hydroxybutyrate cycle for CO_2 fixation (as opposed to fixation

by the ribulose bisphosphate carboxylase/oxygenase of the Calvin-Bassham-Benson cycle used by characterized bacterial ammonia oxidizers) (Stein et al. 2007, Norton et al. 2008). Thus, the current information of carbon metabolism suggests that ammonia oxidation by this group derive energy and electrons primarily from the oxidation of ammonia but can supplement carbon from CO_2 fixation using a limited set of simple compounds that feed directly into central metabolism. Physiological properties and reaction kinetics of AOA is based on the available cultural representative *N. maritimus.* Stoichiometry studies of ammonia oxidation are indistinguishable from that of AOB (Nicol et al. 2008):$1NH_3 + 1.5O_2 \Rightarrow 1\ NO_{-2} + H_2O + H^+$. Moreover, coming to the distribution of AOA, Francis et al. (2005) first reported the wide spread occurrence of ammonia oxidizing crenarchaeota in marine system and suggest that Archaea can contribute significantly to global nitrogen cycles. Phylogenetic analysis based on amoA gene sequence showed that these organisms are phylogenetically diverse than ammonia oxidizing bacteria. After this understanding, number of studies have been carried out in different geographic locations and the results showed that AOA is more abundant than AOB at particular natural environment (Leininger et al. 2006, Wuchter et al. 2006). Later Mincer et al. (2007) found that archaeal amoA was several orders of magnitude more abundant than AOB 16S rRNA at certain depths from the North Pacific Ocean. These discoveries have significantly increased the curiosity on AOA and numerous studies had been carrying out in different laboratories and most of the studies suggested AOA is more abundant than AOB in terrestrial and marine ecosystem while estuaries are different, where AOB dominance over AOA were reported (Dollhopf et al. 2005, Chen et al. 2008, Erguder et al. 2009, Bernhard and Bollmann 2010). The ratio of abundance between AOA and AOB is varying with varying environments. Most of the marine environment showed higher abundance of AOA than AOB. While in estuaries some studies showed greater abundance of AOB than AOA (Caffrey et al. 2007, Mosier and Francis 2008), but contrasting results are also reported (Bernhard et al. 2010).

2.1.4 Detection methods for nitrifying microorganisms

2.1.4.1 Detection methods for AOB and NOB

Classical detection and characterization methods involve culture based isolation and cultivation techniques, such as selective plating, series dilution in batch culture and chemostat. Characterization and phylotyping methods were depends on morphology, lipid profile, GC content and immunofluorescence assays. The estimation of numbers of AOB in the environment by culture based techniques typically trusted on most probable number method (MPN) (Alexander and Clark 1965, Dai et al. 2008, Rodina, 1972). Though they are helpful in ecology as a simple technique for enumeration of the microbial population, this method has important disadvantages; it is biased towards particular strains which can grow best under the given growth conditions and fails in detecting cells which grow as aggregates or are attached to particles in the environment. PCR, DGGE and cloning approaches are the most widely accepted and applicable methods for determining the diversities of AOB and quantitative PCR have been used for calculating the abundance of AOB (Okabe et al. 2004, Schmid et al. 2005, Dai et al. 2008, Mosier and Francis 2008, Jin et al. 2011). In the recent times, several studies have reported the use of molecular probes by Fluorescence *In situ* Hybridization (FISH) for detection and enumeration of the microbial community as well nitrifying community (Wagner et al. 1996, Schmid et al. 2007). 16S rRNA probes for FISH have been successfully used for identification and quantification of nitrifiers populations. Along with quantitative PCR method, direct *in situ* observation using FISH and its improved versions like CARD-FISH are also widely used in the quantitative analysis of AOB. The great acceptance and wide application of FISH techniques together with the development of improved modification on FISH (MAR FISH, GENE FISH, mRNA FISH and Clone FISH etc.), now facilitate the rapid and accurate *in situ* detection of AOB in natural environment and can be used for the quantitative estimation of metabolically active cells (Okabe et al. 2004, Kindaichi et al. 2007, Pratscher et al. 2011).

2.1.4.2 Detection methods for AOA

The three subunits of archaeal enzyme ammonia monooxygenase (amoA, amoB, amok) mediate the process of chemo autotrophic ammonia oxidation. amoA gene copy number is used for the quantitative estimation of AOA currently in most of the abundance estimation studies. Sequence analysis of ammonia amoA gene is also used for the phylogenetic study of AOA. amoA sequence analysis showed a great diversity among AOA and it can be consider as a better tool for the phylogenetic analysis. Quantitative PCR which detects the amoA gene copy number is the only technique that widely used for the estimation of AOA population. Till date no 16S primer specific for AOA has been documented in literatures, whereas primers are available for total Archaea and total Crenarchaeota. Due to the low copy number of amoA gene compared with that of 16S gene making normal FISH techniques as a not preferable method for the estimation of AOA. Advanced modifications of FISH techniques like GENE FISH and mRNA FISH are recently coming out to solve this limitation (Moraru et al. 2010, Li and Gu 2011). But these tools are yet to be proved as suitable for ecological studies.

2.2 Nitrification in Estuarine and Coastal Environment

Estuaries are regions of active inorganic nitrogen transformation (Watson et al. 1981, Vitousek and Howarth 1991, Ogilvie et al. 1997, Dong et al. 2000, Cornell et al. 2003). The major nitrogen pools in estuaries are total nitrogen (mostly organic nitrogen) and available inorganic nitrogen in waters. In water, ammonium and nitrate nitrogen is the predominant form of inorganic nitrogen whereas nitrites occur in trace quantities and is mainly derived through mineralization of organic nitrogen and further oxidation. Human activities have considerably increased the availability of nitrogen in the biosphere (Vitousek et al. 1997), this excess nitrogen can leach from soils and enter to the natural aquatic systems (Galloway et al. 2003), and finally to estuarine and coastal system.

The incidence of hypoxia in coastal waters and estuaries has increased recently in response to anthropogenic nutrient inputs (Levin et al. 2009, Naqvi et al. 2009). Nitrogen is considered as one of the major limiting factors in estuaries and

coastal waters, making the nitrogen dynamics in estuaries particularly significant. (Howarth 1988, Bernhard and Peele 1997). Estuaries serve as a significant nitrogen sink, owing to biotic removal by assimilation, denitrification or by burial processes, and also act as a source of nitrogen via direct discharge or degradation of organic matter. Nitrifications in estuaries are particularly important as 30% of globally fixed nitrogen loss occur in estuarine and coastal environment (Voss et al. 2011). In estuarine and coastal environment, nitrification is often coupled to denitrification (Jenkins and Kemp 1984, Sebilo et al. 2006) resulting in the ultimate return of nitrogen to the atmosphere. More recent studies using ^{15}N tracer experiments have detected anammox reaction in estuarine and offshore sediments (Dalsgaard and Thamdrup 2002, Risgaard-Petersen et al. 2004, Dalsgaard et al. 2005, Hulth et al. 2005, Trimmer et al. 2005). Nitrification is an important bioremediation process in human-perturbed estuarine and coastal ecosystems, where it may function as a detoxification process for excess ammonia (Camargo and Alonso 2006). Researchers are now approaching nitrogen cycle in another way by combining nitrogen cycle and carbon cycle and suggest that the nitrogen cycle may affect the global carbon cycle, and thereby transformation processes of nitrogen may also affect the climate changes also (Grommen et al. 2005, Gruber and Galloway 2008). Bacterial nitrifiers may also co-oxidize a variety of xenobiotic compounds (Kowalchuk and Stephen 2001, Arp and Stein 2003).

The mixing of freshwater and saltwater in estuaries and coastal environment create strong physico-chemical gradients. Influence of physico-chemical conditions along an estuarine gradient will influence the nitrification rate and growth of nitrifying organisms. Shifts in the resident microbial communities due to the variation of environmental conditions were also reported (De Bie et al. 2001, Bernhard et al. 2007). Among various factors, salinity plays a significant role. Different salinity range has been reported for the optimum growth and activity of nitrifiers, that extending from limnetic to moderate saline water (Jones and Hood 1980, Helder and De Vries 1983, Berounsky and Nixon 1993). Whereas nitrification found to be decreased at higher salinity (Seitzinger 1988, Rysgaard et al. 1999, Bernhard et al. 2007, Bernhard and Bollmann 2010). However, the precise reason of the reduction in nitrification is less clear. It is known that salinity plays a major role

in controlling NH_4 adsorption ability (Boatman and Murray, 1982), with increased NH_4 efflux as salinity increases (Boynton and Kemp, 1985) or /and may be due to substrate limitation or, alternatively, a shift in the nitrifying community in response with salinity (De Bie et al. 2001, Bollmann et al. 2002) and functionally distinct communities of AOB have been reported along a salinity gradient (Bernhard et al. 2007). Besides salinity, the distinct seasonal variations of temperature, irradiance and fresh water discharge from rain and river also influence and control the community structure of nitrifying microbes in estuaries. A consistent relationship with other environmental parameters like dissolved oxygen, ammonium and nitrite concentration have not been observed in estuarine system (Erguder et al. 2009, Bouskill et al. 2011, Bouskill et al. 2012). Carbon dioxide, moisture, pH as well as the presence of grazing bacteriovorous protozoa will also regulate the activity by affecting the organisms. Different enzyme mediated process in nitrogen cycle is carried out by metaloenzymes so the presence of trace element may also have a significant influence on activity. For example nitrous oxide reductase is a copper containing enzyme and limitation of copper will affect its activity and thereby denitrification rate can decrease. Likewise, nickel (Ni), sulfide concentrations (Joye and Hollibaugh 1995), and Fe (III) contents are also considered as a regulating factor (Dollhopf et al. 2005). But only little information is available about the linkages between dissolved sulfide concentrations, Fe (III) minerals, and microbial nitrogen transformations. But the possible reason is that Fe(III) compounds react rapidly with sulfides, thereby scavenging sulfides formed through SO_4 reduction and protect nitrifying bacteria from sulfide inhibition, otherwise sulfides bind to ammonia monooxygenase, irreversibly inhibiting this key nitrification enzyme.

2.2.1 Abundance and distribution of AOB and AOA

Previous studies of AOB diversity in freshwater and marine systems documented some overlap of AOB species distribution, but these systems seems to be dominated by distinctly different communities (Hiorns et al. 1995, Stephen et al. 1996, Hastings et al. 1998, Speksnijder et al. 1998, Phillips et al. 1999). Spatio-temporal changes in fresh and marine water in estuaries make the species distribution of AOB more complex. In estuaries, regular disturbance events such as

tidal fluctuations, nutrient pulses and salinity intrusions play a critical role in defining the habitat, and undoubtedly play a role in defining AOB species distribution and diversity as well. Only recently AOB and AOA communities in estuaries have been targeted for molecular studies (De Bie et al. 2001, Cébron et al. 2003, Sahan and Muyzer 2008, Beman et al. 2012, Hatzenpichler 2012), and the factors that regulate their diversity and distribution in these systems remain unclear (Bernhard and Bollmann 2010).

Both bacterial and archaeal ammonia oxidizers are present in estuarine systems, but the specific distribution pattern in natural environment is still remains under investigation. Most of the studies on the abundance of estuarine nitrifiers were limited to sediments. Unfortunately the water column of the estuaries are least studied. These studies have estimated the abundance of AOA and AOB by measuring the abundance of the amoA gene using real-time or quantitative PCR (QPCR). Abundance of beta proteobacterial amoA gene copy number in estuaries are ranging from 10^4 to 10^8 copies per gram of sediment (Bernhard et al. 2007, Mosier and Francis 2008, Santoro et al. 2008, Moin et al. 2009). A wide range of distribution pattern, for AOA ranging from10^4 to 10^9 gene copies per gram of sediment has been reported in estuaries (Mosier and Francis 2008, Moin et al. 2009, Dang et al. 2010). When considering the contribution of bacterial and archaeal ammonia oxidizers towards total bacterial and archaeal abundance, AOA contribute more to the total archaeal composition while AOB contribute very less to the total bacterial abundance. Auguet et al.(2011) showed that AOA may contribute more than 50% to the archaeal community in oligotrophic alpine lakes, while AOB portion in wetlands fluctuated from less than 1% to about 10% of the bacterial community (Sims et al. 2012).

The ratio of abundance between AOA and AOB varies with varying environment. The dominance of AOA over AOB was reported in most open-ocean and terrestrial environment (Di et al. 2009, Beman et al. 2012, Bouskill et al. 2012). The estuarine systems are quite different and it forms unique environment where AOB often outnumbered AOA (Caffrey et al. 2007, Mosier and Francis 2008, Santoro et al. 2008). Abell et al. (2011) reported archaeal amoA, mRNA and DNA

were both approximately one order of magnitude less abundant than that of bacteria in Derwent Estuary in Australia. They observed mean AOB and AOA amoA gene abundances of 3.1 to 4.4 × 10^8 and 2.4 to 3.4 × $10^7 g^{-1}$ dry weight and the mean abundance of AOB and AOA amoA transcripts of 4.2 × 10^7 to 1.1 × 10^8 and 1.3 to 2.6 × 10^6 g $sediment^{-1}$, respectively. This result suggests that AOA play a minor role in nitrification than AOB in estuaries. Mosier and Francis (2008) and Santoro et al (2008) found a shift in the AOA to AOB ratios related to changes in salinity, with AOB outnumbering AOA as salinity increased, mainly due to the changes in the abundance of AOB. Caffrey et al. (2007) also found higher AOB abundance over AOA at few estuarine sites in Weeks Bay. In Plum Island samples, the direction of the change in the ratio of AOA to AOB followed the same pattern as observed in the San Francisco Bay estuary by Mosier and Francis (2008), with ratios generally decreasing with increasing salinity, Bernhard et al (2010).

Diversity of microorganisms in estuaries might be greater than that in the adjacent open oceans due to terrestrial inputs. Some studies have proposed that the AOA community in terrestrial environments are distinct from those in marine environments while other reported that estuaries might harbor mixed populations from soil/sediment and marine water column/sediment (Dang et al. 2008, Mosier and Francis 2008). The absolute abundance of nitrifiers differs among systems, and likely controlled by the differences in physico-chemical properties that change along the estuarine gradient. However, the limitations in methodology used should also be considered. Since some short-comings of the commonly used bacterial primers have recently been identified (Hornek et al. 2006) and in the case of AOA different primers, nearly six set of primers or primer combinations used for quantification by different studies. So primer specificity may affect the absolute numbers of organism detected (Groeneweg et al. 1994).

2.2.2. Factors controlling AOB and AOA

The abundance of ammonia-oxidizing prokaryotes changes with the gradient of some environmental parameters. The pattern of AOB diversity distribution and activity in natural environment is regulated by physiological variables along with hydrodynamic characteristics, such as river discharge and

water residence times. The major physiological controlling factors include ammonia concentration, salinity, dissolved oxygen and temperature. Though the actual environmental determinants that regulate the AOB and AOA distribution in estuaries are still need to be explored, the influence of various biotic and abiotic have been reported. A correlation between increasing salinity and increasing number/proportion of AOB have been reported by various studies (Mosier and Francis 2008, Santoro et al. 2008, Santoro and Enrich-Prast 2009). Salinity is a particularly important parameter for ammonia oxidation because of its influence on availability of ammonia to microorganisms (Boatman and Murray, 1982, Boynton and Kemp, 1985). Temperature and salinity are the two major factors controlling the AOA community structure (Mosier and Francis 2008). The responds of AOA towards salinity is similar with AOB. However, in some geographical locations AOA is negatively and AOB is positively correlated with salinity (Bernhard et al. 2010).

Like other microorganisms temperature positively correlated with AOA and AOB abundance (Groeneweg et al. 1994, Tourna et al. 2008). The higher temperature always supports the maximum diversity and when the temperature decrease the diversity is also decreased (Urakawa et al. 2008). A community composition shift with changing environmental especially salinity along with temperature has also been reported by (Sahan and Muyzer 2008). Dissolved oxygen is also a factor which can positively influence the population structure of AOB but minimum amount of oxygen is sufficient for ammonia oxidation. About 1 to 1.5 mg L^{-1} is sufficient for maintaining the growth and activity of AOB and NOB (Garnier et al. 2007). As AOB utilizing reduced nitrogenous compound as their nutrient substrate they have to compete with heterotrophs for available reduced nitrogen. Previous study reported that AOB can be considered as poor competitor in natural environment and their growth is positively correlating with ammonia concentration (Jones and Hood 1980). Among the various species of AOB, there are also extensively differing substrate affinities, with *Nitrosomonas oligotropha* and *Nitrosomonas ureae* showing the lowest Ks values (1.9 - 4.2 mM NH_3), and *N. europaea* and related species having the maximum Ks values (30 - 61 mM NH_3) (Koops and Pommerening-Röser 2001).

Other major factors that showed influence in the ratio between the AOA and AOB in their systems are oxygen availability and C/N ratio, in which increasing oxygen availability and decreasing C/N ratio led to an increase of the number of AOA and AOB (Beman and Francis 2006, Mosier and Francis 2008). Since many other factors may co-vary with salinity, it is likely that salinity is not the only factor determine the distribution of AOA and AOB. For example AOA abundance has also been strongly correlated with pH (Moin et al. 2009), percentage of clay and lead concentrations (Mosier and Francis 2008) and pore water sulfide (Caffrey et al. 2007). However, because salinity often co-varies with many of these variables in estuaries, it is difficult to identify the real causative factors. Interestingly, strong correlations between dissolved inorganic nitrogen species and AOB abundance have been documented in estuarine studies, whereas this correlation is absent for AOA, but enrichment studies in soils have reported increased AOA abundance when ammonium is added to the sample (Chen et al. 2008, Sahan and Muyzer 2008).

2.2.3 Relationship between nitrifying organisms and activity

In light of the immense microbial diversity observed in natural systems, the question remains as most important is whether the diversity and, more specifically, functional diversity of particular gene plays a role in controlling nitrification rates? A strong positive correlation between nitrification rate and abundance of both AOA and AOB were reported by previous studies (Stehr et al. 1995, He et al. 2007). Strong site specific correlations between ammonia oxidation rates and AOB abundance were detected and that suggested functionally distinct AOB communities along the environmental gradients, (O'Mullan and Ward 2005, Bernhard et al. 2007). Wuchter et al. (2006) provide evidence for this via enrichment cultures and found a correlation between ammonia oxidation rates and abundance of the marine Crenarchaeota, supporting the hypothesis that marine crenarchaeota are nitrifying in pelagic waters. Beman et al. (2008) reported evidence for AOA abundance and nitrification rate relationship in the Gulf of California. Bernhard et al. (2010) reported correlation of nitrification with abundance of AOB at the low and mid salinity sites in the Plum Island estuary and

also found no relation with AOA abundance and suggesting that AOA are not actively nitrifying in the Plum Island estuary, although relatively high numbers are present. In a recent study of AOA abundance in several south eastern estuaries, AOA abundance were positively and significantly correlated with potential nitrification rates at only 2 of 6 sites (Caffrey et al. 2007). It can be hypothesized that the distribution of AOA will explain the varying correlations between rates and AOB abundance along the estuarine salinity gradient. However these conflicting results suggesting that either our understanding of the relationship between nitrification rate and the organisms responsible is incomplete or limitation of the methodology. Although the high abundance of AOA is present in estuarine system this abundance generally not correlates with potential nitrification rates in that particular environment. The possible justifications reported for this result is that most of AOA may be inactive in estuarine system and the high rates of nitrification is due to the activity of very active AOB, or it may be a methodological artifact or may reflect the possibility of alternative energy sources used by AOA (Bernhard et al. 2010).

Microcosm study have shown increased archaeal *amoA* gene expression after ammonium additions but the specific role of the putative archaeal ammonia monooxygenase remains to be confirmed (Treusch et al. 2005). It is also possible that some nitrifiers are not active at the time of sampling, and thus might show a lag phase in activity during incubation and also upon the addition of ammonium or oxygen. Significant differences in recovery after starvation have been demonstrated in some cultivated AOB (Bollmann et al. 2002) A recent study reported extremely high substrate affinity of the cultivated AOA, *N. maritimus* (Martens-Habbena et al. 2009), while it did not show inhibition at ammonium concentrations typical of potential rate experiments (i.e. 0.3 - 0.5 mM). However, due to vastly different oxidation kinetics among AOA and AOB and the recently-reported sensitivity to agitation of *N. maritimus* (Martens-Habbena et al. 2009), it is doubtful that the potential nitrification rates reflect the involvement of all nitrifiers present. Based on the current understanding it can be say that archaeal amoA gene abundance may not be suitable indicator of their nitrifying activity and further studies are necessary to confirm these findings.

Most of the studies used the term potential rates that are normally measured by adding ammonium to the samples and incubating them with shaking for hours to days, and rates are calculated by following the generation of nitrite or nitrate. Potential nitrification rates are typically much higher than *in situ* rates, extra substrate and non-limiting oxygen added to the system. Whether it is the addition of ammonium or non-limiting oxygen conditions that is responsible for stimulating nitrification is not clear. It is thought that potential nitrification rates should be correlated with the abundance of ammonia oxidizers (Henriksen 1980). Thus, inaccurate measurements of either the potential rates or the abundance of ammonia oxidizers will lead to a collapse in the expected relationship. Recent studies have reported great genetic diversity among AOA and AOB and it may be lead to great variation in cell specific reaction rate and great diversity in physiological optima among the organisms. Moreover, the relationship between functional gene diversity and nitrogen conversion rates regulated by the corresponding enzyme also remains poorly understood (Bannert et al. 2011, Levičnik-Höfferle et al. 2012).

Literature review has demonstrated that, AOB and AOA act as a major player in ammonia oxidation in most of the natural systems. In association with ammonia oxidation both these groups generate significant amounts of nitrous oxide or for some species to fully reduce nitrite to N_2O through a poorly characterized partial denitrification pathway. The role of AOB and AOA on determining the fate of nitrogen removal is significant in estuarine and coastal environment as marine system is source for about 30% of global N_2O inputs to the atmosphere (Shaw et al. 2006). There is no single factor is expected to shape the nitrification rate and the community composition and abundance of nitrifying microbes and it is possible that combined environmental factors might be collectively responsible for the diversity, abundance and activity of nitrifying organisms. Though the higher abundance of AOA is proved in most of the natural environment, the actual contribution of AOA to global nitrogen cycle is still indistinguishable particularly from tropical estuarine and coastal waters.

2.3 Indian Scenario

Nitrification process in Arabian Sea acquired enough scientific attention and nitrification, denitrification and anammox process in have been well studied. Arabian Sea is well known for the presence of upwelling region, oxygen minimum zone and anoxic regions. Various studies elaborated nitrification process in these environments. Krishnan and Bharathi (2009) have studied on nitrification and denitrification process in the anoxic zone of Arabian Sea coast. It suggested that in Arabian Sea nitrification operates at the base of this suboxic system, and it efficiently feeding denitrification. Moreover the alarming nitrous oxide input into the atmosphere could be relatively more due to a tighter intrinsic coupling of nitrification–denitrification, rather than denitrification driven by extraneous nitrate alone. Ammonia oxidizing organism and ammonia oxidation rates in the Arabian Sea has been studied recently by Newell et al. (2011) and the observed ammonia oxidation rates ranged from undetectable to 21.6 $\pm$ 0.1 nM L^{-1} d^{-1}. Dominance of AOA over AOB and absence of relationship between gene abundance and ammonia oxidation rate has also been reported. Ward et al. (2009) explained that denitrification is the dominant nitrogen loss process in the Arabian Sea. The upwelling regions of Arabian Sea are well known for the anaerobic ammonia oxidation–anammox. Various studies were focused on this aspects documented the distribution activity and diversity of anammox. However classical nitrification and denitrification process is the most dominant nitrogen removal process in the Arabian Sea (Chang et al. 2012, Trimmer and Purdy 2012).

India houses a total of 14 major, 44 medium and 162 minor estuaries, and the total surface area of Indian estuaries comes around to 27000 km^2 calculated from the mouth of the estuary to the region where tidal fluctuations are almost insignificant (Qasim 2003). Indian estuaries are generally called monsoonal estuaries and have characteristic runoff periods and exhibit non-steady state behavior (Vijith et al. 2009). Seasonal discharge into these monsoonal estuaries far exceeds the total volume of the estuary during peak runoff period and whole estuary assumes limnetic condition (Sarma et al. 2009, Sarma et al. 2010, Sarma et al. 2011). Discharges of variable magnitudes occur for a period of 4 - 6 months and the

upstream rivers almost dries up during the other periods allowing for the dominant seawater influx in to the estuary. The mean river discharge from the Indian estuaries varied from 28 to 3505 m^3 s^{-1} (Sarma and Rao 2013). The magnitude of discharge determines the amount of organic matter and nutrients entering the estuary and also the stability of water column that governs the interaction with the microbial processes (Sarma et al. 2011, Sarma et al. 2012). Hence, the biogeochemical processes in monsoonal estuaries during discharge period could be completely different from those in dry period (Sarma et al. 2011). More over high atmospheric temperature in Indian estuaries also play a significant role in the biogeochemical cycling of material and to make the system different from other word estuaries.

Considerable amount of nutrient enters in to the Indian estuaries from various sources extending from domestic sewage to fertilizers industry along the shores of the estuary. Large amount of untreated or partially treated domestic and industrial sewage enter the system due to the increased human settlement in the near shore areas. The fertilizer industry is another important source of organic nutrient in to the Indian estuary. India is ranked second globally in terms of consumption of nitrogen and phosphate as fertilizer and consumes as a report from the fertilizer association of India, New Delhi.

Studies on nitrification especially nitrifying microorganisms from Indian estuaries are limited and most of the little available works are mainly from estuary associated mangrove ecosystem. However in a recent elaborative study by (Sarma and Rao 2013) estimated the N_2O emission from the major monsoonal estuaries of India and in this work they estimated the nitrification rate from these estuaries also. The nitrification range observed in that study ranged from (0.07 and 0.87 µM N day^{-1}) and the rate was comparatively lower than the rate of other world estuaries (0.2 to 336 µM N day^{-1}). Sarma and Rao (2013) reviewed in the same work and suggested that the annual mean nitrification rate observed from the Indian estuaries were significantly less than the European and American estuaries such significant difference might had been caused by variation in DIN loading in to the estuaries. The DIN concentrations in the European and American estuaries were in the range of 5 to 500 µM with nitrification rates of 0.2 to 336 µM N day^{-1}. The

concentrations in the Indian estuaries were 0.6 to 16.6 μM during the wet period, whereas they were 0.6 to 13.5 μM during the dry period and these concentrations are significantly less than the world estuaries. (Sarma et al. 2009, Sarma et al. 2010) suggested reason for this low accumulation of nutrient in Indian estuary, that is monsoonal estuaries received significant amount of nutrients during the peak discharge period, however, nutrients are not utilized in the estuary due to high flushing rates and high suspended load (Sarma et al. 2009, Acharyya et al. 2012). As a result, nutrients are flushed to the coastal regions where they support primary production. Hence, low nitrification rates were found in the Indian estuaries.

Most of the studies in Indian sub-continent on nitrification mainly restricted to mangroves and soil ecosystem close to estuaries. Nitrifiers distribution and activity and influencing factors have been studied at different mangrove ecosystem of India. (Krishnan and Bharathi 2009) studied on nitrification in sediment cores collected from mangrove swamps along the Chapora and Mandovi estuaries. They had given a special emphasis on organic carbon and iron modulated nitrification and reported nitrification rate of 2 to 20 ng N g sediment^{-1} and also inferred that the quality of organic carbon and quantity of iron govern the nitrification rates in mangrove swamps. A depth wise diversity of free living N_2 fixing and nitrifying bacteria and its seasonal variation with nitrogen containing nutrients in the mangrove sediments of Sundarban, was reported by (Das et al. 2013). The studies reported abundance of nitrifying bacteria in the order of 10^4 and documented a decreasing trend of total microbial load, nitrifying and nitrogen fixing bacteria with increase in depth throughout the year.

Aquatic environment especially estuaries are the least studied sites for nitrifying microorganisms. These regions are also highly under-sampled with reference to time and space. Most of the available studies on nitrifying organisms are based on conventional culture techniques. Only few studies are available from Indian sub-continent on nitrification rate and nitrifying organism in soil. Subrahmanyam et al. (2014) studied the response of ammonia-oxidizing archaea and bacteria to long-term industrial effluent-polluted soils, Gujarat, Western India. They reported one order higher abundance of AOA amoA gene compared to AOB

amoA gene were reported in uncontaminated and moderately contaminated soil samples and higher abundance of AOB in contaminated soil sample. Phylogenetic analysis of bacterial amoA gene sequences of their studies suggested that AOB was dominated by *Nitrosospira*-like sequences. Thaumarchaeal "group 1.1b was found to be the dominant group of AOA involved in the nitrification.

One of the studies Kumari et al. (2011) studied the distribution pattern of nitrifying bacteria and its seasonal variation in fish pond ecosystem. Based on culture dependent analysis microplate-most probable number method they reported the abundance of nitrifiers, ranged from 2.1 to 12.2 × 10^3 cells ml^{-1}. Their observation showed a linear relationship between abundance and activity of nitrifiers. This study also revealed the critical role of ammonia concentration in nitrification rate.

2.4 Cochin Estuary

The CE is one of the most productive estuarine system located along the south west coast of India. It is a well-studied estuary with respect to hydrography, chemistry and biology. The hydrography/flow regime/geochemistry has been well described by (Sankaranarayanan and Stephen 1978, Ramamirtham and Muthusamy 1986, Balchand et al. 1990, Joseph and Kurup 1990, Ouseph 1990, Nair et al. 1993, DineshKumar et al. 1994, Menon et al. 2000, Srinivas et al. 2003, Jyothibabu et al. 2006, Balachandran et al. 2008, Gireeshkumar et al. 2013). However studies on the microbial ecology of the CE has been initiated only recently (Chandran et al. 2008, Thottathil et al. 2008, Chandran et al. 2013, Joseph 2013, Parvathi et al. 2013, Parvathi et al. 2014). In recent years, this ecosystem has come under immense anthropogenic pressure due to increased urbanization, pollution and land reclamations and it also receive large amount of anthropogenic input from various source extending from domestic sewage and fertilizer industry along the shore of the estuary. Increased anthropogenic activities started from the mid-1970s, now generating 104 × 10^3 m^3 of industrial and 260 m^3 of domestic wastes per day, are being released directly in to the estuary without treatment (Balachandran et al. 2005, Martin et al. 2008, Martin et al. 2011). Severe oxygen depletion and several incidences of fish mortality are reported in the estuary due to these indiscriminate

discharges of anthropogenic waste (Venugopal et al. 1980, Nandan and Azis 1995, Naqvi et al. 1998). Increased anthropogenic inorganic nitrogen loading has been well documented in the system and it can further intensified in future. Despite the increased nitrogen load in the CE only a single study has been carried out on nitrification (Miranda et al. 2008). In this, the seasonal changes in the nitrification rate along the estuarine gradient were estimated. The reported nitrification rate was non-detectable to 3.98 μM day^{-1} with increased nitrification during pre-monsoon season. However this study did not study the microorganisms which mediate the process. Studies on distribution of nitrifying microorganisms and the nitrification rates are very limited and unfortunately not received enough attention. The present study in Cochin estuary (CE), forms the first report on distribution and seasonality of nitrifying microorganisms and its activity from an estuary in the Indian region.

Chapter - 3
MATERIALS AND METHODS

Contents

3.1 Description of the Study Area

3.1.1 Cochin estuary

The Cochin estuary (CE) is the second largest wetland ecosystem along the southwest coast of India, covering an area of ~25600 ha (Menon et al. 2000) extending from 9˚ 30' - 10˚ 12' N to 76˚ 10' - 76˚ 20' E. This estuary is running parallel to the Arabian Sea covering a distance of 80 kms, extending from Munambam at north to Alleppey at south and two permanent openings connect the CE to the Arabian Sea. Constant mixing with seawater through tidal exchanges has given it the characteristics of a tropical estuary. Since it is geographically located in the tropical region, the mean temperature of surface water is ~ 28°C during monsoon and 30°C during pre-monsoon seasons (Madhupratap et al. 1992). The depth of the estuary varies from 2 to 7 m, but the ship channels at the Cochin harbor region are dredged and maintained at 10 to 13 m. The annual rainfall in Cochin is around 3200 mm of which, nearly 75% occurs during the South-West (SW) monsoon (Qasim 2003). SW monsoon is the main cause for the seasonal variation in the CE. This complex micro tidal estuary (Joseph and Kurup 1989) undergoes a characteristic transformation from a river-dominated system during the monsoon

season (June–September) to a tide- dominated system during the pre-monsoon season (February–May). The most important hydrological variable encountered in the CE is salinity, similar to the conditions encountered in estuaries with a gradual declining of salinity from 30 at the entrance i.e. mouth of the estuary to nearly fresh water at the head of the estuary i.e. point of entry of the rivers.

The nutrient composition of the estuary is greatly influenced by terrestrial anthropogenic inputs, fresh water influx from six rivers, Periyar, Pampa, Manimala, Minachil and Achankovil mainly during the prolonged monsoon, and seawater influx from two bar mouths (Menon et al. 2000, Madhu et al. 2010). Previous studies have estimated that the CE receives high concentrations of industrial effluents of about 104×10^3 m^3 day^{-1} and untreated domestic wastes of ca 260 m^3 day^{-1} (Balachandran et al. 2005). These studies revealed that the system bears excess of inorganic nitrogen irrespective of seasons and the nutrient recycling suggests that this system is very sensitive to increased nutrient loadings.

3.1.2 Description of sampling sites

For this study, 4 stations were selected across a salinity gradient. Station positions were fixed using the Global Positioning System (Magellan NAV DLX 10, USA) and the station positions are given in Figure 2.1 Geographic position of stations are given below.

Stations	Latitude	Longitude
Station 1	10˚ 01, 850` N	76˚15.918` E
Station 2	9˚59.147` N	76˚15.7` E
Station 3	9˚58.252` N	76˚14.278` E
Station 4	10˚00` N	76˚ 05` E

Station 1 is close to river entry and in this study designated as “low saline” with an average salinity of below 10. Stations 2 and 3 are considered as “intermediate saline” stations with salinity range of 10 to 25. These stations are estuarine in nature located close to the entry point of river and receive

anthropogenic inputs from domestic sewage and industrial effluents. Station 4 is a coastal station, about 20 kms away from station 3 with minimum of fresh water input except from precipitation during SW monsoon. This station is designated as "high saline" station with salinity range between 25 and 35. Average depth of the estuarine stations (Stns. 1, 2 & 3) is > 10 m and coastal station (Stn. 4) is around 25 m.

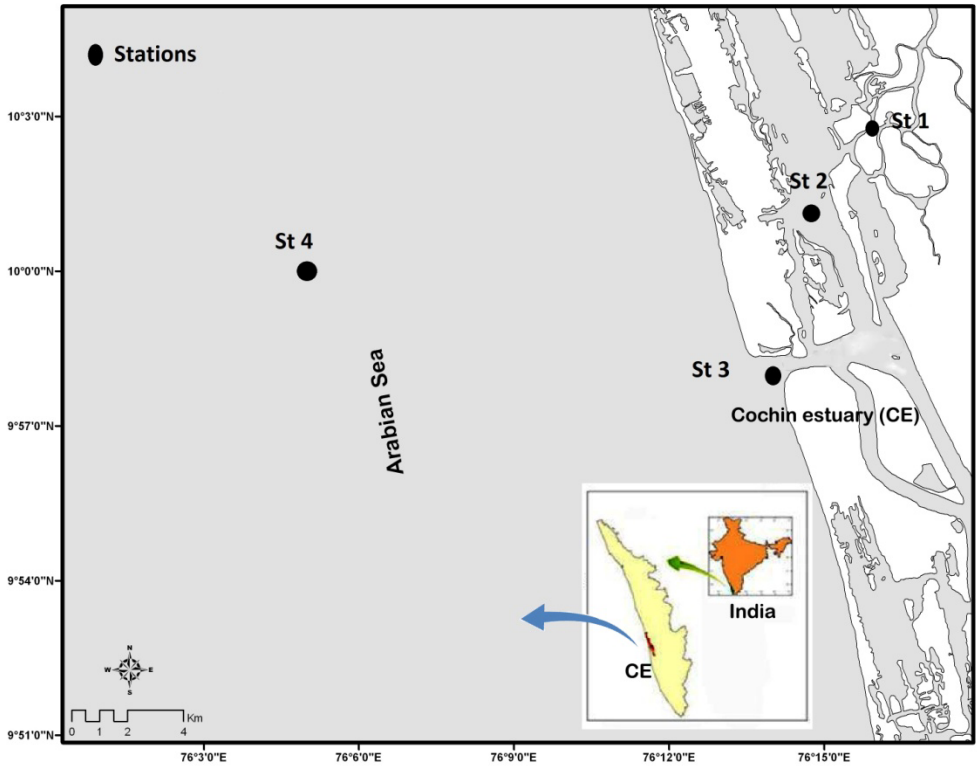


Figure 3.1 Location of sampling stations in the Cochin Estuary

3.2 Sampling Strategy and Transportation

Bimonthly sampling was carried out from all stations for a period of one year (2011) covering three different seasons: – pre-monsoon (January - April), monsoon (June - August) and post-monsoon (October - December). Two sets of water samples were collected from each station using a 5 L Niskin sampler, surface sample (~1m depth) and bottom sample (close to the bottom). Water samples for estimating physicochemical parameters were sub-sampled in polypropylene bottles and preserved at 4° C during transportation and for microbiological analysis, the samples were transferred to sterile glass bottles and stored at 4-5° C till analysis. All samples were processed immediately after transportation.

3.3 Analytical Studies

3.3.1 Environmental parameters

3.3.1.1 Temperature and salinity

Water temperature was measured by dipping the stainless steel temperature probe of a hand held traceable mini digital thermometer with a precision of 0.1°C into ambient water. Salinity of the samples was determined using a Digi Auto Salinometer (Model TSK, accuracy ±0.001) immediately after reaching the laboratory.

3.3.1.2 pH

pH was estimated based on a pH measurement at constant temperature method (Gieskes 1969, 1970). A digital pH meter (Thermoelectron Corporation, USA) was used for determining pH of water samples after calibrating it with the standard buffers of pH 4, 7, and 9.2 (Fluka), respectively.

3.3.1.3 Dissolved oxygen (DO)

DO concentration was estimated using Winkler's titrimetric method (Carpenter 1965). Water samples were carefully siphoned into 125 ml acid washed (10% HCl) glass stopper bottles without formation of air bubbles. Winkler A (1 ml of 3 M manganous chloride) and Winkler B (1 ml of 8 M alkaline iodide) were added to the samples and mixed properly. The precipitate formed was dissolved using 1 ml of 10 N H_2SO_4 and titrated with 0.01 N sodium thiosulphate using starch as indicator. Concentration of oxygen is expressed as mg L^{-1}.

3.3.1.4 Suspended particulate matter (SPM)

For determination of SPM, 250 ml of water samples were filtered through previously weighed millipore filter paper (0.45 µm pore size) subsequently, the filter was dried at 80° C to 90° C to constant weight to eliminate the water content and re-weighed. The differences in weight indicate the amount of suspended solids and the value expressed in mg L^{-1}.

3.3.2 Nutrient concentrations

3.3.2.1 Ammonia-N (NH_4)

Ammonia-N was determined according to the indophenol blue method of Koroleff, (1983). The measurement of ammonia included both free dissolved ammonia gas and the ammonium ions. This method estimates the sum of NH_4^+ and NH_3 and is denoted here as NH_4-N. In a moderately alkaline medium, ammonia reacts with hypochlorite to form monochloramine, which in presence of phenol, catalytic amount of nitroprusside ions and excess of hypochlorite forms indophenol blue. The formation of monochloramine requires a pH between 8 and 11.5. At higher pH, ammonia is incompletely oxidized to nitrite. Calcium and magnesium ions precipitate in seawater as hydroxide and carbonate respectively above pH 9.6. However, their precipitation can be prevented by complexing them with citrate buffer. Adequate care was taken to ensure that samples, blanks and standards were not contaminated during the course of analysis. The samples were 'fixed' by the addition of reagents immediately after collection and the absorbance, after the colour development (after 6 hours) was measured at 630 nm using U V - Vis spectrophotometer (Shimadzu, 1650 PC Japan). The concentration was calculated based on the standard ammonium chloride (NH_4 Cl) solution (precision: ±0.05). Concentration is expressed in μM N-NH_4.

3.3.2.2 Nitrite-N (NO_2)

Nitrite was measured by the method described by Bendschneider and Robinson (1952). In this method, nitrite in the sample was allowed to react with sulphanilamide in an acid solution. The resulting diazo compound reacted with N-(1-naphthyl)-ethylene diamine to form a highly coloured azo dye. The absorbance was measured at 543 nm using a U V - Vis spectrophotometer. The concentration was calculated based on the standard Sodium nitrite ($NaNO_2$) solution (precision: ±0.05 μM). Concentration is expressed in μM N-NO_2.

3.3.2.3 Nitrate-N (NO_3)

The method described by Grasshoff (1970) was used for the estimation of nitrate. In this method the nitrate present in the sample was reduced to nitrite

using a reducter filled with copper coated-cadmium granules. The condition of reduction was adjusted so that nitrate is almost quantitatively converted to nitrite and not reduced further. Nitrite thus formed was estimated as the method mentioned in section 3.3.2.2 above. Potassium nitrate (KNO_3) was used for standardization. Concentration is expressed in μM N-NO_3.

3.3.2.4 Phosphate

The method of Murphy and Riley (1962) was adopted for estimating inorganic phosphate. Phosphate and ammonium molybdate were allowed to react in acid solution to give phosphomolybdic acid, which was reduced by ascorbic acid. Optical density was measured using a spectrophotometer (Shimadzu, Japan) after 10 min at 882 nm. Potassium dihydrogen phosphate (KH_2PO_4) was used as standard and the concentration is expressed in μM.

3.3.2.5 Silicate

Silicate was estimated using protocol of Grasshoff (1964). Sample was allowed to react with ammonium molybdate resulting in the formation of silicomolybdate, phosphomolybdate and arsenomolybdate complexes and oxalic acid was added to reduce to silicomolybdous acid and the absorbance of blue colour was measured at 810 nm. Sodium fluorosilicate (Na_2SiF_6) solution was used as standard and the he concentration is expressed in μM.

3.4 Microbiological Enumeration

3.4.1 Direct total counts (DTC)

Total prokaryotic abundance was determined by DAPI (4,6-Diamidino-2-Phenylindole) (Porter and Feig 1980). Samples for total bacterial counts were fixed with buffered formalin and stored at 4°C. These samples were stained with DAPI (Fluka) (final concentration 0.01% w/v) for five minutes before filtering it through 0.2- μm polycarbonate nuclepore filter (Millipore, USA). Samples were enumerated at 100X magnification under a Nikon epifluorescence microscope, and at least 10 fields of >30 cells per field were counted. Total prokaryotic abundance was expressed as cells ml^{-1}.

3.4.2 Fluorescent *In Situ* Hybridization (FISH)

FISH is a molecular tool used for rapid independent monitoring of phylogenetically defined bacterial populations in environmental samples using nucleic acid probes. In the present study, abundance of total eubacteria, archaea, ammonia oxidizing bacteria and nitrite oxidizing bacteria in the water were estimated using FISH.

3.4.2.1 Sample fixation and preservation

About 200 ml of water sample from each station was fixed with formaldehyde to a final concentration of 2-4% and kept for 1 hour at room temperature (27± 2°C). The samples were sonicated for 20 to 40 seconds in order to detach the particle associated cells. Then 10-25 ml of sample was filtered onto a white 0.2-µm polycarbonate membrane filter (Millipore GTTP04700) that was placed over cellulose nitrate pre-filter (Millipore AP1504700). Low pressure was applied during filtration to avoid cell damage. Filters were washed with 30 ml of sterile phosphate-buffered saline (pH 8) followed by 30 ml of sterile distilled water and air-dried over absorbent paper in individual sterile petri dishes. The filters were immediately hybridized or stored at -20˚ C until hybridization.

3.4.2.2 Hybridization and DAPI staining

Modified protocol of Glöckner et al. (1999) was used for hybridization. Replicate filters were used for hybridization with suitable probes labeled with fluorescent dye CY3. Each filter was cut into four sections with a razor blade, and each section was placed onto a microscope slide. A slit was made in the right edge of each filter section to ensure that the side containing the bacteria was facing upwards. The filter sections were hybridized with 20 µL of hybridization buffer, containing 0.9 M NaCl, 20 mM Tris- HCl (pH 8), 35 to 60% formamide (formamide % varies depending on probe), 0.01% sodium dodecyl sulphate (SDS), and 50 ng of oligonucleotide probe. Each slide with filter sections was placed into a polyethylene tube (in a horizontal position). A piece of blotting paper was put into the polyethylene tube and soaked it with 1ml hybridization buffer to create a humid atmosphere. These caped polyethylene tubes incubated in the dark for 90 to 120

minutes at 46˚ C hybridization chambers. Following incubation, filter sections were quickly transferred into pre-warmed (48°C) washing buffer prepared in individual falcon tubes. The pre warmed wash buffer contained 20 mM Tris-HCl, 5 mM EDTA, 0.01% SDS and 20 to 60 mM NaCl (5M NaCl depending on % formamide in hybridization buffer), and incubated for 15 min at 48°C in a stirred water bath. The wash buffer with filter section was poured into a Petri dish and the filter section was picked and rinsed by placing them into a Petri dish with distilled H_2O for several seconds. Each filter section was dried at room temperature over absorbent paper, placed on a glass slide. Dried filters were counter-stained with 50 µl of DAPI (1 µg ml^{-1}) for 3-8 minutes. After staining, each filter section was washed with 1 ml of filtered Milli Q water, dried over absorbent paper and mounted on a glass slide in glycerol medium (Citifluor #1; Citifluor). Hybridization and counting were completed within one month after preparation. The details of FISH probes used, its sequences and reference are given in the Table 3.1. Target sites of the probes, formamide concentration in hybridization buffer and NaCl concentration in the wash buffer are given in the Table 3.2. (Preparation of all buffers and solutions are given in Appendix section.)

Table 3.1 Sequence of the probes used for FISH

Organisms	Probe Name	Sequence	Reference
Bacteria	EUB 338	GCTGCCTCCCGTAGGAGT	(Amann et al. 1990)
Archaea	ARCH 915	GTGCTCCCCCGCCAATTCCT	(Stahl and Amann 1991)
AOB			
β-AOB	Nso 190	CGATCCCCTGCTTTTCTCC	(Obarry et al. 1996)
N. mobilis	NmV	TCCTCAGAGACTACGCGG	(Pommerening-RÃ¶ser et al. 1996)
NOB			
Nitrobacter sp.	NIT	CCTGTGCTCCATGCTCCG	(wagner et al. 1996)
Nitrospira sp.	Ntspa 712	CGCCTTCGCCACCGGCCTTCC	(Daims et al. 2000)

Table 3.2 Target sites of the probes, formamide concentration in the hybridization buffer and NaCl concentration in the wash buffer.

Probe Name	Concentration		
	Target Sites	Formamide (%) in hybridization buffer	NaCl (mM) in the wash buffer
EUB 338	16S (338–355)	55	20
ARCH 915	16S (934–915)	40	56
Nso190	16S (190–208)	55	20
NmV	16S (174–191)	40	56
NIT	16S (1035– 1048)	45	40
Ntspa 712	16S (712–732)	40	56

3.4.2.3 Counting of FISH cells

The slides were either examined immediately using an Olympus BH-2 epifluorescent microscope, equipped with a 100X oil objective, Cy3 filter (41007-HQ) and DAPI filter (UG-1), or were stored at -20ºC for a maximum of 2 days before microscopic analysis. As Cy3 fluorescence fades much more rapidly than DAPI fluorescence (Pernthaler et al. 2001) direct counts of hybridized cells were completed first, followed by DAPI counts (under UV light), for total bacteria, in the same field of view.

3.5 Community Structure of AOB and AOA using DGGE

3.5.1 Sample preparation

3.5.1.1 DNA extraction

DNA extraction was performed following the protocol of Boström et al. (2004) with slight modification. Different steps involved in DNA extractions are briefly given below.

1. One to two liters of water samples were filtered through 0.2 µm cellulose nitrate filter and stored at -20°C until DNA extraction.

2. Frozen filters were cut into small pieces and put into 2 ml microcentrifuge tube containing 525 µl of lysis buffer.
3. Into the tube 11 µl of lysozyme (1mg/ml) was added and incubated at 37˚ C for one hour in water bath.
4. After incubation 60 µl of 10% SDS and 3 µl of proteinase (K 100 µg ml^{-1}) were added and incubated at 55°C for 6 hours.
5. An approximately equal volume (0.7 to 0.8 ml) of chloroform/isoamyl alcohol (24:1) was added to the above, mixed thoroughly by inverting the tube, and spinned for 4 to 5 minutes in a microcentrifuge. A white interface was visible after centrifugation.
6. The aqueous and viscous supernatant was removed to a fresh microcentrifuge tube, leaving the interface behind.
7. About 0.6 volume of isopropanol was added and mixed by inverting the tube to precipitating the nucleic acids.
8. The tube was centrifuged for 10 minutes at 10000 rpm. The supernatant was discarded. The pellet was washed with 1000 µl of 99% ethanol and centrifuged for 10 minutes at 1000 rpm to purify the DNA.
9. The supernatant was discarded and the purified DNA pellet was dried under vacuum.
10. The DNA pellet was then dissolved in 50 µl of 1X TE buffer for analysis. Preparation of all buffers, solutions and reagents are given in Appendix section.

3.5.1.2 DNA detection by agarose gel electrophoresis

Accurately weighed 1.5 g of electrophoresis grade agarose was added to 100 ml of electrophoresis buffer (1X TAE). The agarose was melted in a microwave oven (IFB, USA) and was swirled in between to ensure even mixing. The molten agarose was allowed to cool down to 50-60°C. Prior to the preparation of molten agarose the gel caster (BioRAD, USA) was leveled using the leveling feet and leveling bubble. The comb was placed on the appropriate slots of the tray. The molten agarose was poured into the gel tray. The gel was allowed to solidify for 20-30 minutes at room temperature. After the gel was hardened, the comb was

withdrawn and the gel tray removed carefully. The gel casting tray was placed in the electrophoresis tank and sufficient 1X TAE buffer was added to cover the gel to a depth of 1mm. The extracted DNA samples were mixed with 6X loading dye in the ratio 1:5 and loaded into the gel wells with a micropipette at the negative electrode. Three µl of 500 bp DNA molecular weight marker was loaded as reference. The leads were connected and voltage applied so that DNA could migrate toward the anode. The gel was electrophoresed at 100 V until the dye front had migrated to about three-fourth of the gel. The DNA fragments were visualized using the gel documentation system (BioRAD excel, USA) and the image was captured using image lab software (BioRAD, USA).

3.5.1.3 Estimation of DNA by spectrophotometry

DNA was quantified by measuring absorbance at 260 nm using Nanodrop Spectrophotometer (Nanodrop Technologies, USA). An absorbance value of 1 at 260 nm is equivalent to the concentration of 50 µg ml^{-1} for double stranded DNA. Purity of DNA was checked by measuring the ratio of absorbance at 260 nm and 280 nm. If the ratio A260/A280 is approximately equal to 1.8, it indicates pure DNA. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants.

3.5.2 PCR amplification of DNA for AOB

The purified DNA was subjected to PCR in a Thermal cycler (Eppendorf, Germany) for the amplification. All PCRs for DGGE were carried out with an equimolar mixture of three forward primers (Kowalchuk et al. 1997) (CTO189fA-GC, CTO189f BGC, and CTO189 f C-GC), each with a GC clamp and a reverse primer containing a single ambiguous base without GC clamp. The forward primers CTO189fA and CTO189fB (CCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAGRAAAGCAGGGGATCG [GC clamp underlined]) and CTO189fC-GC (CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAGGAAAGTAGGGGATCG) were synthesized separately and collectively referred to as CTO189f-GC.

The reverse primer sequence was CTO 654r (CTAGCYTTGTAGTT TCAAACGC). The primers amplify 465 bp partial rDNA sequences of β-subdivision ammonia oxidizer. Amplification was carried out in a 50 µl reaction mixture in duplicate. Each mixture containing 50 ng of purified DNA (DNA samples were diluted in milliQ water for required ng concentration), 3 mM concentration of $MgCl_2$, 2.5 mM of each dNTP, 10 pM of each primer and 3 U/µl of Taq polymerase in 1X of Taq buffer (NEB Canada). The PCR conditions used were: initial denaturation at 95°C for 5 minutes, 30 cycles consisting of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 45 seconds and final extension at 72 °C for 10 minutes. Two reactions, a positive control with DNA template known to be successfully amplified and a negative control lacking template DNA was set up in each PCR run. Amplification was confirmed by gel electrophoresis. Duplicate PCR products of 465 bp length were mixed to minimize PCR bias and this was used for DGGE.

3.5.3 PCR amplification of DNA for AOA

Ammonia monoxygenase gene (amoA) of AOA was amplified for the community structure analysis of AOA. A combination of GC clamp attached forward primer amoAF (CTGAYTGGGCYTGGACATC) and reverse primer without GC clamp amoAR (TTCTTCTTTGTTGCCCAGTA) were used as primer sets (Wuchter et al. 2006). The GC clamp was attached to the 5' end of the primer. Amplification was carried out in a 50 µl reaction mixture in duplicate. Each mixture containing 50 ng of purified DNA, 3 mM concentration of $MgCl_2$, 2.5 mM of each dNTP, 10 pM of each primer and 3U/µl of Taq polymerase in 1X of Taq buffer (NEB Canada). The PCR conditions used were: initial denaturation at 95°C for 5 minutes, 30 cycles consisting of denaturation at 94 °C for 1 minute, annealing at 57° C for 1 minute and elongation at 72 °C for 30 seconds and final extension at 72° C for 10 minutes. Amplification of 256 bp length of AOA gene was confirmed by gel electrophoresis.

3.5.4 Denaturing Gradient Gel Electrophoresis (DGGE) analysis

DGGE of PCR products generated were performed by the method described by (Muyzer and Smalla 1998) with the use of a D-Gene system (Bio-Rad Laboratories).

3.5.4.1 Preparation of acrylamide gel

For DGGE of AOB Polyacrylamide gradient gels (8% polyacrylamide; 1.5 mm thick; 0.53 TAE; 37:1 acrylamide-bisacrylamide; 35 to 50% denaturant) using gel sandwich sizes of 16x16 cm were poured with the aid of a gradient maker (CBS, Del Mar, Calif.). Denaturing acrylamide (100%) was defined as 7 M urea with 40% formamide (24). A 10% poly acrylamide gel with 30 to 55 % denaturant was used for AOA. Gels were poured from bottom to top. The gel was allowed to set for about one hour at room temperature. Ammonium Persulfate (APS) and Tetramethylethylenediamine (TEMED) were used for the polymerization of the gels as per the instructions in the equipment's manual. Meanwhile the electrophoresis tank was filled with 7 L of 1X TAE buffer. The casted gel was inserted into the core after removing the comb. The other side of the core was also fitted with a gel sandwich so that the upper buffer chamber is formed. The wells of the gel were washed several times with same buffer to remove partially polymerized particles. The core along with the gels was carefully placed in the buffer tank. About 350 ml of running buffer was added to the upper buffer tank. The temperature control module was placed over the tank, switched on and the temperature was set at 60° C.

3.5.4.2 Sample loading

The heater and power were switched off and the sample was loaded (35 µl) after mixing with 5 µl of 6X loading dye. The power was switched on; the heater and the pump were also switched on. As soon as temperature 60° C was attained, the electrophoresis unit was switched on. Gels were run for 6.5 hours at 200 V in 0.53 TAE buffer at a constant temperature of 60° C or 16 hours at 75 V. After electrophoresis run was completed, the power was switched off. The core sandwich assembly was removed from the buffer tank. The gel sandwiches were carefully detached and the gel removed.

3.5.4.3 Viewing the Gel

The gels were stained in MilliQ (Millipore B. V., Etten-Leur, Netherlands) water containing 0.5 mg of ethidium bromide/liter and destained twice in 0.53 TAE buffer prior to UV transillumination. The bands separated were observed in a gel documentation system (BioRAD, USA) and the images were taken using image lab software.

3.5.4.4 Analysis of DGGE profiles

DGGE gel images were analyzed with Image lab software from Bio-Rad (ver. 4.65). The software carries out a density profile analysis, detects the bands from each lane and calculates the relative contribution of each band to the total lane intensity. The numbers of operational taxonomic units (OTU) in each sample were counted as number of DGGE bands. Gels were cross-checked visually as well for number of bands per lane. An intensity matrix was constructed based on the relative contribution of the band to the total intensity of the lane. DGGE bands were detected and transformed into a presence/absence binary matrix. Cluster analysis of DGGE bands based on square root transformed community data matrix through Bray-curtis similarity were performed with PRIMER v.6 software package (Plymouth Marine Laboratory).

3.6 Phylogenetic Analysis of AOB

3.6.1 Excision of bands and re-amplification

The documentation of the DGGE gel was followed by printing of the gel picture. The bands were carefully marked and labeled. The gel was placed on a UV transparent acrylic plate. The UV lamp was switched on and unique bands were excised with a sterile surgical blade. The UV lamp was immediately switched off in order to minimize the damage to the DNA bands in the gel. The gel pieces were transferred to the labeled 1.5 ml micro-centrifuge tube with 25 µl of sterilized deionized water. The tubes were incubated at 4° C for overnight. The tubes were spinned for about 30 seconds. The supernatant was aspirated and stored. About 2 µl of the supernatant was used as a template for re-amplification using the same

primers but without GC clamp. PCR was performed at similar condition mentioned in the DGGE PCR. The resulting PCR products were run on 1.5% agarose gel for assessment of quality and quantity (as mentioned above).

3.6.2 Cloning of PCR product

The PCR product was cloned using TOPO®- Vector System as per the manufacture's instruction and contains following steps:

1. TOPO vector and salt solution (1 µl each) were added to 4 µl of fresh PCR product. The mixture was incubated for 30 minutes at 24°C after gently mixing, centrifuged for a few seconds after incubation to collect the contents at the bottom.
2. Frozen JM109 high efficiency competent cells were taken from storage and placed in an ice until just thawed (about 5- 30 minutes). Mixed the cells by gently flicking the tube, as the competent cells were extremely fragile excessive pipetting was avoided.
3. Ligation reaction of 5 µl each was transferred to 50 µl One Shot® Chemically Competent *E. coli* cells taken in 1.5 ml microcentrifuge tube on ice and mixed gently. Another tube on ice with 0.1 ng uncut plasmid also set up for determination of the transformation efficiency of the competent cells was. The tubes were gently flicked and placed on ice for 30 minutes.
4. After 30 minutes incubation, heat-shocked the cells for 45-50 seconds in water bath at exactly 42°C (without shaking) and immediately returned the tubes to ice for 2 minutes.
5. LB broth (950 µl) of room-temperature was added to the tubes containing cells transformed with ligation reactions and 900 µl to the tube containing cells transformed with uncut plasmid and incubated for 1.5 hours at 37°C with shaking (~150rpm).
6. Each transformation culture of 100 µl was plated on to duplicate kanamycin added LB plate. For the transformation control, a 1:10 dilution with SOC medium was plated. Incubated the plates overnight (16-24 hours) at 37°C.

7. The colonies were counted and picked for further analysis after 8-12 hours. The bacterial colonies were streaked for single colony on LB plates containing 100 μg ml^{-1} kanamycin and incubated at 37°C.
8. A single colony was isolated and inoculated into 5 ml of LB containing 100 μg ml^{-1} kanamycin and grown overnight with shaking at 37°C.
9. Mixed 0.85 ml of culture with 0.15 ml of sterile glycerol and transferred into a cryo-vial and stored at -80°C for long term storage. The remaining broth was pelletized and this pellet was used for plasmid extraction.

3.6.3 Plasmid extraction

Triplicate positive clones were selected at random and plasmids were extracted using plasmid extraction kit. Extracted plasmid was quantified by measuring absorbance at 280 nm using Nanodrop Spectrophotometer (Nanodrop Technologies, USA) and diluted to concentration of 200 ng μl^{-1}.

3.6.4 Sequencing PCR

Genes in the plasmids were amplified using the vector primers M13F-GTAAAACGACGGCCA and M13R-CAGGAAACAGCTATGAC. Amplification was carried out in a 10 μl reaction mixture. Each mixture containing 200 ng of template, 10 pM of primer and 1 μl of Big dye. The PCR conditions used were: initial denaturation at 95°C for 30 seconds, 25 cycles consisting of denaturation at 95°C for 15 seconds, annealing at 65°C for 15 seconds and elongation at 72°C for 30 seconds and final extension at 72°C for 5 minutes. The PCR are products were clean up using PCR clean up kit as per manufacturer's instructions and the PCR products were then sequenced (Sci genome, Cochin).

3.6.5 Phylogenetic tree

Sequence data obtained were analyzed and edited using Sequencher V4.10.1 (Gene Codes). Subsequently, the sequences were compared with those in the Gen Bank database using the Basic Local Alignment Search Tool (BLAST) algorithm to determine approximate phylogenetic affiliations. The nucleic acid sequences showing the closest similarities were used as reference sequences while

constructing neighbour-joining tree using the software MEGA (version 5.0). Bootstrap analysis was carried out using 1000 iterations.

Submission of sequences in GenBank

The partial 16S rRNA gene environmental clone sequences obtained from this study were deposited in the Genbank database (Genbank accession numbers KM386955 to KM386977).

3.7 Estimation of Activity

3.7.1 Nitrification rate

Chemical inhibitor method was used for determining the nitrification rates in the water column of the CE followed by the protocol of Bianchi et al. (1994).

Principle:- Nitrification rates were obtained by measuring the increase or decrease of nitrite concentrations in subsamples containing allylthiourea (ATU) or sodium chlorate ($NaC1O_3$), the well-known inhibitors of the oxidation of ammonium and nitrite, respectively.

Protocol:- Five hundred ml of sample was dispensed in to nine one liter capacity bottles and the bottles were divided into three sets as shown in Figure 3.2. First set was kept as control without any inhibitor, the second set received ATU, at 100 mg L^{-1} final concentration and the third set received $NaClO_3$ at final concentration of 10 mg L^{-1}. Preliminary experiments were carried out to prove that the inhibitor concentration was sufficient to inhibit the oxidation process before the actual analysis. Samples were incubated in dark for 36 hours at room temperature. Sub-samples (25 ml) were taken out at 6 hours intervals from 0 to 36 hours for analyzing the nitrite concentration. Ammonia oxidation activity was demonstrated by the increase of nitrite concentration in the triplicate receiving $NaClO_3$, as the increase in nitrite concentration in these bottles were only due to ammonia oxidation. The nitrite oxidation was demonstrated by the

decrease in nitrite concentration in the triplicate receiving ATU as the decrease these bottles were due to nitrite oxidation in sample of no further ammonia oxidation ay the action of ATU. Rates were estimated during the exponential phase of the nitrite decrease or increase and the results were expressed as µM N day^{-1}. All the analysis were performed in triplicate.

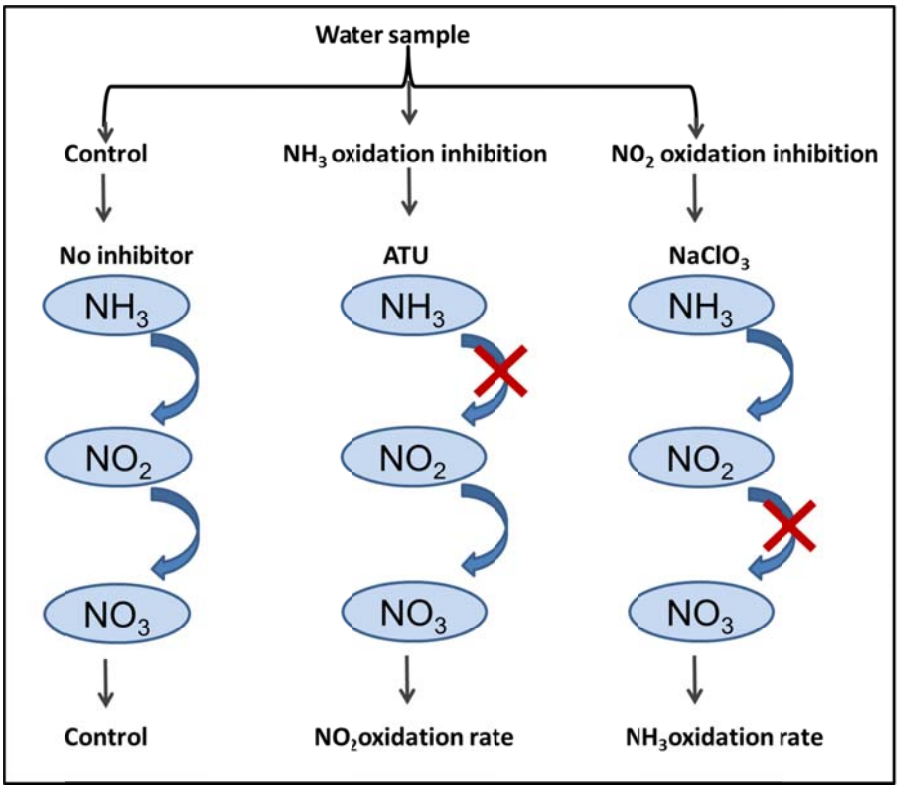


Figure 3.2 Schematic representation of analytical approach used for measuring nitrification rate.

3.7.2 Differential Contribution of AOA and AOB in Ammonia Oxidation

Recovery of Nitrification Rate (RNR) analysis was used to understand the differential contribution of AOA and AOB in ammonia oxidation process in the CE using the protocol described by Taylor et al. (2010)

Principle: - Chemical inhibitors and protein synthesis inhibiting antibiotics were used so that bacterial and archaeal ammonia oxidation were reversibly inhibited by acetylene which irreversibly inactivates the

ammonia monoxygenase enzyme. But upon the removal of acetylene, NH_3 oxidation could be resumed in ammonia oxidizers after a 1 to 2 hours delay by re-synthesis of the protein ammonia monoxygenase (AMO) as described by Hyman and Arp (1992) and the antibiotics that prevent protein synthesis could be used to discriminate between ammonia oxidation activities of AOA and AOB during the recovery of nitrification rate after acetylene exposure.

Protocol: - For assays of the recovered ammonia oxidation rate, 3 sets of 500 ml of water samples were dispensed in one L bottles with black phenolic caps fitted with gray butyl stoppers. A schematic diagram of experimental procedure is given in Figure 3.3. Another three sets of samples were also arranged at the same time for actual nitrification rate measurement as shown in the above section.

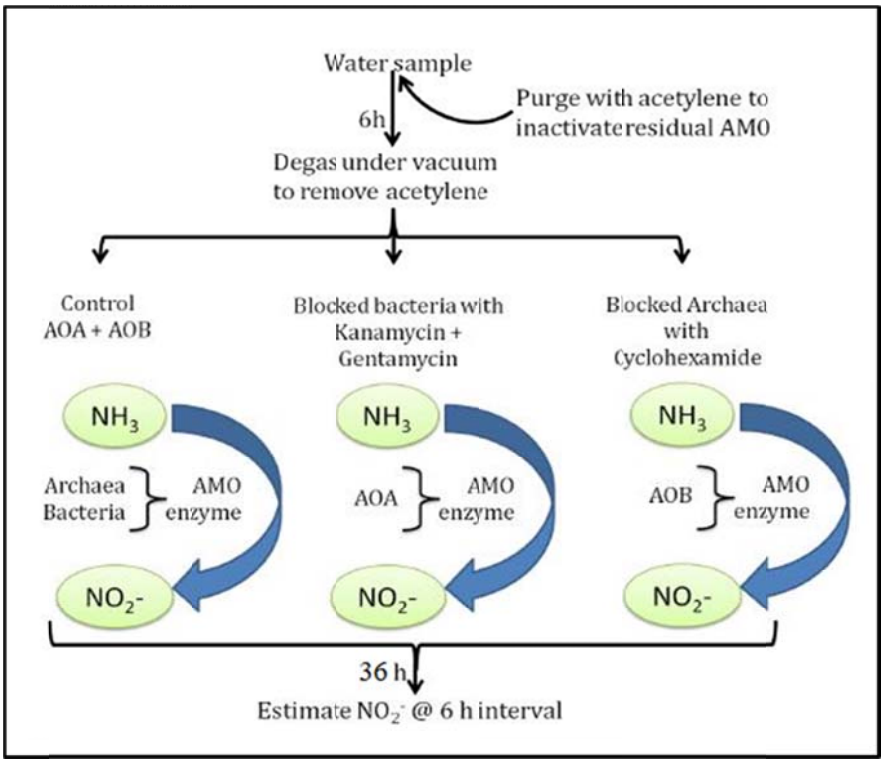


Figure 3.3 Schematic representation of analytical approach used for measuring differential contribution of AOB and AOA in ammonia oxidation rate.

Acetylene was purged to the headspace for 6 hours (0.025%, vol/vol, or 0.025 kPa). Preliminary experiments were conducted to prove a 6 hours acetylene

exposure was sufficient to inactivate all NH_3 oxidation in the samples used in the study. Acetylene inhibition and recovery steps were carried out at 32˚C. These conditions in the absence of any enzyme inhibitors were considered to be the standard. Samples incubated without any acetylene purging was considered as nitrification rate controls and water samples were amended with sodium chlorate (20 ppm) to inhibit the conversion of nitrite to nitrate. Acetylene removal after purging was achieved by placing the bottle under vacuum and degassing for 10 minutes. After degassing, all bottles were incubated with caps loosened to permit aeration. Aqueous solutions of antibiotics and nitrification inhibitors were added to the samples after acetylene was displaced. Preliminary experiments were carried out with a range of concentrations of the antibiotics to determine their minimum effective concentrations. The water-soluble bacterial protein synthesis inhibitors kanamycin and gentamicin (final concentration 750 μg ml^{-1} each) were added to inhibit bacterial protein synthesis. Water soluble eukaryotic protein synthesis cycloheximide (final concentration 650 μg ml^{-1}) was used for protein synthesis in AOA. Nitrite accumulation was monitored at 6 hours interval for 36 hours following standard spectrophotometric technique as given above. The values were expressed as μM N day^{-1}. All the analysis were performed in triplicate.

3.8 Statistical Analysis

Following statistical analysis was performed using suitable software package in order to better explanation of microbial and environmental data sets. The data were normalized by log transformation before analyses.

3.8.1 Karl Pearson's correlation

Karl Pearson's correlation was used for understanding the statistically significant relationship or associations between environmental variables, microbial abundance and nitrification activity.

3.8.2 Three-way ANOVA

The environmental and bacterial abundance data were subjected to statistical analysis for significant variation across the sampling period, over the study stations and between surface and bottom along with their first order interactions using three-way ANOVA. Thereafter with student's t test for paired comparison between stations and between months to estimate the significance of the spatial and temporal variations of these parameters along with biological parameters with SPSS, V13 software (Jayalakshmy,1998).

3.8.3 Canonical Discriminant Analysis (CDA)

CDA was performed for the discrimination between stations based on (1) environmental data and activity and (2) based on environmental parameters and abundance data for ecologically identifying the station locations in the CE. The classification success of the discriminant analysis was checked using jackknifed cross-validation (SPSS Inc., 1999) technique. To visualize the station differences, first and second factors discriminant scores were plotted.

3.8.4 Principal components analysis (PCA)

PCA was carried out for environmental variables and AOB and NOB abundance and also for environmental parameters and nitrification rate. All the variables were normalized and analysis was done based on the correlation matrix using the statistical program PAST version 2.02. The biplot was drawn according to the correlation biplot of (Legendre 1998).

3.8.5 Step up multiple regression model (SMRM)

The significance of the environmental and biological parameters in influencing the bacterial abundance were also determined with step up multiple regression model with interaction effects SPSS, V13, (Jayalakshmy,1998) after normality testing and applying the appropriate transformation using Tuckey's test of additivity (Federer 1968).

Chapter - 4
RESULTS

Contents

4.1 Environmental Characteristics

4.1.1 Temperature

Spatio-temporal variations were visible in the water temperature of the study area and it ranged between 24 and 32° C during the study period (Figure 4.1). However, it was above 30° C at all the stations during most the sampling period. Surface waters showed relatively higher temperature compared to bottom waters. The water temperature was higher during pre-monsoon (30 - 32°C) compared to other seasons.

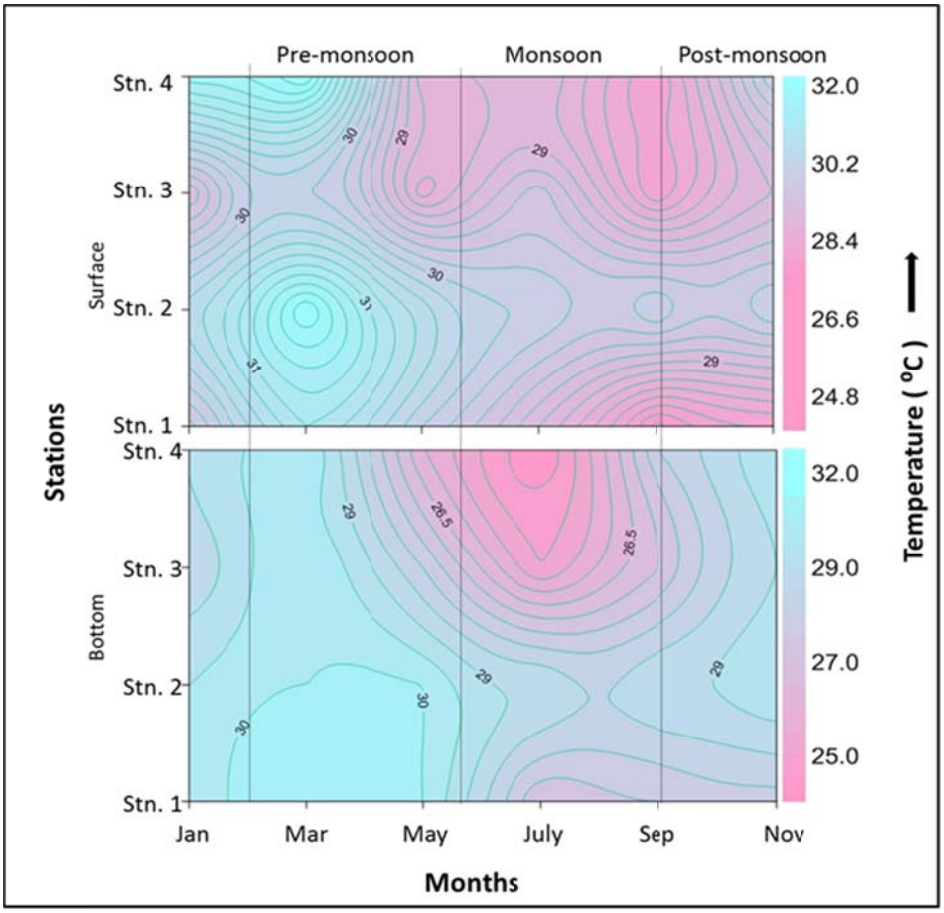


Figure 4.1 Distribution of temperature in surface and bottom waters in the CE.

4.1.2 Salinity

Water salinity showed marked variation (0 - 35) in the CE during the study period (Figure 4.2). Average salinity at Station 1 (low saline station) was 3.8 ± 4.3, while at stations 2 and 3 (intermediate saline stations) it was 15.9 ± 9.8. In the high saline station (Stn. 4), salinity was 24.3 ± 8.1. Bottom waters at most of the stations showed relatively higher salinity than the surface waters. Salinity decreased with the onset of monsoon and reached its minimum during the peak monsoon (July) and it slowly increased during post-monsoon and reached its maximum at the end of this season (January) or at the starting of pre-monsoon (March). Though the coastal station was 20 km away from bar-mouth, the salinity decreased from ca 35 to 14 and 18 in the surface and bottom waters respectively, during the peak monsoon period. Seasonal salinity variation of surface and bottom waters are given in Table 4.1.

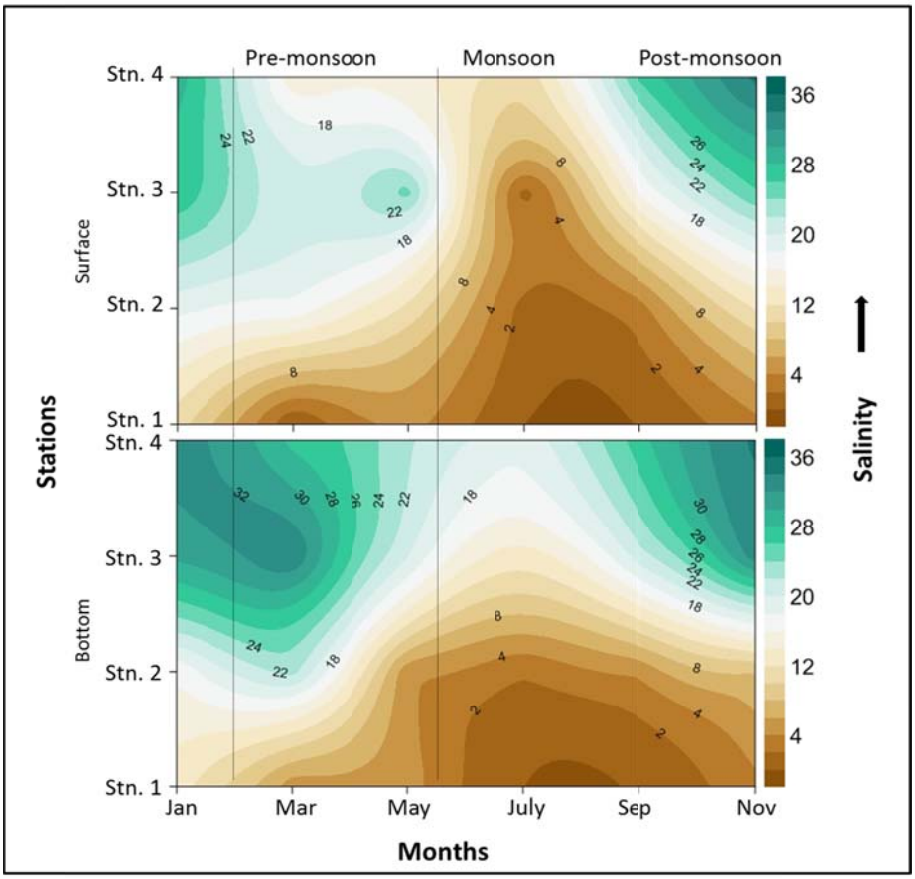


Figure 4.2 Distribution of salinity in surface and bottom waters in the CE.

Table 4.1. Seasonal salinity variation of surface and bottom waters.

Stations	Pre-monsoon		Monsoon		Post-monsoon	
	Surface	Bottom	Surface	Bottom	Surface	Bottom
Stn. 1 (Low saline)	2.4 ± 3	4.9 ± 0.6	0.06 + 06	0.14 ± 0.1	7.1 ± 5	8.2 ± 6
Stns. 2 & 3 (Intermediate saline)	18.5 ± 5	20 ± 12	5.5 ± 6	10.2+8	21 ± 7	22 ± 11
Stn. 4 (High saline)	24.6 ± 4	25.8 ± 4	18.5 ± 11	14.7 ± 6	31.7 ± 4	33.5 ± 1

4.1.3 pH

pH in the study area ranged from 6.98 to 8.25 as shown in Figure 4.3. pH was relatively low during monsoon months due to the increased fresh water influx in to the estuary. Higher pH observed in coastal station (7.7 to 8.25) compared to other stations. Stations 1 and 2 maintained the pH close to neutral throughout the study period except in the month of March. pH in the bottom water was relatively higher than surface water.

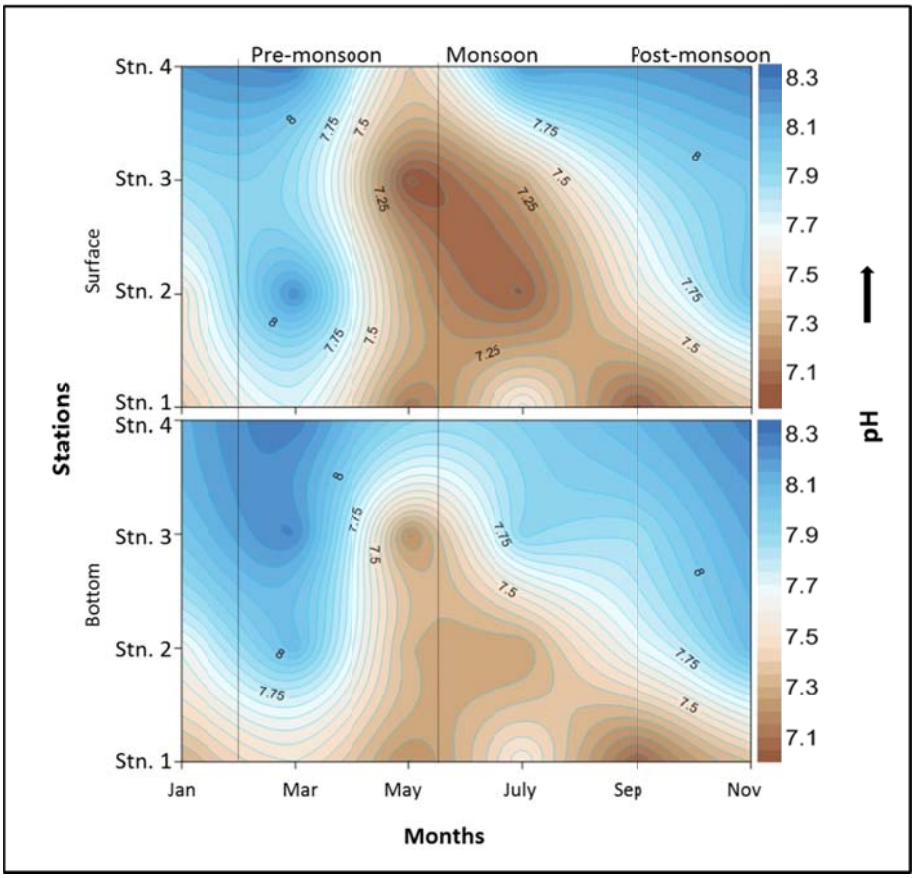


Figure 4.3 Distribution of pH in surface and bottom waters in the CE.

4.1.4 Dissolved Oxygen (DO)

Water column in the study area was well oxygenated throughout the year except for few sampling occasions (average 5.04 $\pm$ 1.4 mg L^{-1}). DO concentration in the CE ranged from 2.16 to 7.47 mg L^{-1} as shown in Figure 4.4. Low levels of DO ranged from 2.6 to 6.0 mg L^{-1} were recorded in the intermediate saline station, (Stn. 3). DO in the CE did not follow any seasonality but, it was comparatively higher during the month of January.

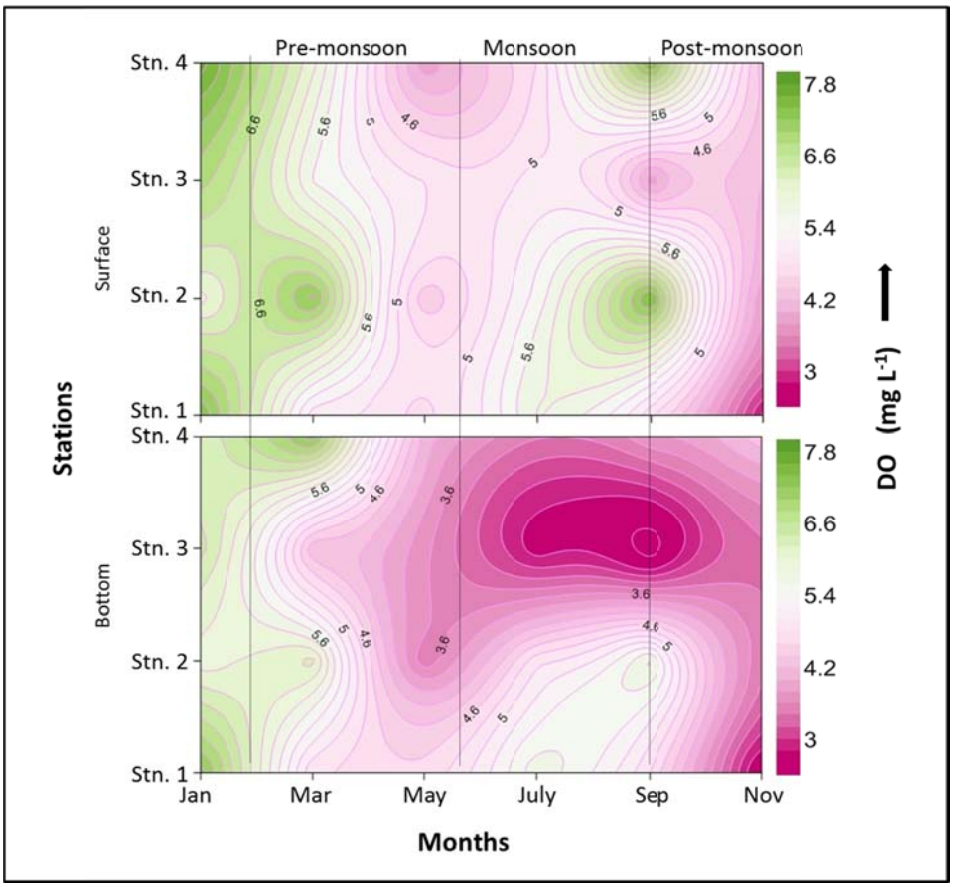


Figure 4.4 Distribution of DO concentration of surface and bottom waters in the CE.

4.1.5 SPM

Spatio-temporal variations were visible in the distribution of SPM in the study area (Figure 4.5) and it ranged from 3.2 to 155 mg L^{-1} (Figure 4.5). Low saline station showed comparatively lower SPM (3.2 to 30 mg L^{-1}) than the intermediate saline stations (10 to 155 mg L^{-1}) and high saline station (12 to151 mg L^{-1}). SPM was high in the bottom waters during monsoon compared to other seasons.

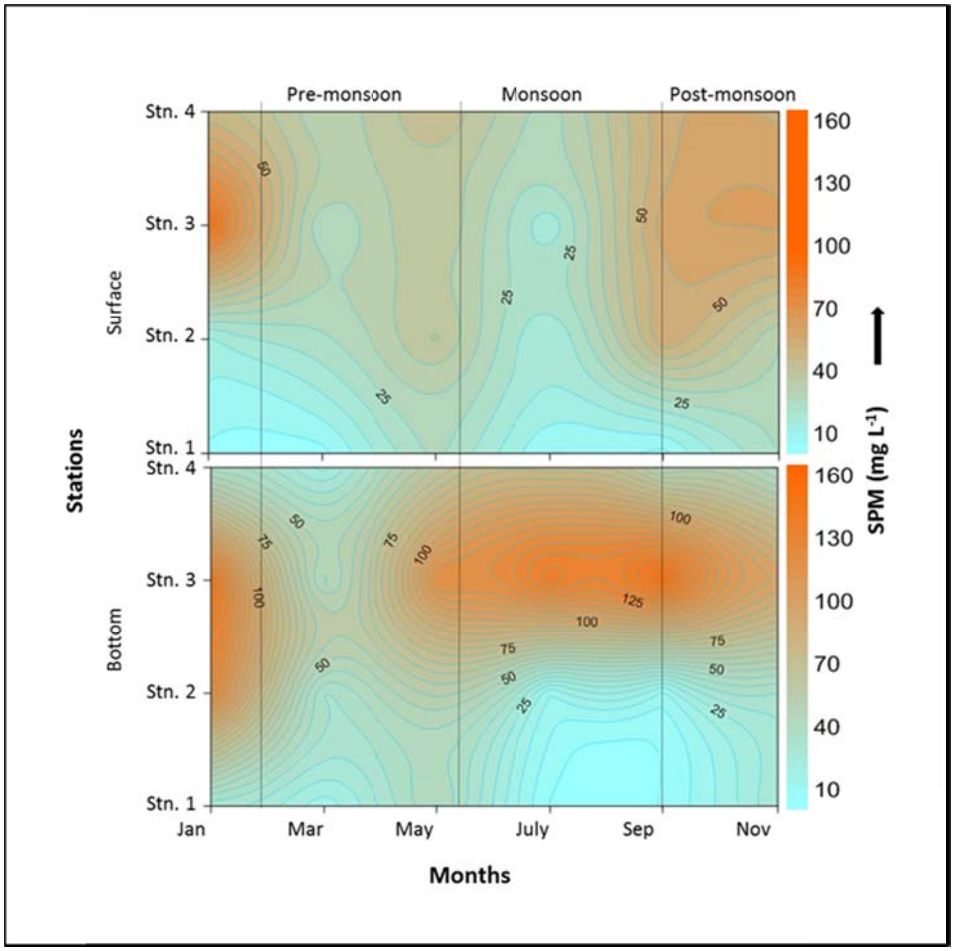


Figure 4.5 Distribution of SPM in surface and bottom waters in the CE.

4.1.6 Nutrients

4.1.6.1 Ammonia

Ammonia concentration ranged from 0.17 to 49.01 µM during the study period. It ranged from 5.65 to 47.72 µM in the low saline station, 1.32 to 49.01 µM in the intermediate saline stations and 0.17 to 28.35 µM in the high saline station. Figure 4.6 shows the spatio-temporal variations of ammonia concentration during the study period. Irrespective of station, the average concentrations of ammonia in the CE were 25.1 $\pm$ 7 µM during pre-monsoon, 6.5 $\pm$ 0.5 µM during monsoon and 17.6 $\pm$ 4 µM during post-monsoon. Ammonia was maximum at the end of pre-monsoon month (May) and, minimum during the peak monsoon month (July). Surface and bottom samples did not show variation in concentration.

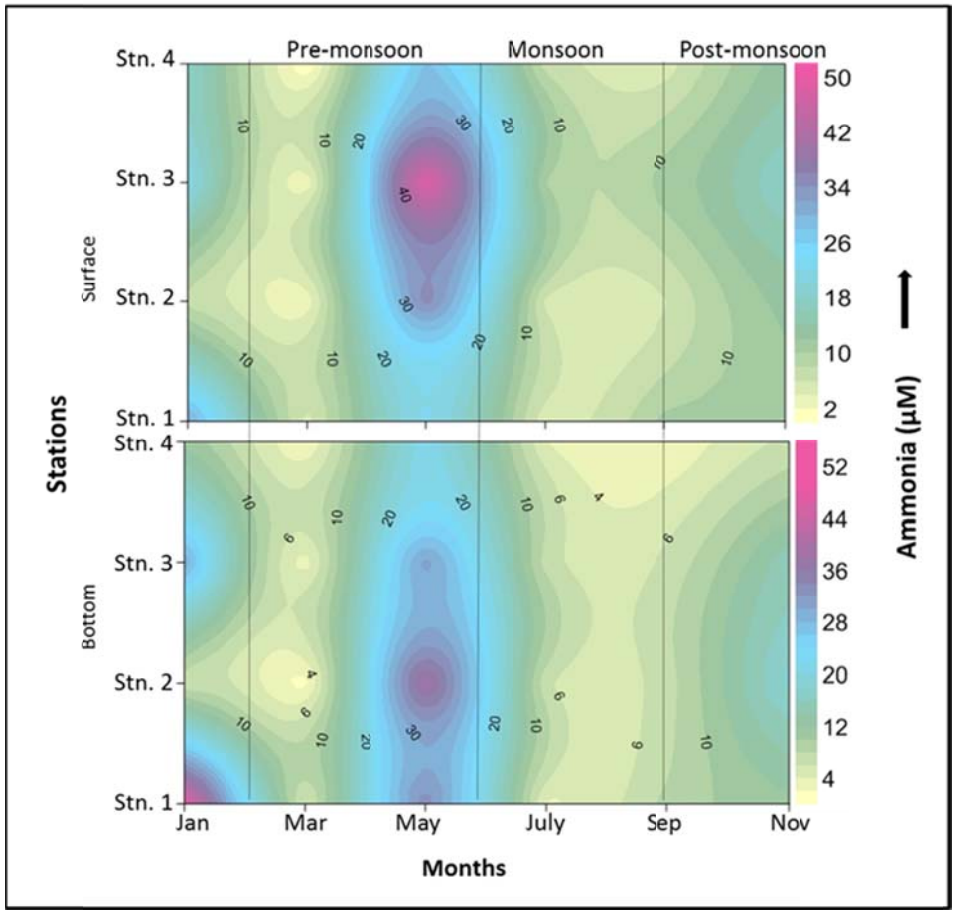


Figure 4.6 Ammonia concentration in surface and bottom waters in the CE.

4.1.6.2 Nitrite

Nitrite concentration in the CE ranged from 0.04 to 1.04 µM during the study period. The nitrate concentration ranged from 0.04 to 0.84 µM in low saline station (Stn. 1), 0.11 to 1.04 µM in intermediate stations (Stns. 2 and 3) and 0.05 to 0.57 µM in high saline station (Stn. 4). Figure 4.7 shows the spatio-temporal variations of nitrite concentration along the sampling stations. Unlike ammonia concentrations, nitrite was relatively higher during monsoon and lower during pre-monsoon season. Higher concentration of nitrite was observed in the bottom samples compared to the surface samples.

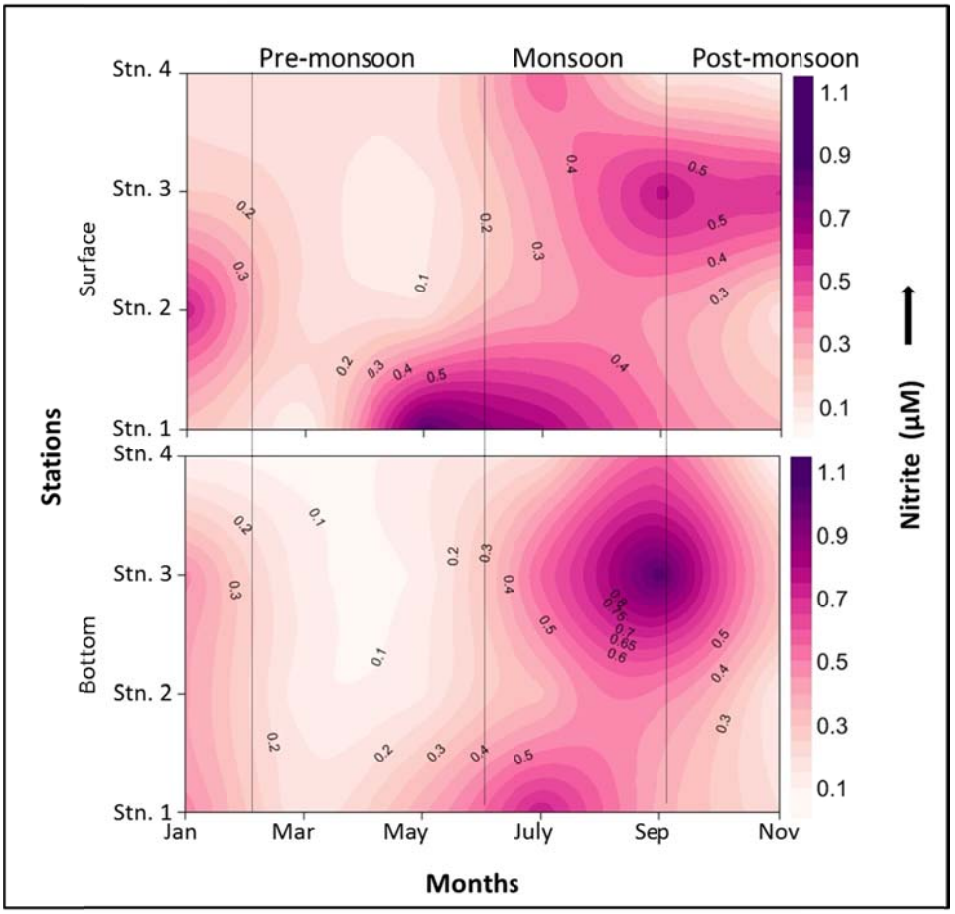


Figure 4.7 Nitrite concentration in surface and bottom waters in the CE.

4.1.6.3 Nitrate

Nitrate concentration ranged between 0.32 to 28.35 µM, during the study period. Figure 4.8 shows the spatio-temporal variation of dissolved nitrate in sampling stations. Nitrate concentration in low saline station ranged from10.32 to 26.21 µM and in intermediate saline stations 1.13 to 28.5 µM whereas in high saline station the concentration ranged between 0.34 and 5.32 µM. Similar to ammonia, nitrate levels were relatively higher during pre-monsoon and lower during post-monsoon season. Higher concentration of nitrate was observed in the bottom samples than in the surface samples. Ammonia was the major component of dissolved inorganic nitrogen (>60%) of the total dissolved nitrogen whereas nitrate was close to 40% of the total dissolved nitrogen concentration. Nitrite was the minor component in dissolved inorganic nitrogen concentration and it contributed only less than 1%.

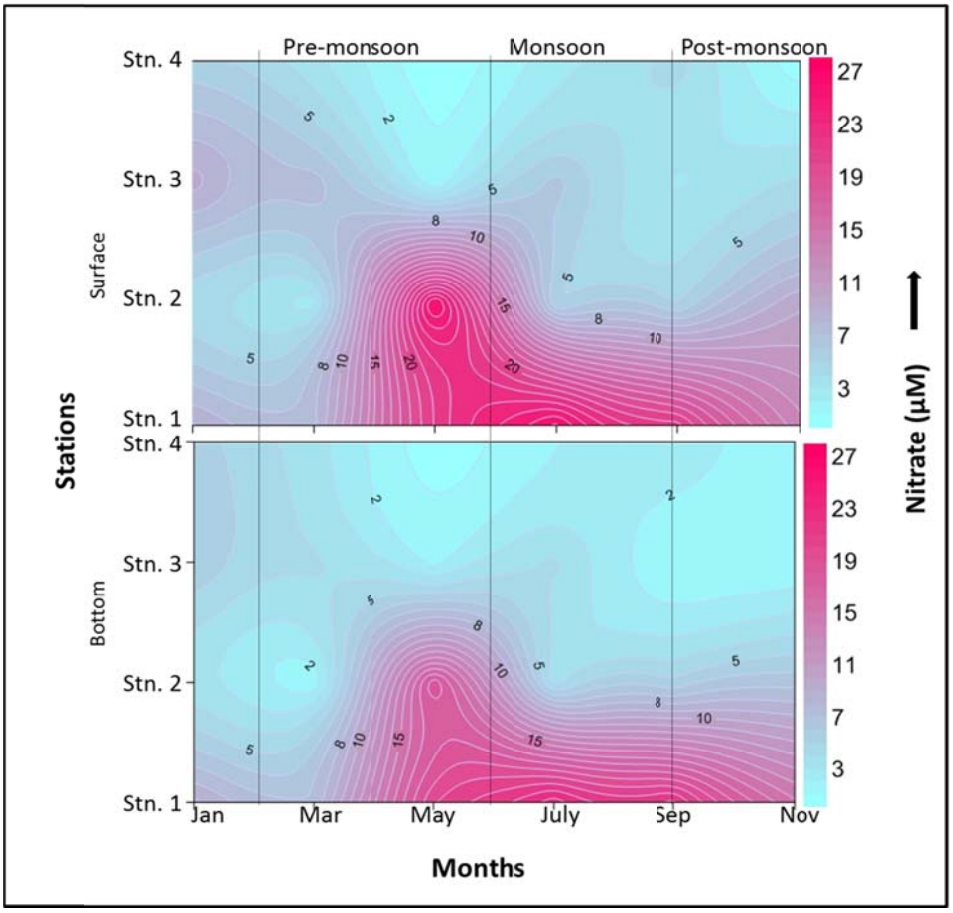


Figure 4.8 Nitrate concentration in surface and bottom waters in the CE.

4.1.6.4 Phosphate

During the study period, phosphate concentration in the CE ranged from 0.11 to 2.82 µM (Figure 4.9). It ranged from 0.42 to 2.52 µM, 0.12 to 2.82 µM and 0.11 to 2.78 µM in low, intermediate and high saline waters, respectively. A clear seasonality was visible in the pattern of phosphate concentration with higher concentration during monsoon and minimum concentration during post-monsoon. N/P ratio was calculated by dividing the sum of all the inorganic nitrogen concentration by phosphate concentration. The N/P ratio was higher during pre-monsoon (average 71.99 ± 62.4) and post-monsoon (average 44.1 ± 32.5), but relatively lower during monsoon (11.5 ± 8.7).

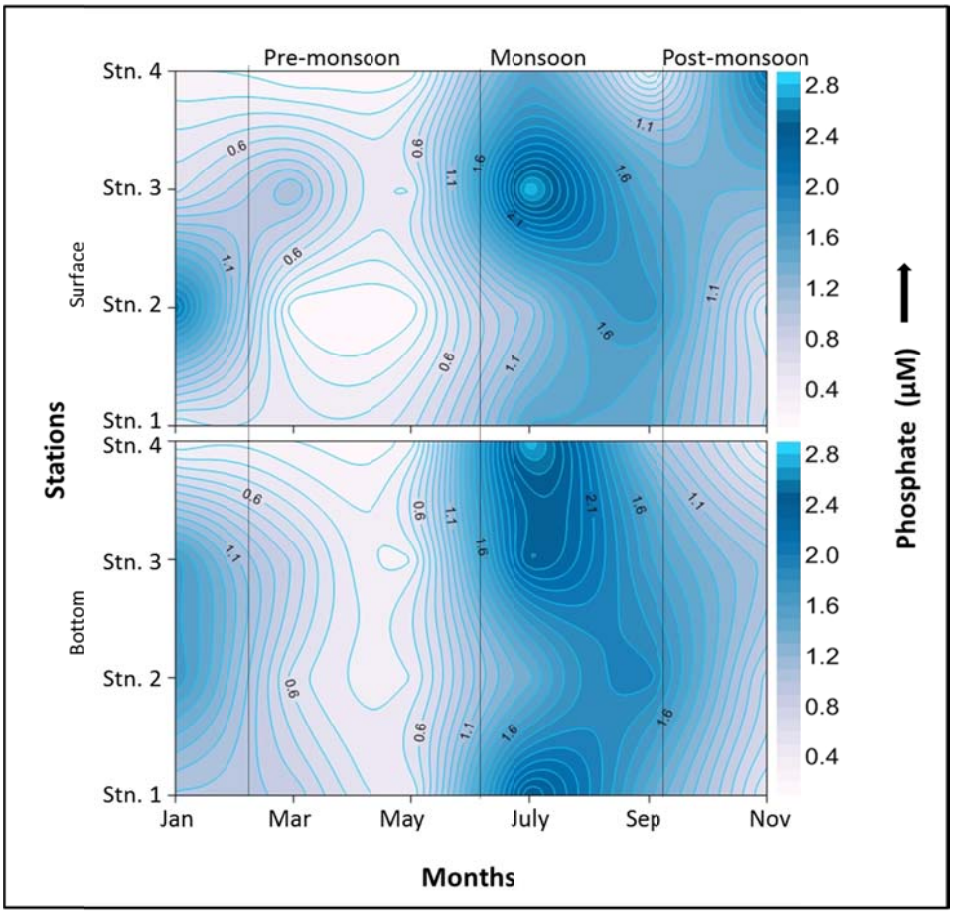


Figure 4.9 Phosphate concentration in surface and bottom waters in the CE.

4.1.6.5 Silicate

Silicate concentration in the CE ranged from 0.01 to 83.53 µM (Figure 4. 10). In that, it ranged from 9.02 to 83.5 µM, 4.41 to 49.41 and 0.012 to 25.88 in low saline, intermediate saline and high saline station respectively. High concentration of silicate was observed during monsoon and relatively low concentration during pre-monsoon. Surface and bottom samples did not showed much variation.

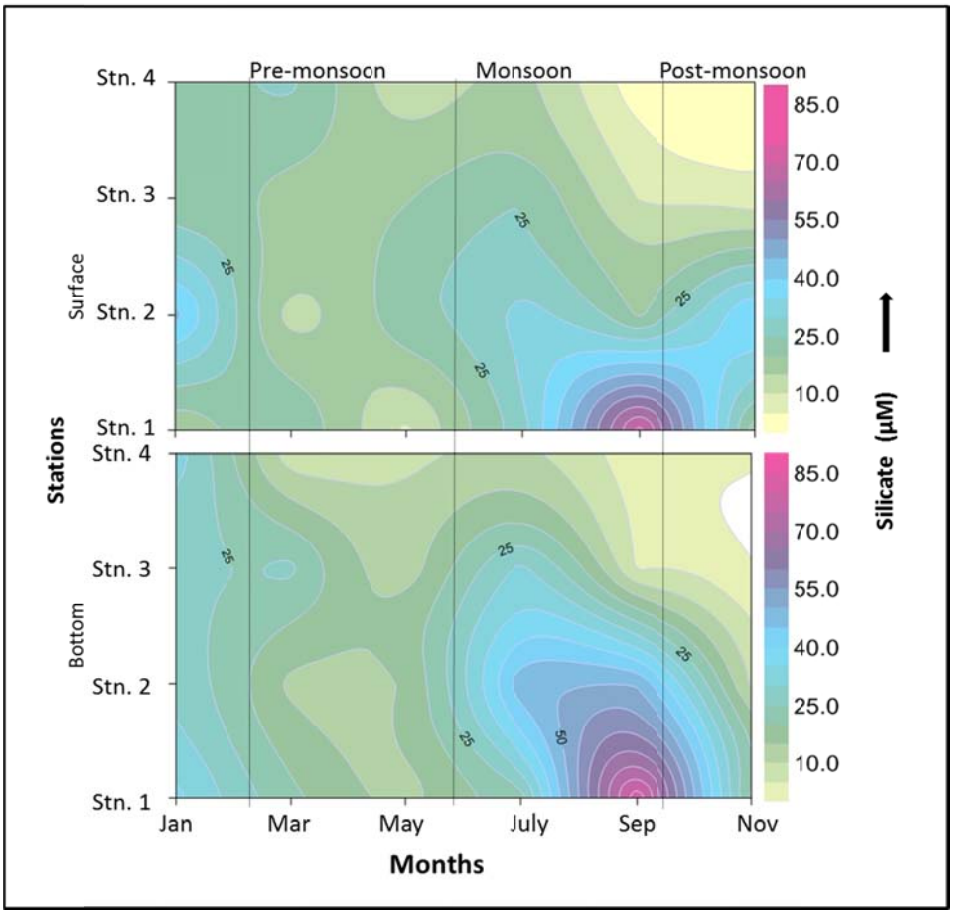


Figure 4.10 Silicate concentration in surface and bottom waters in the CE.

4.1.7 Statistical analysis

Significance in seasonal and spatial variations of environmental parameters and nutrients were estimated using three way ANOVA. The results showed that there was significant spatial and temporal variation for all the studied environmental parameters (Table 4.2). In the case of depth wise variation (between surface and bottom) salinity, DO, SPM, nitrate and silicate showed significant variation. Interaction effects of the variables were not significant except for SPM between stations and depth, and for nitrate and silicate between station and months.

Table 4.2 Three way ANOVA and their first order interaction effects between stations (A), depth-surface and bottom (B) and months (C)

	Between			Interaction effect		
	A	B	C	A & B	B & C	A & C
Saliniy	38.5**	16.6**	44.3**	5.3	4.2	6.9
Ammonia	5.1**	0.8	17.1**	2.1	0.8	1.9
Nitrite	4.5*	0.7	8.5**	0.5	1.5	2.7
Nitrate	53.4**	9.0**	20.7**	2.1	0.3	31.1**
Silicate	5.7**	66.6**	5.5**	0.3	1.0	4.8**
Phosphate	4.9*	1.2	14.5*	0.1	1.1	2.1
DO	5.9**	14.4**	15.8**	1.4	1.5	3.5
SPM	4.7*	14.5**	4.9**	3.2**	1.1	2.4

$– A (stations; df 3,15) B (surface and bottom samples; df 1,15) C (months; df 5,15)

*- Calculated F statistic is significant at 5% level of significance ($p<0.05$)

** - Calculated F statistic is significant at 1% level of significance ($p<0.01$)

4.2 Distribution of Microorganisms

4.2.1 Total count

Total prokaryotic abundance in the CE estimated as DAPI count and it ranged between 0.75×10^6 and 1.91×10^6, cells ml^{-1} (Figure 4.11). Estuarine stations showed one order increase in the abundance than the coastal station. Prokaryotic abundance in station 1 ranged from 1.12 to 1.52×10^6, cells ml^{-1}, in station 2 it ranged from 1.01 to 1.92×10^6, cells ml^{-1}, in station 3 it ranged from 1.11 to 1.67×10^6, cells ml^{-1} and in station 4 the counts ranged from 0.75 to 0.92×10^6, cells ml^{-1}. During the pre-monsoon season the counts were higher than other seasons. Surface-bottom variations were not observed in the DAPI count.

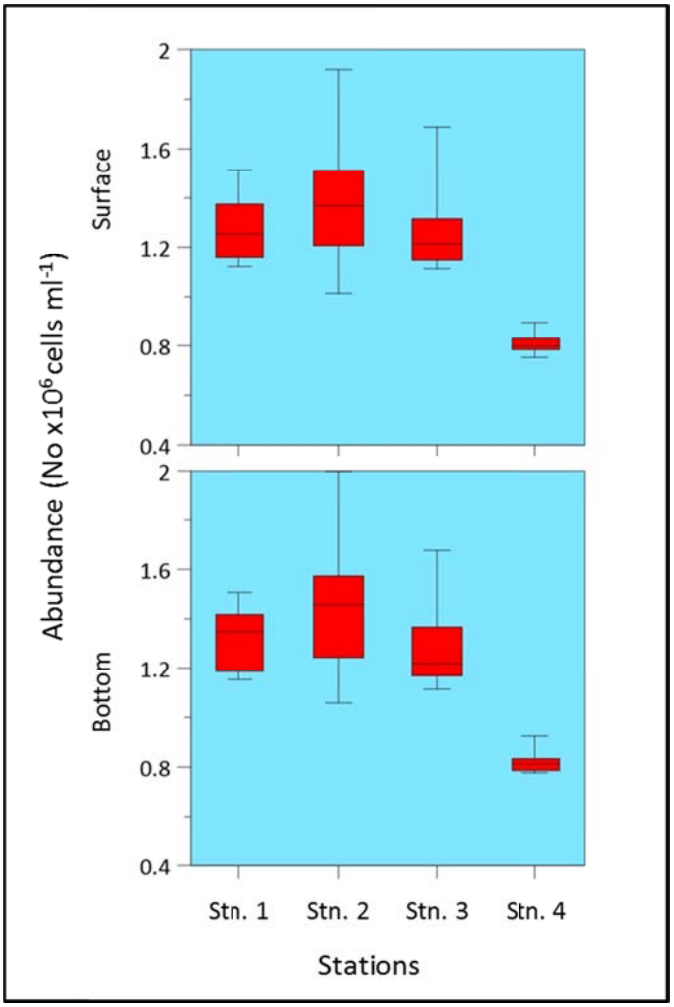
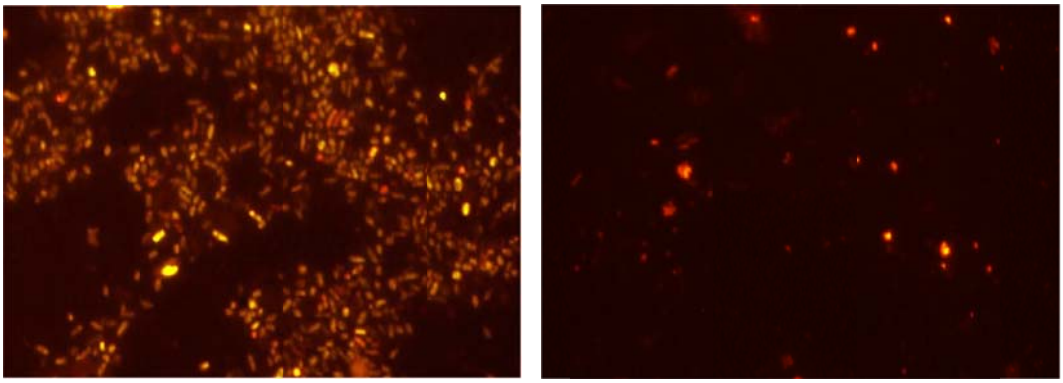


Figure 4.11 Box Wisker plot showing quartile deviation in distribution of total prokaryote counts.

4.2.2 Eubacteria

Representative images of FISH probe hybridized cells of Eubacteria and Archaea are shown in Figure 4.12. Spatio-temporal variations were observed in the distribution of Eubacteria in the CE and the abundance ranged from 3.3 to 6.9 $\times 10^5$ cells ml^{-1} (Figure 4.13). In low saline water, it ranged from 5.5 to 6.9 $\times 10^5$ cells ml^{-1}, in intermediate saline waters it ranged from 4.4 to 6.6 $\times$ 10^5 cells ml^{-1} and in high saline water it ranged from 3.3 to 4.5 $\times$ 10^5 cells ml^{-1}. Temporal variations in abundance were also visible for Eubacteria in the CE irrespective of the stations. The abundance ranged from 3.65 to 6.91 $\times 10^5$ cells ml^{-1} during pre-monsoon, 3.9 to 6.5 cells ml^{-1} during monsoon and 3.31 to 6.32 cells ml^{-1} during post-monsoon. Average surface count (5.53 $\times$ 10^5 cells ml^{-1}) and bottom count (5.41 $\times$ 10^5 cells ml^{-1}) did not show much variation.



Eubacteria **Archaea**

Figure 4.12 Representative FISH images of Eubacteria and Archaea.

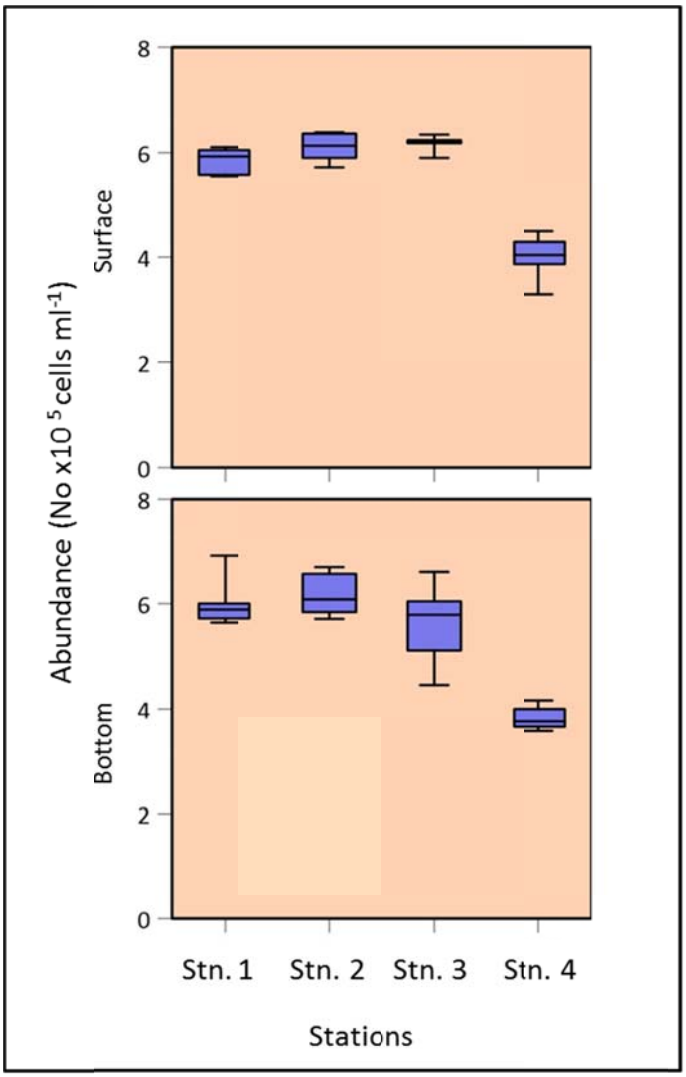


Figure 4.13 Box Wisker plot showing quartile deviation in distribution of Eubacteria.

4.2.3 Archaea

Archaeal abundance in the CE ranged from 1.9 to 5.48 × 10^5 cells ml^{-1} (Figure 4.14) and was always less than the bacterial abundance. Abundance of Archaea ranged from 3.2 to 4.4 × 10^5 cells ml^{-1} in low saline station, 2.3 to 5.4 × 10^5 cells ml^{-1} in intermediate saline stations and 1.9 to 2.5 × 10^5 cells ml^{-1} in high saline station. Higher abundance of Archaea was observed during pre-monsoon with highest abundance in month of May at intermediate saline station (5.4 × 10^5 cells ml^{-1}) and minimum during the monsoon month of September at high saline station. Similar to Eubacteria, archaeal abundance was always less in high saline coastal station during all seasons and did not show any depth related variation.

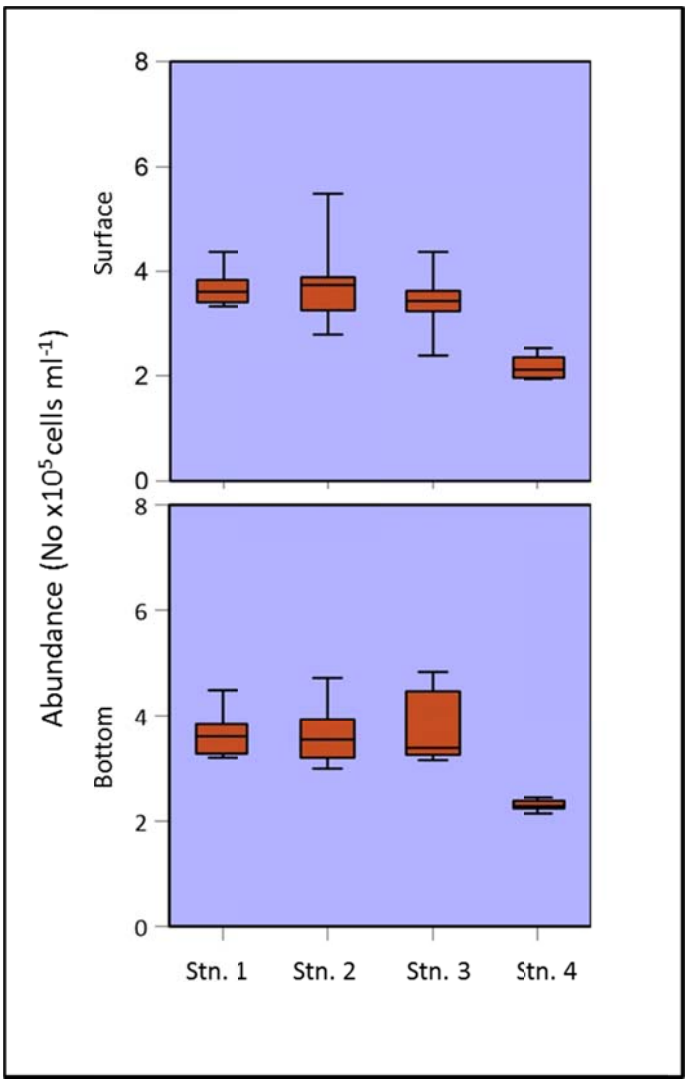


Figure 4.14 Box Wisker plot showing quartile deviation in distribution of Archaea.

4.2.4 AOB and NOB

Spatio-temporal variations were observed in the abundance of AOB (β-AOB and *N. mobilis*) and NOB (*Nitrospira* sp. and *Nitrobacter* sp.) in CE during the study period. Irrespective of the stations and seasons, nitrifiers abundance was in the order of 10^4. The abundance of β-AOB and *N. mobilis* ranged from 3.15 to 9.3 $\times 10^4$ and 1.01 to 4 $\times 10^4$ cells ml^{-1}, respectively. In NOB, *Nitrobacter* sp. and *Nitrospira* sp. abundance ranged from 2.69 to 7.63 $\times$ 10^4 and 2.51 to 6.17 $\times$ 10^4 cells ml^{-1}, respectively. Monsoon season showed relatively lower abundance of both AOB and NOB compared to other seasons irrespective of stations. Representative images of FISH probe hybridized cells of AOB and NOB are shown in Figure 4.15

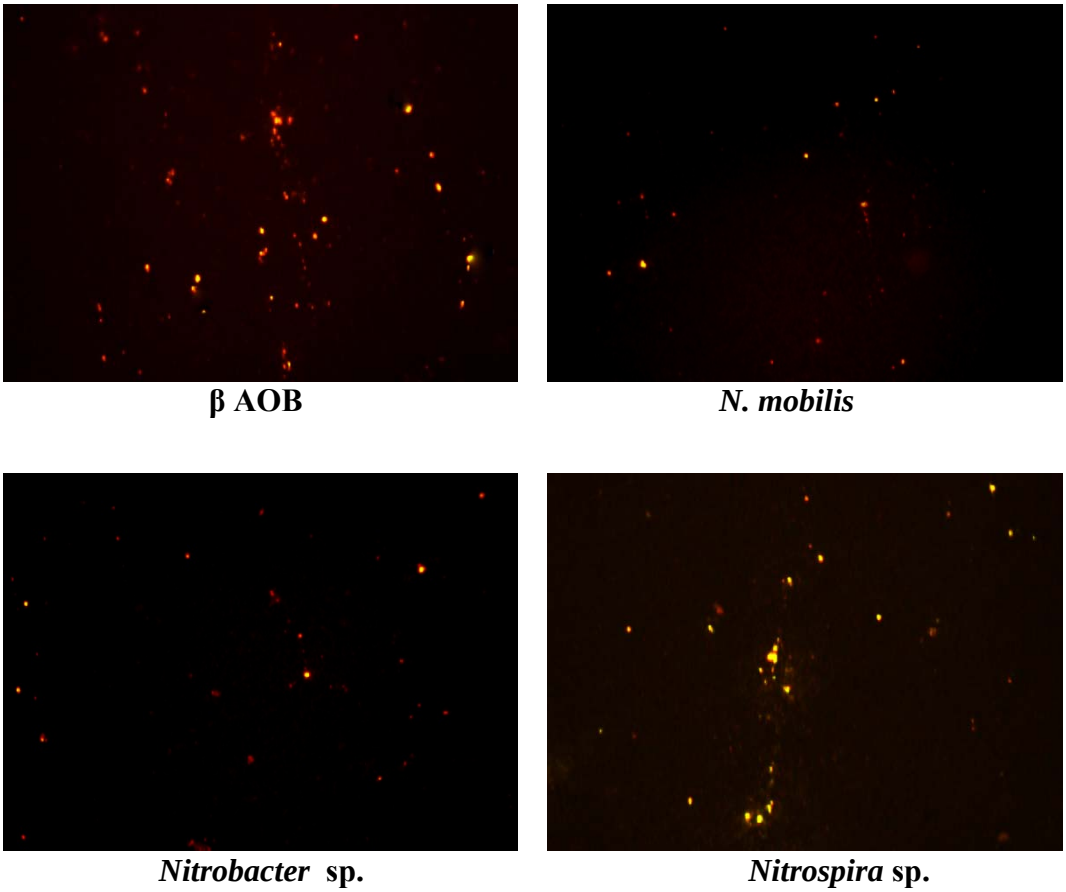


Figure 4.15 Representative FISH images of AOB and NOB.

Figure 4.16 shows the variation in abundance of nitrifiers in low saline station (Stn 1). In this station β AOB varied from 4.77 to 7.24 $\times$ 10^4 cells ml^{-1}, low

and high abundance were observed in the month of September and January respectively. Whereas *N. mobilis* varied from 1.02 to 4.01 × 10^4 cells ml^{-1}, low and high abundance were observed in the month of March and November. In the case of NOB, *Nitrobacte*r sp. and *Nitrospira* sp. varied from 2.84 to 5.63 × 10^4 cells ml^{-1} and 3.27 to 5.18 ×10^4 cells ml^{-1}, respectively. Low and high abundance for NOB were seen in July and May respectively. Temporal variation observed in the abundances of AOB and NOB in station 1 is shown in Table 4.3.

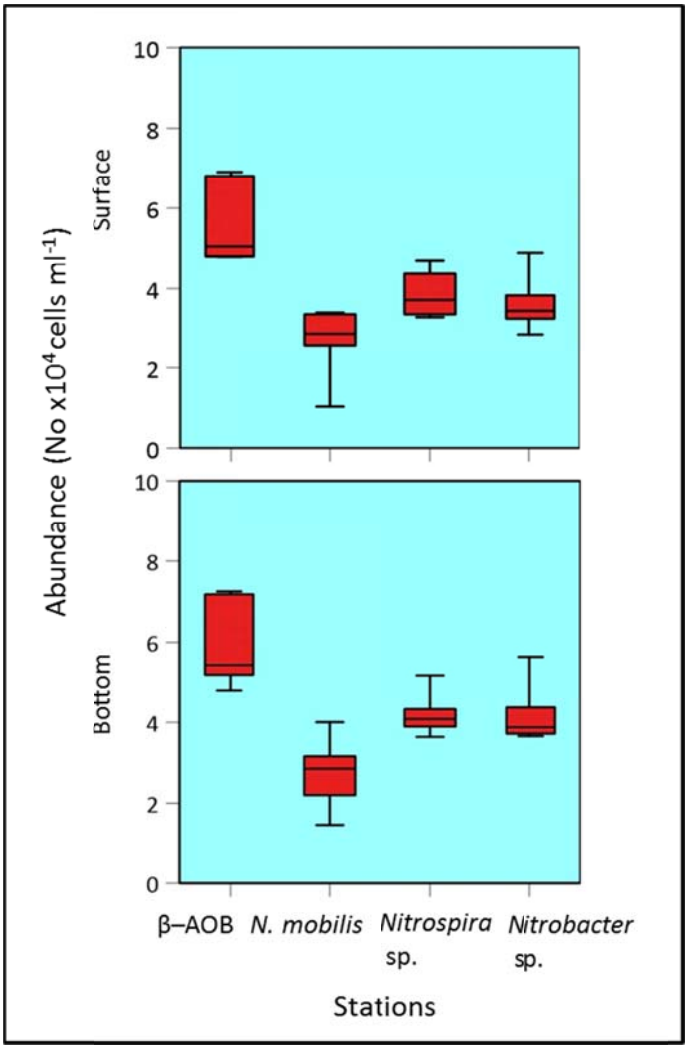


Figure 4.16 Box Wisker plot showing quartile deviation in the distribution of AOB and NOB in the water column of low saline station (Stn. 1).

Table 4.3 Seasonal variation of AOB and NOB (cells ml^{-1}) at Station 1.

Organism		Pre-monsoon	Monsoon	Post-monsoon
AOB	β-AOB	4.78 - 7.17×10^4	4.77 - 5.01×10^4	5.05 - 7.24×10^4
	N. mobilis	1.02 - 3.35 ×10^4	2.63 - 3.09×10^4	2.21 - 4.01×10^4
NOB	*Nitrospira* sp.	4.33 - 5.18×10^4	3.59 - 4.37 ×10^4	3.27 - 4.68×10^4
	Nitrobacter sp.	3.24 - 5.63×10^4	3.26 - 3.99×10^4	2.83 - 3.37×10^4

In intermediate saline station 2, β AOB varied from 4.51 to 9.34 ×10^4 cells ml^{-1} N. *mobilis* varied from 1.04 to 4.01 × 10^4 cells ml^{-1}. For NOB, *Nitrobacter* sp. and *Nitrospira* sp. varied from 3.24 to 7.63 × 10^4 cells ml^{-1} and 3.49 to 6.17 × 10^4 cells ml^{-1}, respectively (Figure 4.17). High abundance of nitrifiers was observed in the month of May, except for *N. mobilis* which showed high abundance during November. Stations 1 and 2, where the dissolved ammonia concentration was comparatively higher, showed higher abundance of all the nitrifiers throughout the year. Seasonal variation of nitrifiers at station 2 is shown in Table 4.4.

Table 4.4 Seasonal variation of AOB and NOB (cells ml^{-1}) at station 2.

Organism		Pre-monsoon	Monsoon	Post-monsoon
AOB	β-AOB	4.51- 9.34×10^4	5.13 - 538×10^4	5.35 - 6.16×10^4
	N. mobilis	1.04 - 3.35×10^4	2.81 - 3.01×10^4	1.37 - 4.01×10^4
NOB	*Nitrospira* sp.	4.13 - 6.17×10^4	3.49 - 5.58×10^4	3.50 - 519×10^4
	Nitrobacter sp.	3.61 - 7.63×10^4	3.63 - 4.96×10^4	3.24 - 605×10^4

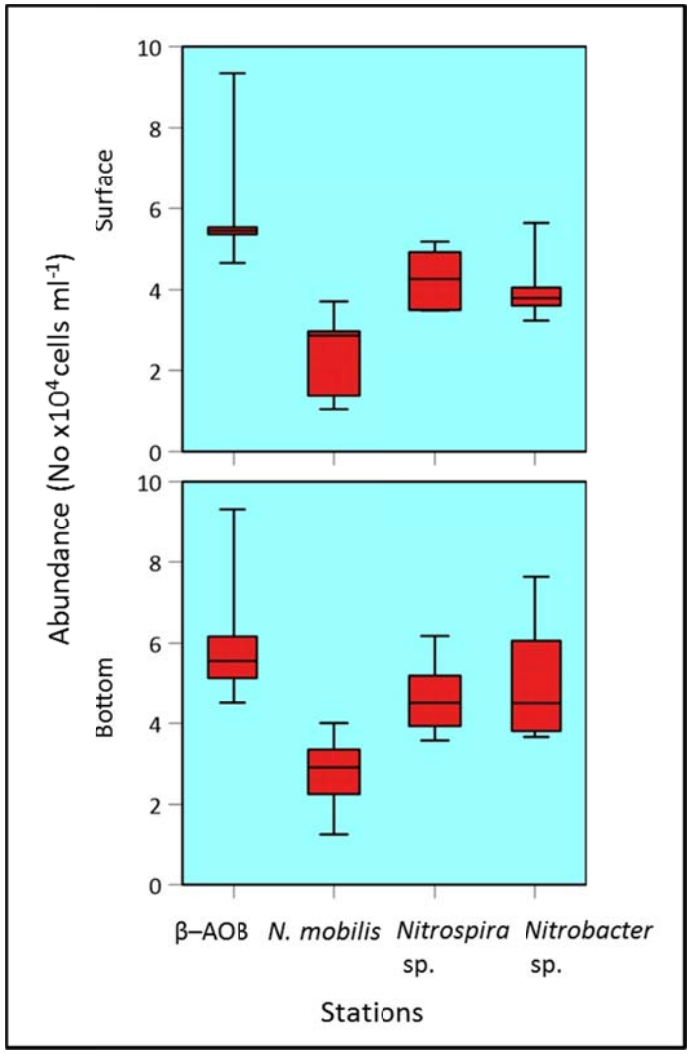


Figure 4.17 Box Wisker plot showing quartile deviation in the distribution of AOB and NOB in the water column of intermediate saline station (Stn. 2).

At station 3, β AOB varied from 3.78 to 8.22 × 10^4 cells ml^{-1}, the low and high abundance were observed in the month of March and May, respectively (Figure 4.18). *N. mobilis* varied from 1.17 to 3.71 × 10^4 cells ml^{-1} during the month of March and November. Among NOB, *Nitrobacter* sp. and *Nitrospira* sp. varied from 2.73 to 5.59 × 10^4 cells ml^{-1} and 2.91 to 5.31 × 10^4 cells ml^{-1}, respecively. *Nitrobacte*r sp. and *Nitrospira* sp. showed higher abundance during May and lower abundance during July and September (Table 4.5).

Table 4.5 Seasonal variation of AOB and NOB (cells ml^{-1}) at Station 3.

Organism		Pre-monsoon	Monsoon	Post-monsoon
AOB	β-AOB	3.78 - 8.22×10^4	3.79 - 5.61 ×10^4	5.42 - 7.38×10^4
	N. mobilis	1.17 - 3.01×10^4	1.41 - 2.79×10^4	2.77 - 3.71×10^4
NOB	*Nitrospira* sp.	3.18 - 5.30×10^4	3.02 - 3.94×10^4	2.91 - 4.71×10^4
	Nitrobacter sp.	3.19 - 5.59×10^4	2.73 - 3.50×10^4	2.75 - 4.26×10^4

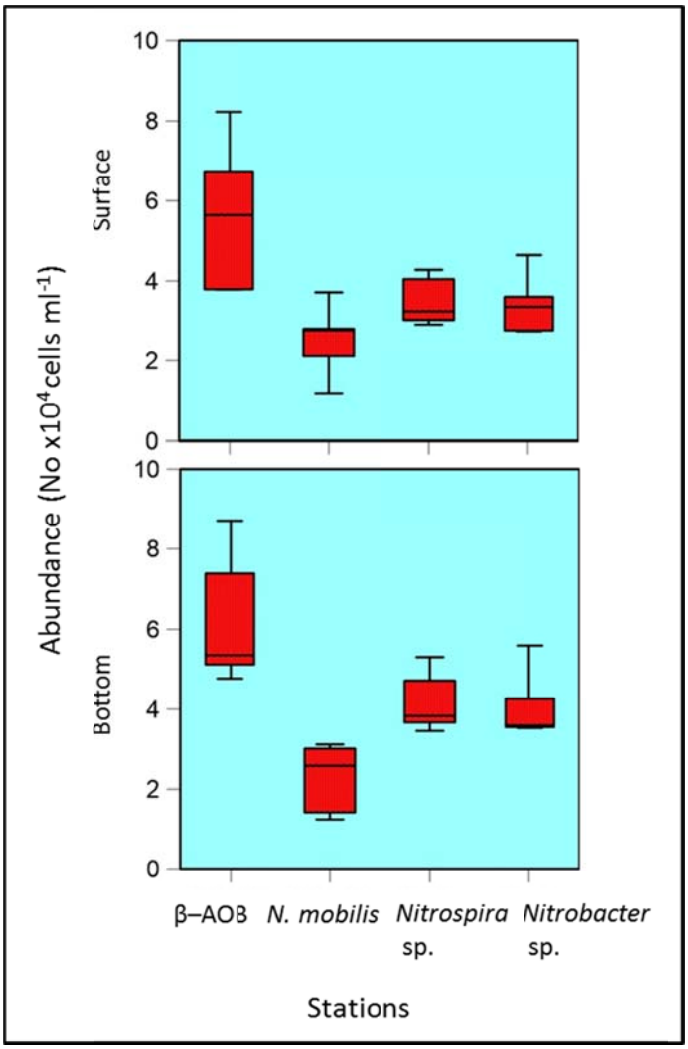


Figure 4.18 Box Wisker plot showing quartile deviation in the distribution of AOB and NOB in the water column of intermediate saline station (Stn. 3).

High saline coastal station (Stn. 4) showed relatively less abundance for all the nitrifiers (Figure 4.19). β AOB varied from 3.15 to 4.93 × 10^4 cells ml^{-1}, low and high abundance in the month of September and May, respectively. *N. mobilis* varied from 1.01 to 2.47 × 10^4 cells ml^{-1}, low and high abundance observed during March and November, respectively. Among NOB, *Nitrobacter* sp. varied from 2.69 to 3.72 × 10^4 cells ml^{-1} in March and May. *Nitrospira* sp. varied from 2.51 to 3.63 × 10^4 cells ml^{-1} in May and July, respectively (Table 4.6).

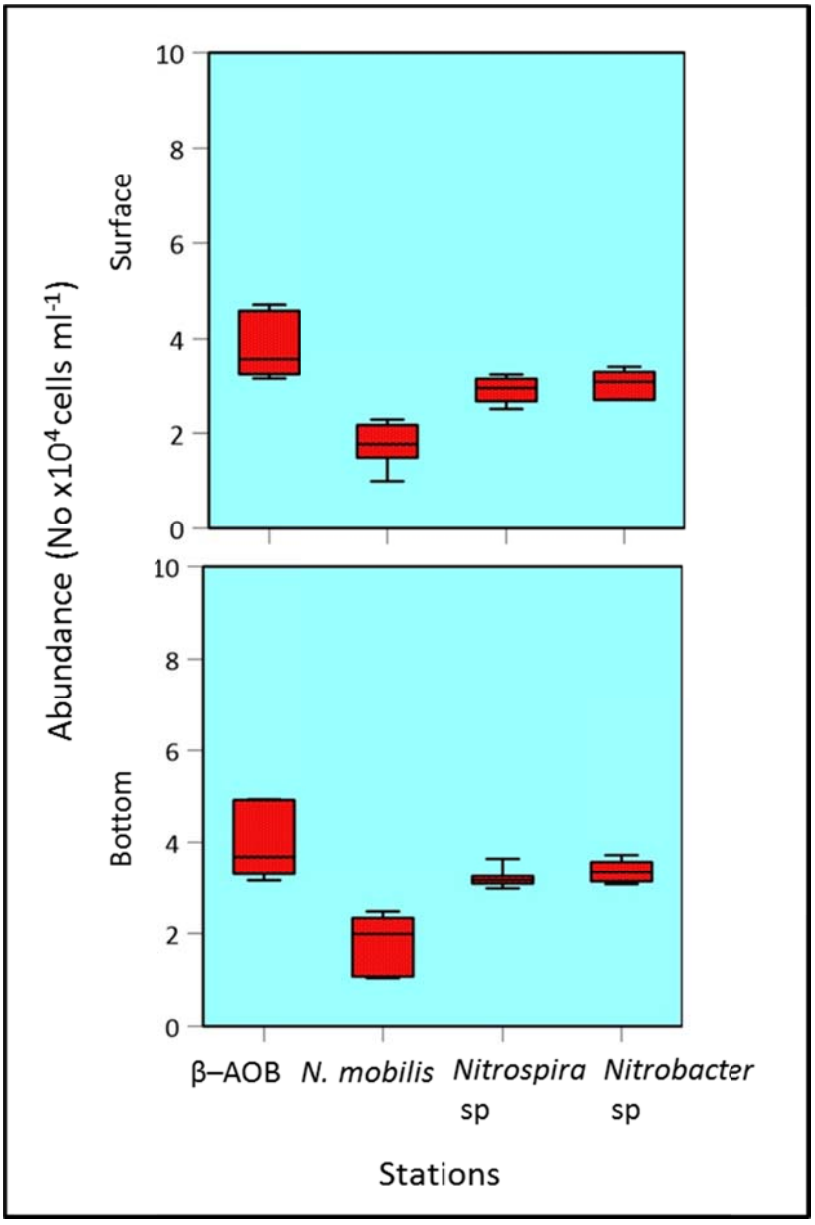


Figure 4.19 Box Wisker plot showing quartile deviation in the distribution of AOB and NOB in the water column of high saline station (Stn. 4).

Table 4.6 Seasonal variation of AOB and NOB (cells ml^{-1}) at Station 4.

Organism		Pre-monsoon	Monsoon	Post-monsoon
AOB	β-AOB	3.15 - 4.92×10^4	3.15 - 4.04×10^4	3.24 - 4.93×10^4
	N. mobilis	1.01 - 2.28×10^4	1.07 - 1.91×10^4	1.81 - 2.47×10^4
NOB	*Nitrospira* sp.	2.91 - 3.63×10^4	2.51 - 3.24×10^4	2.97 - 3.23×10^4
	Nitrobacter sp.	2.70 - 3.72×10^4	2.69 - 3.56×10^4	2.87 - 3.36×10^4

4.2.5 Statistical analysis

4.2.5.1 Spatio-temporal variations of parameters

The abundance of AOB and NOB showed heterogeneity in distribution among the sampling sites of the CE. Three way ANOVA showed significant spatial and temporal variation at 1% level for Eubacteria, *N. mobilis*, *Nitrobacter* sp. and *Nitrospira* sp. while the spatial variation of β-AOB showed significance at 5% level (Table 4.7). Abundance of Archaea showed temporal variation while it did not show spatial variation. Significant difference in abundance in surface and bottom waters was observed for *Nitrospira* sp. and *Nitrobacter* sp. ($F = 40.4$, $df = 1,15$, $p < 0.01$ and $F = 49.1$, $df = 1,15$, $p < 0.01$, respectively). Abundance of AOB and NOB was recorded higher during pre-monsoon and lower during monsoon, indicating seasonal variation ($p < 0.01$). Interaction effects of the variables were significant between stations and months for Eubacteria, β AOB and *Nitrospira* sp.

Table 4. 7 Three way ANOVA and their first order interaction effects between stations, depths and months.

	Between			Interaction effect		
	A	B	C	A & B	B & C	A & C
Eubacteria	24.3**	1.0	25.3**	2.4	0.6	6.1**
Archaea	11.7**	1.3	2.1	1.7	0.3	0.9
β- AOB	51.2**	8.5	125.6**	1.0	0.5	10.1**
N. mobilis	4.4*	0.1	11,01**	0.5	0.8	1.6
***Nitrobacter* sp.**	13.4**	40.4**	34.5**	1.9	0.8	2.9
***Nitrospira* sp.**	25.3**	49.9**	49.1**	1.9	3.7	3.7**

$– A (stations; df 3, 15) B (surface and bottom samples; df 1, 15) C (months; df 5, 15)
*- Calculated F statistic is significant at 5% level of significance p <0.05
** - Calculated F statistic is significant at 1% level of significance p <0.01

4.2.5.2 Influence of environmental variables on the distribution of microorganisms

Environmental factors which have significant correlation on the distribution of microorganisms were determined using Karl Pearson correlation. Eubacteria showed significant positive correlation ($r = 0.40$, $p<0.01$, $n = 48$) with nitrate concentration and negative correlation with salinity (Table 4.8). Other environmental factors did not show any significant correlation with abundance of Eubacteria. Archaea showed significant positive correlation with ammonia and nitrate ($r = 0.31$ and 0.43 $p < 0.01$, $n = 48$). Similar to Eubacteria, Archaea also showed negative correlation with salinity ($r = -0.49$ $p <0.01$ $n = 48$). Both AOB and NOB showed significant positive correlation with dissolved ammonia ($r < 0.28$, $p< 0.01$, $n = 48$). β-AOB showed significant correlation with nitrate also ($r = 0.39$, $p< 0.01$, $n = 48$). *N. mobilis*, *Nitrospira* sp. and *Nitrobacter* sp. showed significant negative correlation with DO ($r = <0.28$, $p< 0.01$, $n = 48$) while β AOB did not show such correlation. AOB and NOB distribution did not show any correlation with other nutrients, salinity, pH and SPM. The scatter plot in the PCA helped to visualize all the data points plotted in the coordinate system given by the two most

important components. Figure 4.20 is the results of PCA analysis showing the influence of environmental parameters in the distribution of Eubacteria and Archaea. PCA analysis also showed that ammonia was the major factor regulating the distribution of AOB and NOB (Figure 4.21).

Table 4.8 Inter relationship between environmental parameters and microbial group.(** p<0.01; * p<0.05)

	Eubacteria	Archaea	β-AOB	*N. mobilis*	*Nitrospira* sp.	*Nitrobacter* sp.
Ammonia	0.12	0.31**	0.63**	0.28**	0.33**	0.42**
Nitrite	0.20	0.09	0.1	0.13	-0.02	-0.03
Nitrate	0.40**	0.43**	0.39**	0.23	0.18	0.21
Salinity	-0.49**	-0.3**	-0.19	-0.23	-0.3**	-0.13
DO	-0.06	-0.1	-0.03	-0.28**	-0.3**	-0.3**
SPM	-0.23	-0.2	0.15	-0.02	0.01	0.04
Silicate	0.26	0.11	0.13	0.16	0.05	-0.03
Phosphate	0.12	-0.1	-0.18	-0.01	-0.2	-0.2

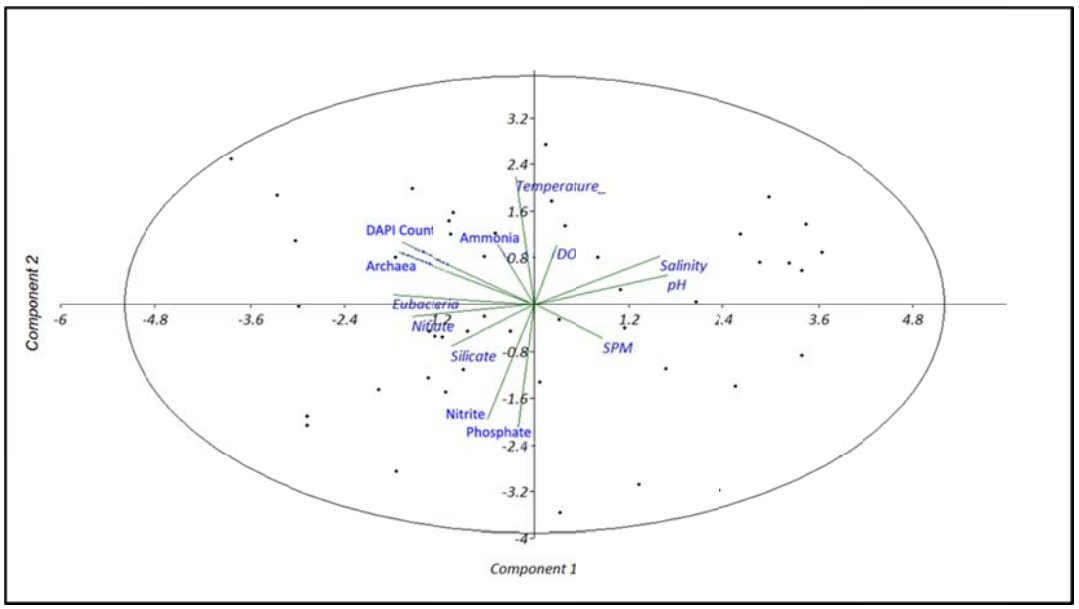


Figure 4.20 PCA analysis showing the influence of environmental parameters on the distribution of total prokaryotes, Eubacteria and Archaea. Sampling locations are displayed by points and the biotic and abiotic variables are shown by arrows. The direction of arrows dictates the direction of increase of that particular variable on spatially assembled sampling locations.

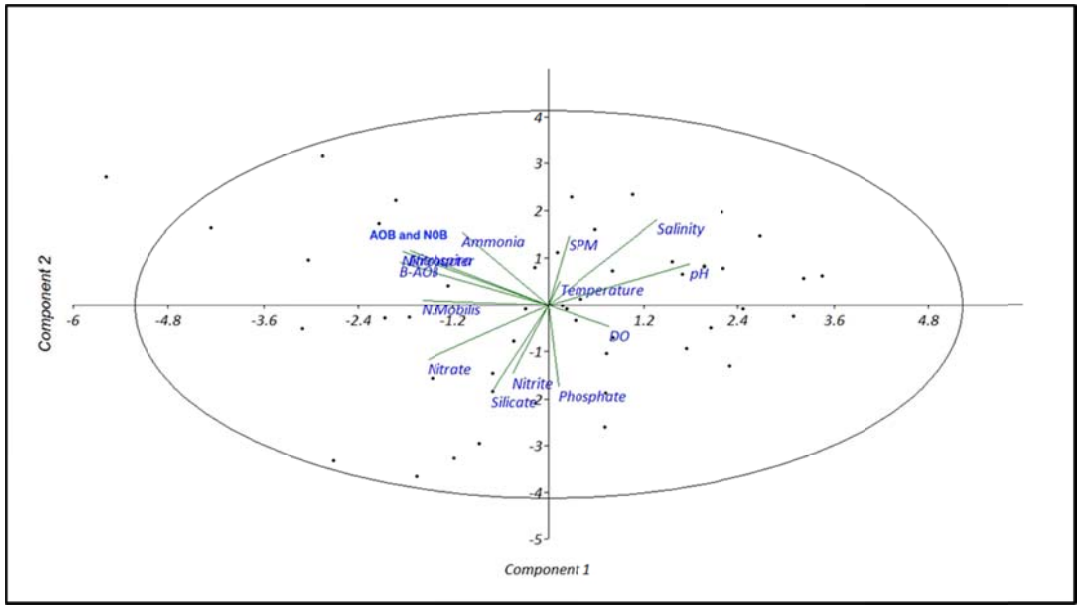


Figure 4.21 PCA analysis showing the influence of environmental parameters on the distribution of AOB and NOB. Sampling locations are displayed by points and the biotic and abiotic variables are shown by arrows. The direction of arrows dictates the direction of increase of that particular variable on spatially assembled sampling locations.

Canonical discriminate analysis (CDA) analysis was carried out to delineate the factors which contribute significantly to discriminate between stations, discarding the difference observed between surface and bottom and difference between seasons. It was observed that all the biological parameters together with all the environmental parameters contribute significantly to discriminate stations (Figure 4.22). Based on CDA, two regions were uniquely demarcated as coastal (Station 4) and estuarine (stations 1-3) (Wilks lamda = 0.00958., calculated $\chi 2$ = 173.55) with 48 degrees of freedom. The two eigen values, λ_1 (13.115) and λ_2 (2.871) together explain 94.1% of the variation in the distribution of the various biological and environmental parameters. CDA could classify about 89.6% of the grouped cases and 83.3% of the cross validated grouped cases correctly.

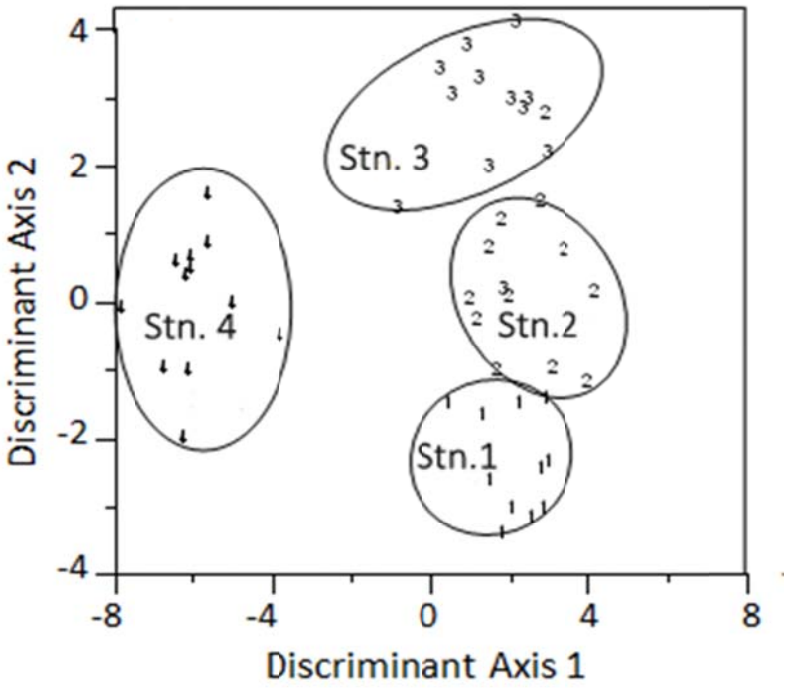


Figure 4.22 Discrimination between stations based on CDA with environmental parameters and nitrifiers abundance.

Step up multiple regression model (SMRM) with first order interaction effects was applied to determine the significantly contributing biological and environmental parameters to the numerical abundance of Archaea, Eubacteria, AOB and NOB. According to this model, in the Cochin estuarine region, the corresponding influencing factors are nitrate, nitrite, ammonia, SPM, silicate and phosphate (72.6% variability explained, VE) for Eubacteria; nitrate, nitrite, salinity, DO, and silicate (83.5% VE) for Archaea; nitrate, ammonia, salinity, and SPM (90.4%VE) for β-AOB; Nitrate, ammonia, salinity, DO, and phosphate (65.9% VE) for *N. mobilis*; ammonia, DO, SPM and phosphate (73.5% VE) for *Nitrospira* sp.; and nitrate, nitrite, ammonia, pH, salinity and SPM (86.3% VE) for *Nitrobacter* sp. In the coastal region the distribution of Eubacteria was controlled by salinity, DO, SPM and silicate (with 94.2 % as (VE) while Archaea was controlled by salinity, and SPM (with 86.3 % VE). Among AOB in the coastal region, β-AOB was controlled by salinity, SPM, and silicate (96.44 % VE), while *N. mobilis* by DO, silicate and PO_4 (96.6% VE). While among NOB in the coastal region, distribution of *Nitrospira* sp. varied depending on nitrite, DO, and SPM which explained 95.5% of the seasonal variation in its abundance and *Nitrobacter* sp. was influenced by

ammonia, DO, and silicate which could extract a considerable amount of variation (91% VE).

4.3 Community Structure of AOA and AOB

4.3.1 DGGE banding pattern of AOB

Spatial and temporal changes in the DGGE banding pattern and Bray-curtis similarity cluster for AOB from the sampling stations are shown in Figures 4. 23 – 4.26. The number of bands were extracted by image analysis which resulted in a binary matrix (presence or absence of bands in different samples). Four to 10 bands/OTU were visible in the DGGE gel image of AOB. The banding pattern did not show any significant temporal variation. The banding pattern of AOB at station 1 showed 4 to 7 bands (Figure 4.23) without any significant temporal variations as Bray-curtis similarity index did not separate them in to clusters. Surface and bottom variation in OTU were also absent for AOB. From the total 77 bands observed with in the gel, 12 bands were unique that represented the number of possible major phylotypes in that station. Unique bands, which excised for sequencing, are labeled in the figure.

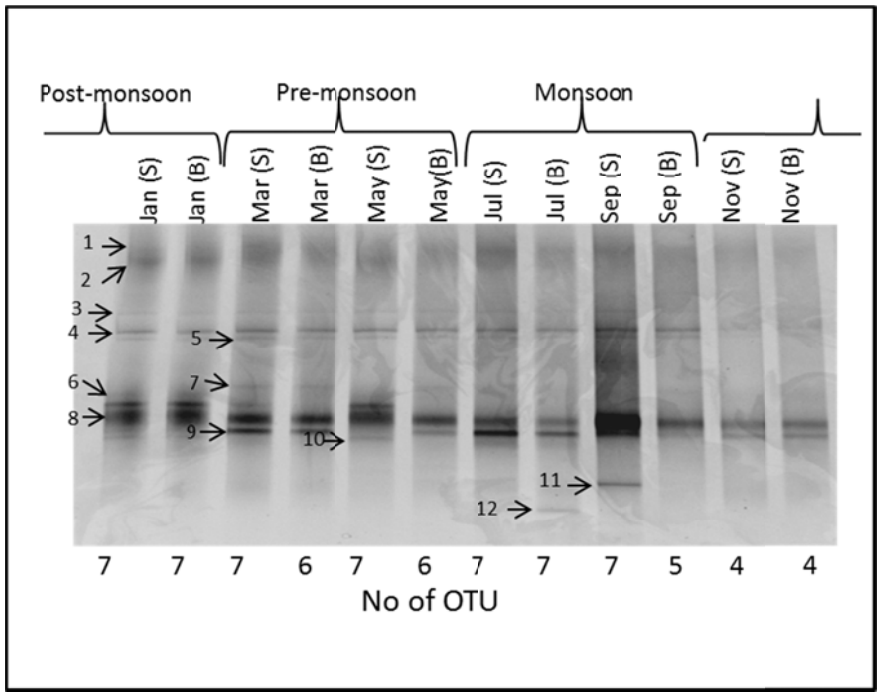


Figure 4.23 DGGE profile showing temporal variation in the banding pattern of AOB at station 1. Arrows represent the excised bands.

In station 2 AOB showed 5 to 7 bands per lane without any significant temporal variations (Figure 4.24) and similar to station 1, no discreet cluster was formed based on Bray-curtis similarity index. Total 69 bands were observed in the gel, in which 7 bands were unique and represent the possible number of major phylotypes in that station. Unique bands were indicated by numbers in the figure.

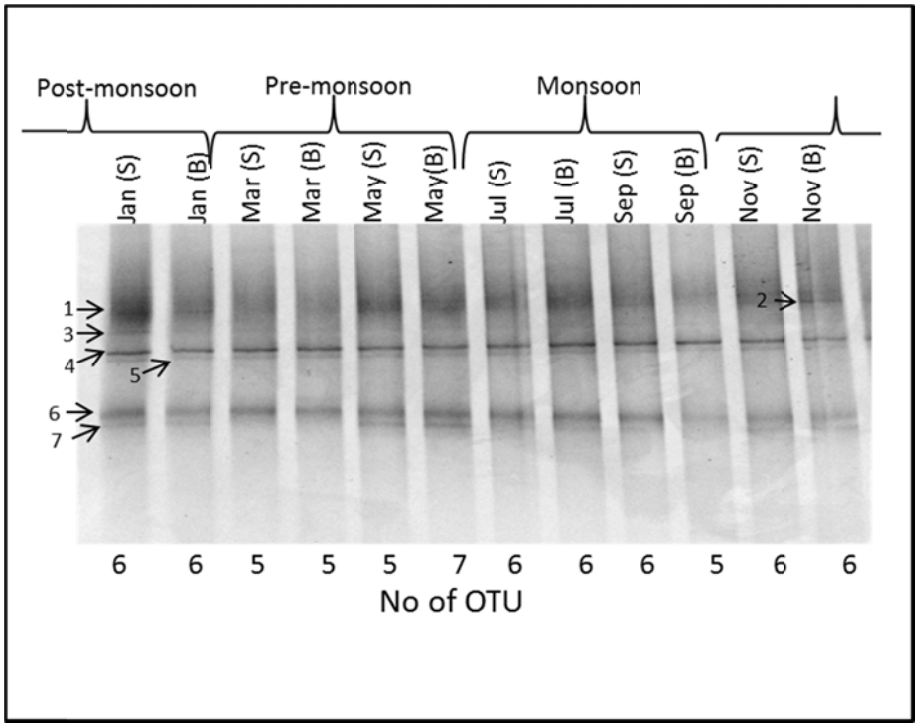


Figure 4.24 DGGE profile showing temporal variation in the banding pattern of AOB at station 2. The excised bands are indicated by numbers in the DGGE gel.

In station 3 DGGE gel of AOB showed 4 to 8 OTU per lane (Figure 4.25). Seasonal and surface-bottom variations were not significant. Though Bray-curtis similarity index grouped the bands in to different clusters, they were not significant as the similarity was close to 85%. In this station total 69 bands were observed, in that 7 unique band classes representing the possible phylotypes were present.

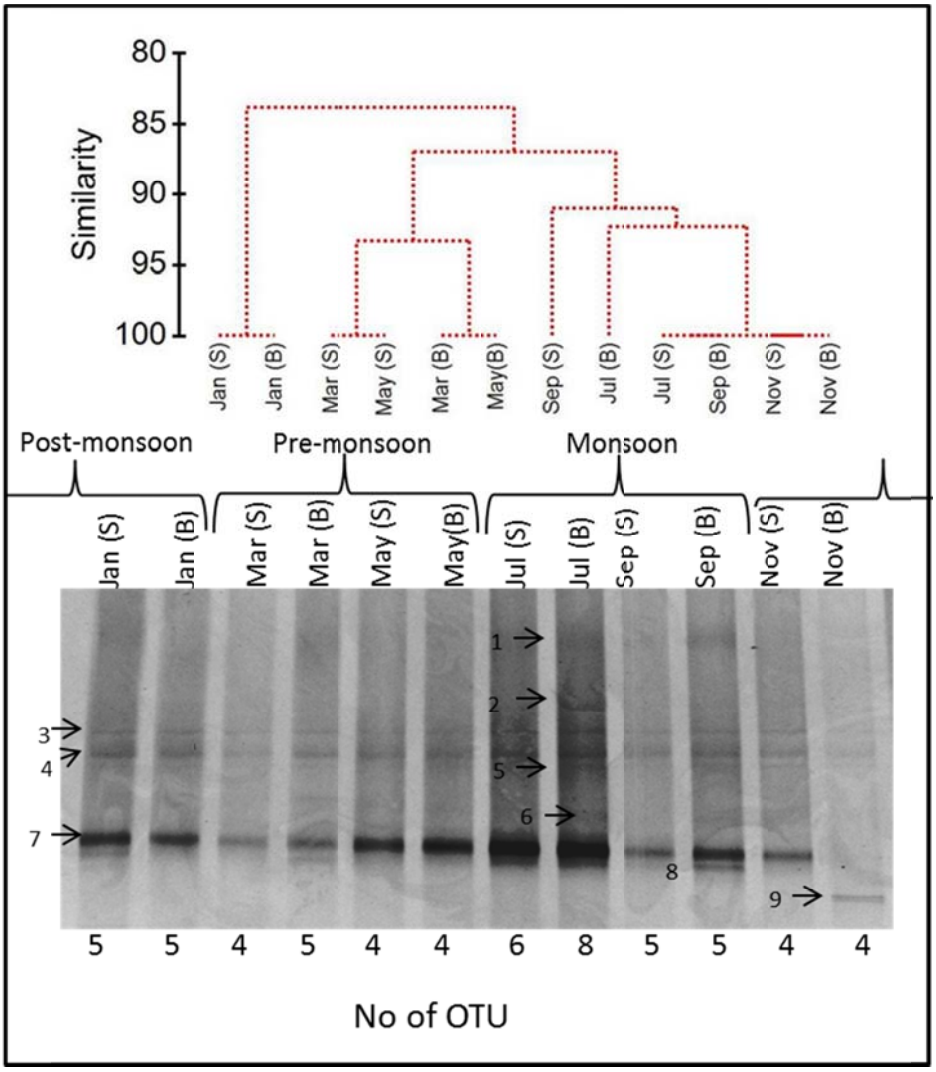


Figure 4.25 DGGE profile and cluster analysis showing temporal variation in the banding pattern of AOB at station 3. The excised bands are indicated by numbers in the DGGE gel.

The banding pattern of AOB from station 4 is shown in Figure 4.26. The number of band per lane varied from 4 to 10. Bottom waters recorded high number of bands in most of the months and the maximum number of bands was in the month of July. The Bray-curtis similarity index grouped the bands in to different clusters, but these were not significant as the similarity was close to 75%. From the total of 62 bands observed 10 phylotypes could be differentiated.

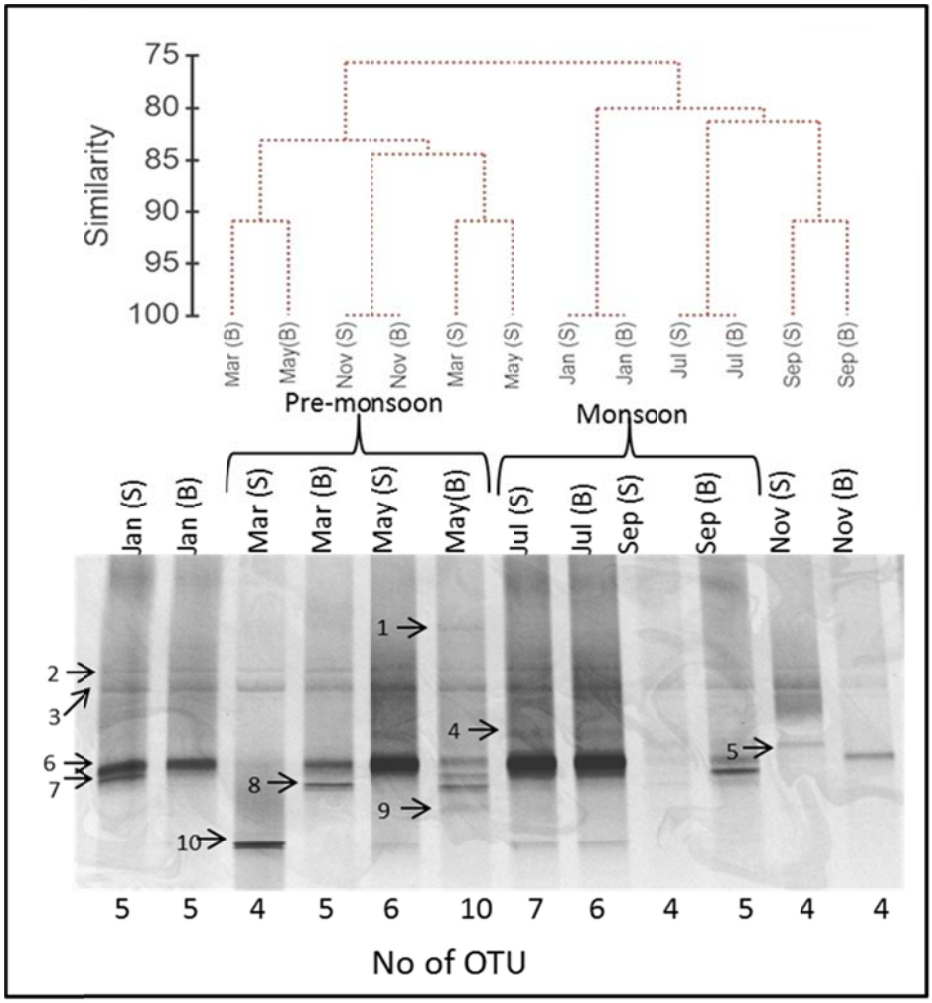


Figure 4.26 DGGE profile and cluster analysis showing temporal variation in the banding pattern of AOB at station 4. The excised bands are indicated by numbers in the DGGE gel.

4.3.2 DGGE banding pattern of AOA

Spatial and temporal changes in the DGGE banding pattern and Bray-curtis similarity cluster for AOA are shown in Figures 4.27 to 4.30. The number of bands varied from 7 to 26 with the highest number of bands during the monsoon months. Common bands were detected in all the stations indicating the presence of wide spread phylotypes at these stations. The DGGE banding pattern of AOA at station 1 showed 13 to 26 bands with higher number of bands in the bottom water. As seen in Figure 4.27, two separate clusters were formed with Bray-curtis similarity index up to 59%. Although monsoon and non-monsoon samples formed separate clusters, there was overlapping of seasons. Bottom waters of May showed

maximum number of OTU (pre-monsoon) and minimum numbers in January (post-monsoon).

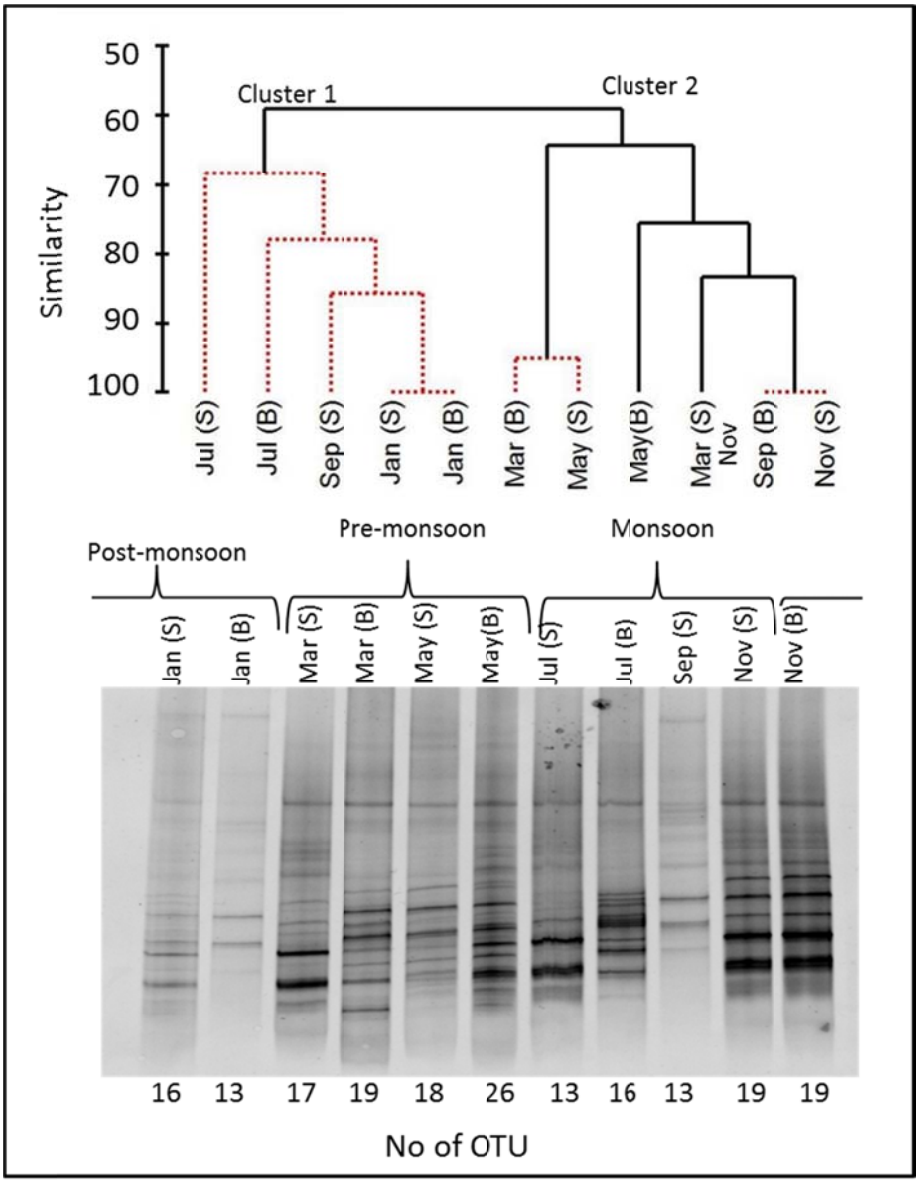


Figure 4.27 DGGE profile and cluster analysis showing temporal variation in the banding pattern of AOA at station 1.

DGGE banding pattern at station 2 showed 13 to 19 bands, similar to station 1 (Figure 4.28). Bottom samples showed higher number of OTU than surface samples. The community structure of AOA also showed seasonal fluctuations, but the Bray-curtis similarity index reached up to 79%. Two clusters were visible, monsoon cluster and non-monsoon cluster.

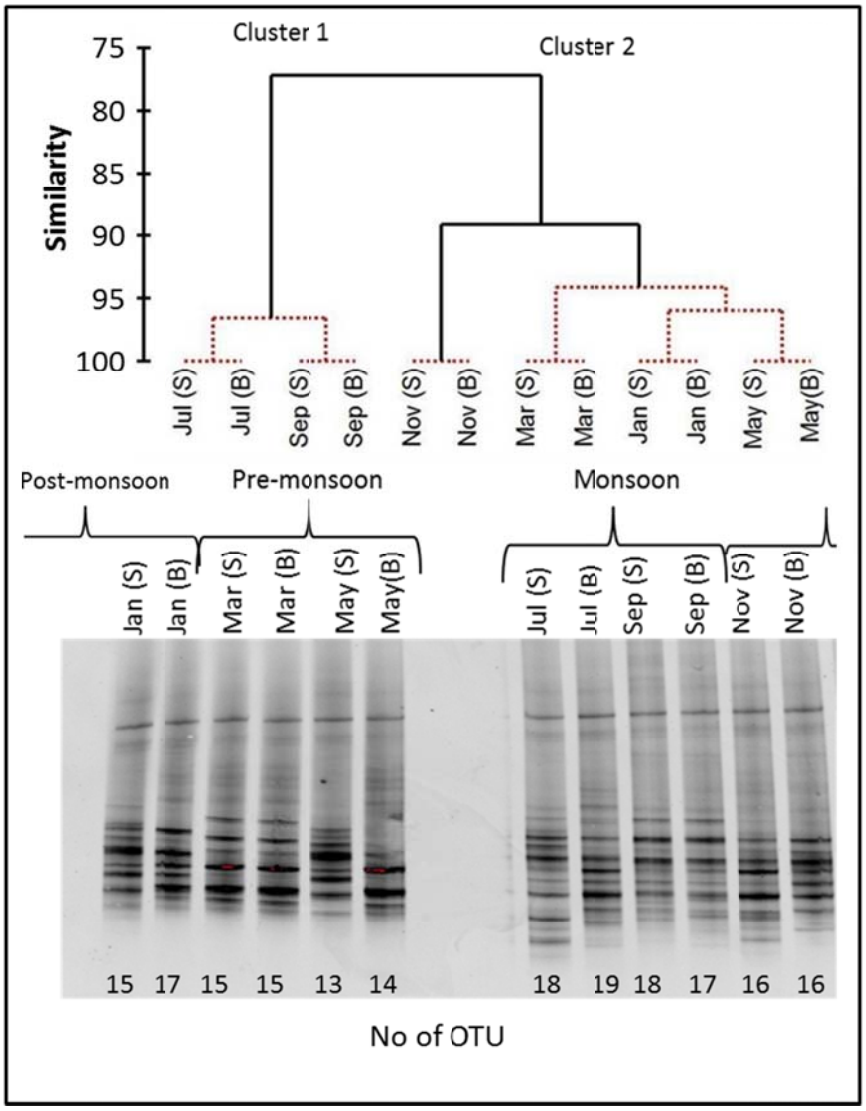


Figure 4.28 DGGE profile and cluster analysis showing temporal variation in the banding pattern of AOA at station 2.

DGGE banding pattern of AOA at station 3 showed 7 to 11 bands (Figure 4.29). Surface and bottom samples did not show much variation in the OTU. The community structure of AOA showed minimum seasonal fluctuations with Bray-curtis similarity index up to 85%. Two clusters were formed; unlike stations 1 and 2, monsoon and non-monsoon separation was not visible at this station.

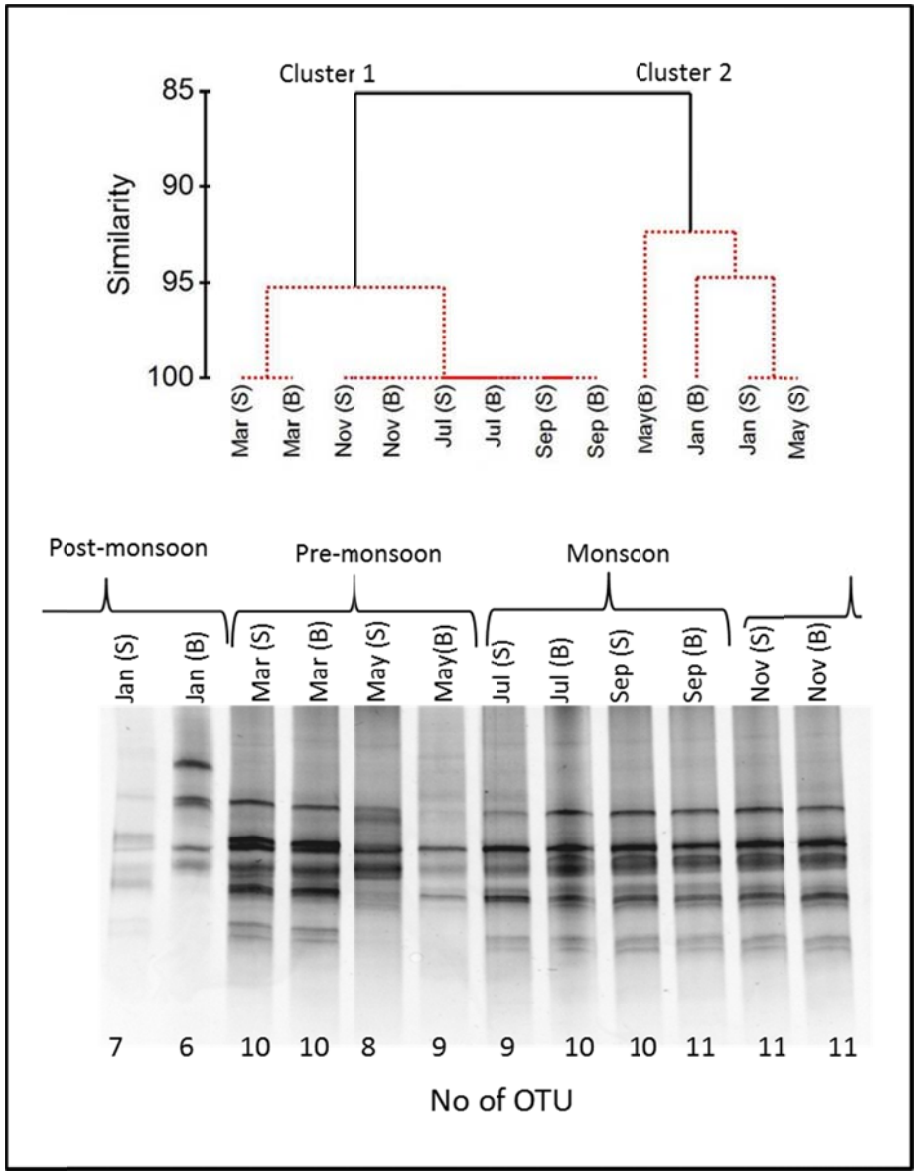


Figure 4.29 DGGE profile and cluster analysis showing temporal variation in the banding pattern of AOA at station 3.

DGGE banding pattern of AOA at station 4 showed 12 to 21 bands with higher number of bands from bottom samples (Figure 4.30). Maximum numbers of OTU were observed in the DGGE gels from the high saline coastal station (Stn. 4). The community structure did not show any significant seasonal fluctuations at station 4. Bray-curtis similarity index reached up to 92%. Total number of OTU observed for AOB and AOA from all the stations are combined and given in the Table 4.9.

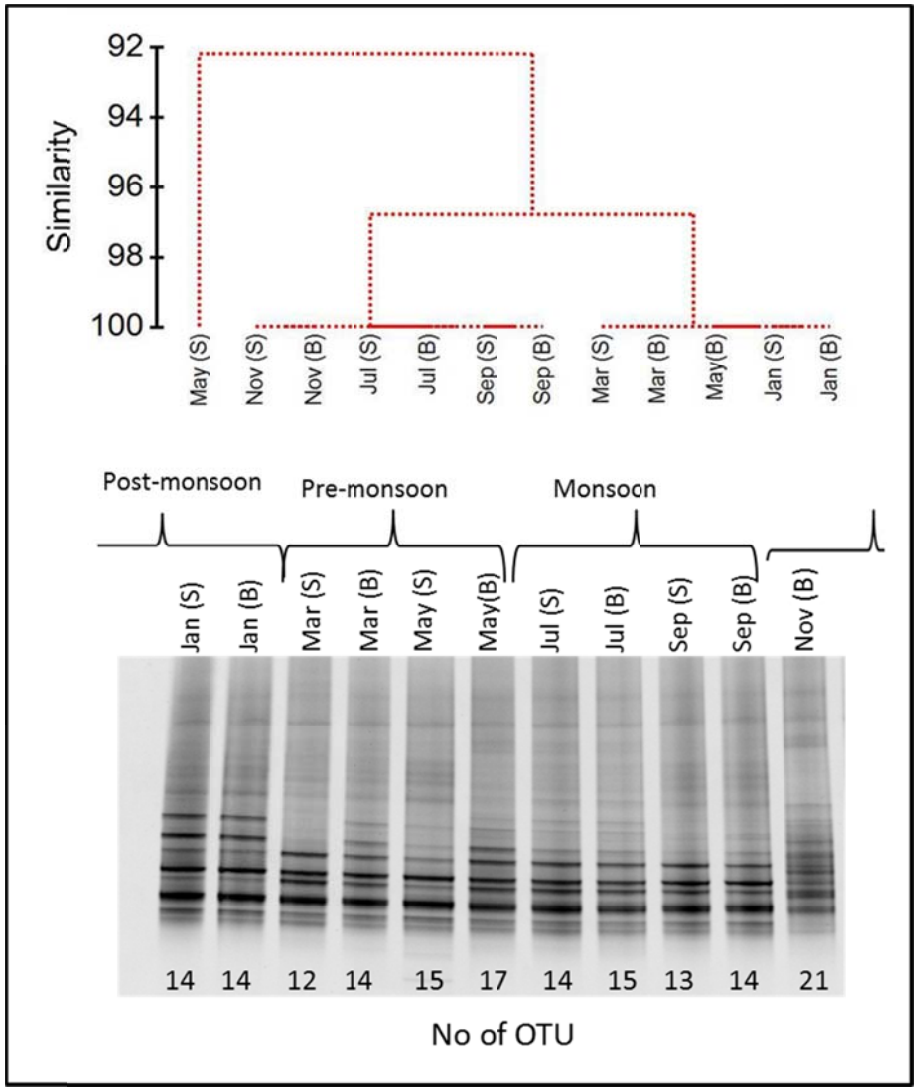


Figure 4.30 DGGE profile and cluster analysis showing temporal variation in the banding pattern of AOA at station 4.

Table 4.9 Number of OTU observed for AOB and AOA in the CE.

	OTU for AOB	OTU for AOA
Station 1	12	27
Station 2	7	24
Station 3	9	14
Station 4	10	25

4.3.3 Diversity of AOB

Unique bands visualized in the DGGE gels were re-amplified and cloned. Forty bands belonging to different band classes were sequenced in order to identify the dominant members in the nitrifiers community. This represented the structure of the most numerically dominant nitrifiers population in the CE during the study period. Phylogenetic analysis of the DGGE bands showed major affiliation of AOB to β-proteobacteria, which mainly includes *Nitosospira* sp. *Nitrosomonas* sp. and several other uncultured β-proteobacterium. (Figure. 4.27) While one band showed similarity with γ-proteobacteria *Nitrosococcus* sp.

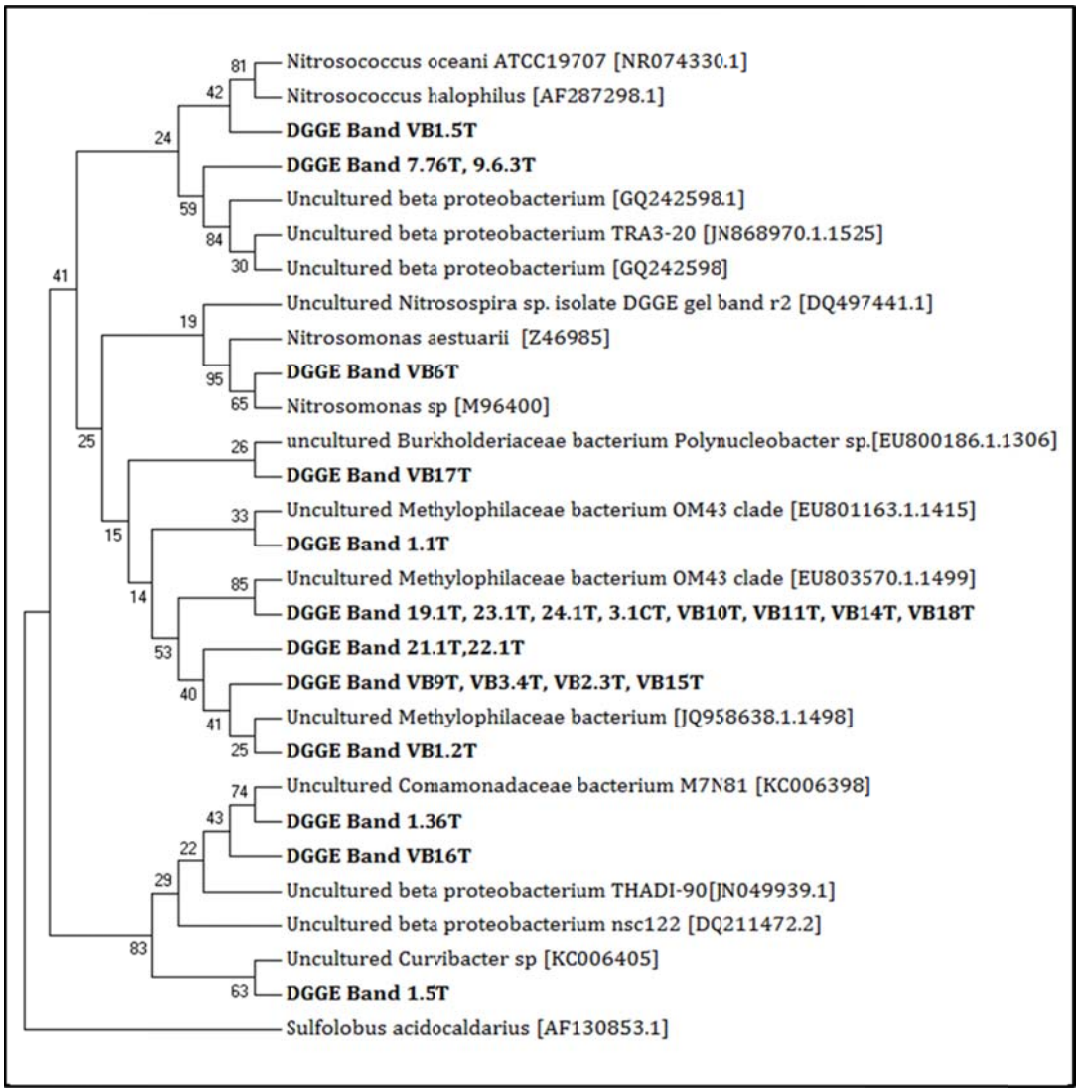


Figure 4.31 Rooted neighbour-joining phylogenetic tree based on 16S rRNA gene of ammonia oxidizing β-proteobacteria sequences retrieved from DGGE bands.

4.4 Nitrification Rate

Nitrification rate was calculated from the accumulation of nitrite in $NaClO_3$ treated bottles. The annual nitrification rate varied from 0.05 to 10.22 μM N day^{-1} in the CE (Figure 4.32). The nitrification rate in the low saline station ranged from 0.24 to 8.3 μM N day^{-1}, in the intermediate saline stations it ranged from 0.11 to 10.22 μM N day^{-1} and in the high saline station from 0.054 to 5.4 μM N day^{-1}. The high saline coastal station showed comparatively low nitrification rate than the estuarine stations irrespective of the season. Nitrification rates were considerably low, just above the detection limits during monsoon irrespective of the stations (0.05 to 0.261 μM N day^{-1}), which increased during post- monsoon (0.576 to 4.66 μM N day^{-1}) and reached the plateau during pre-monsoon (5.5468 to 10.228 μM N day^{-1}). Bottom samples always showed higher nitrification rate than surface waters (Figure 4.28). It was observed that the nitrification rate in the CE was positively correlated with abundance of AOB and NOB ($r < 0.8 < 0.01$, $n = 24$) except *N. mobilis* and dissolved ammonia concentration ($r = 0.65 < 0.01$, $n = 24$). Salinity and other environmental factors did not show any significant correlation with nitrification rate.

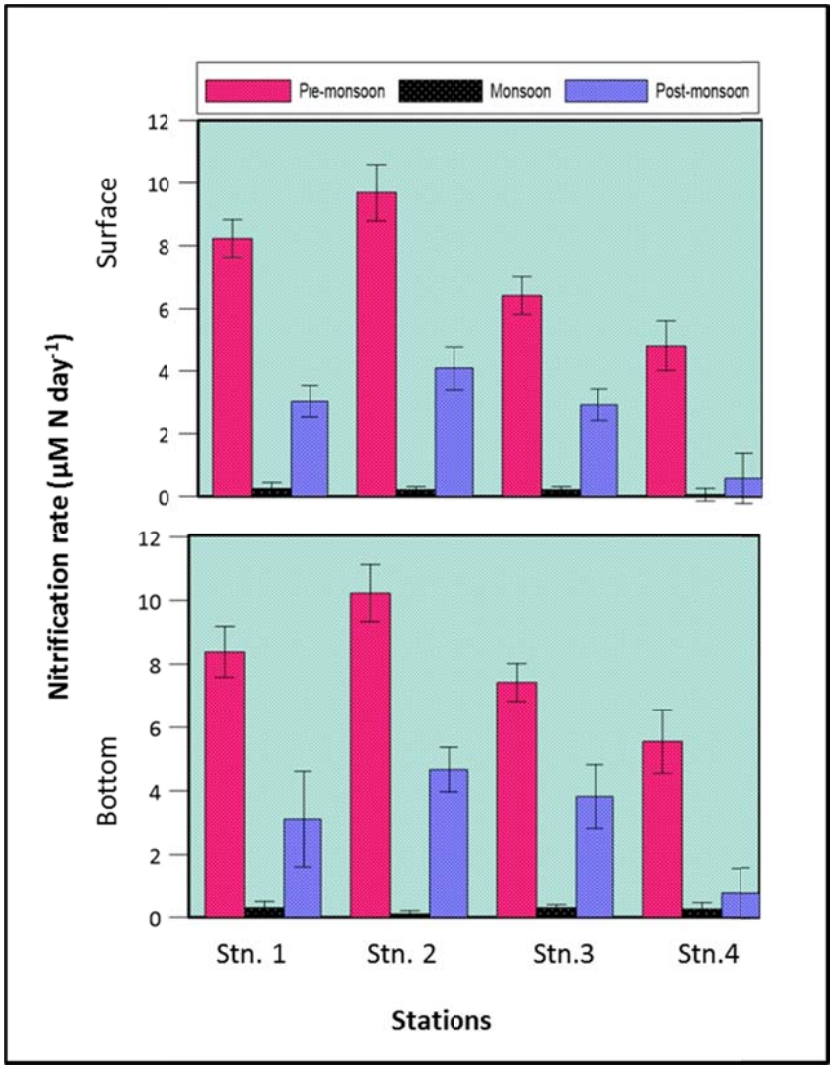


Figure 4.32 Seasonal variation in nitrification rate in surface and bottom waters of the CE.

4.5 Statistical Analysis

CDA analysis performed on the nitrification rates showed that there was no unique station wise discrimination (Figure 22). Monsoon and post- monsoon samples formed a single cluster, while pre-monsoon samples formed a separate cluster. The seasonal discrimination with distinct clusters were observed indicating that, seasonal variations outrate the spatial variations at different levels (Wilks lamda = 0.00558., calculated $\chi 2 = 81.64$; df = 24). CDA could classify 100% of the grouped cases correctly whereas 91.7% of the cross validated grouped cases were correctly classified. The two eigen values, λ_1 (41.769) and λ_2 (3.532) together explained 100% of the variation in the distribution of nitrifiers and environmental parameters.

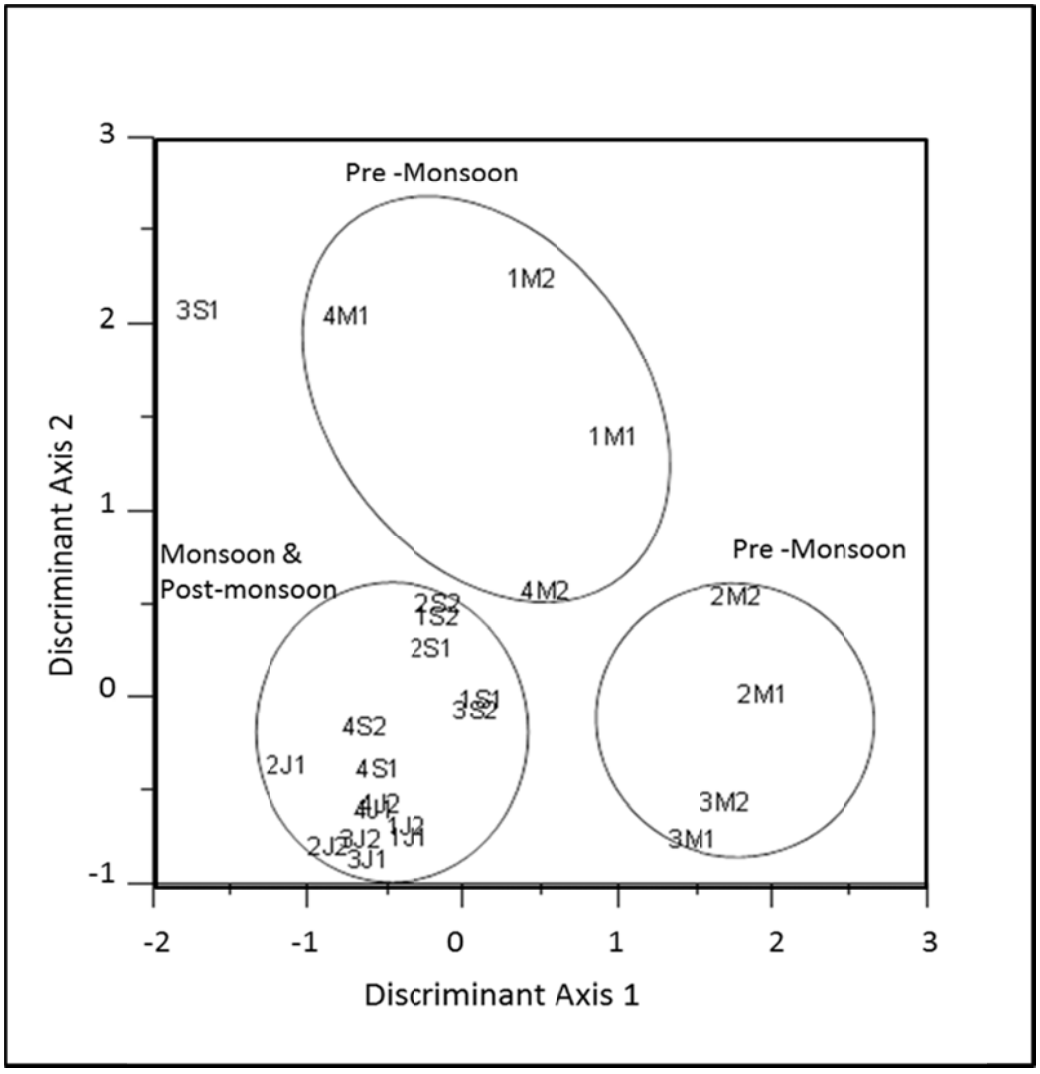


Figure 4.33 Discrimination between stations based on Canonical discriminant analysis with (CDA) environmental parameters and nitrification rate. The alphabets refer to months and the number refers to stations.

Table 4.7 Showing interrelationship between environmental parameters and nitrification rate in the CE. Nitrification rate showed significant positive correlation with abundance AOB and NOB except *N. mobilis* ($r = <0.80$, $p <001$ $n = 24$). Nitrification also showed significant correlation with ammonia concentration ($r=0.65$, $p<0.01$, $n = 24$). Other environmental factors did not show correlation with nitrification rate. Figure 4.34 showing the PCA analysis results of influence of environmental parameters on nitrification rate in the CE.

Table 4.10 Inter relationship between environmental parameters and nitrification rate. (** p<0.01; * p<0.05)

Biotic and abiotic variables		Nitrification rate ($\mu M\ N\ day^{-1}$)
AOB	β AOB	0.84**
	N. mobilis	0.30
NOB	*Nitrospira* sp.	0.81**
	Nitrobacter sp.	0.83**
Environmental parameters	Salinity	0.01
	Temperature	0.327
	DO	-0.23
	pH	0.22
	Ammonia	0.66**
	Nitrite	-0.22
	Nitrate	0.34
	Phosphate	-0.72
	Silicate	-0.22
	SPM	0.22

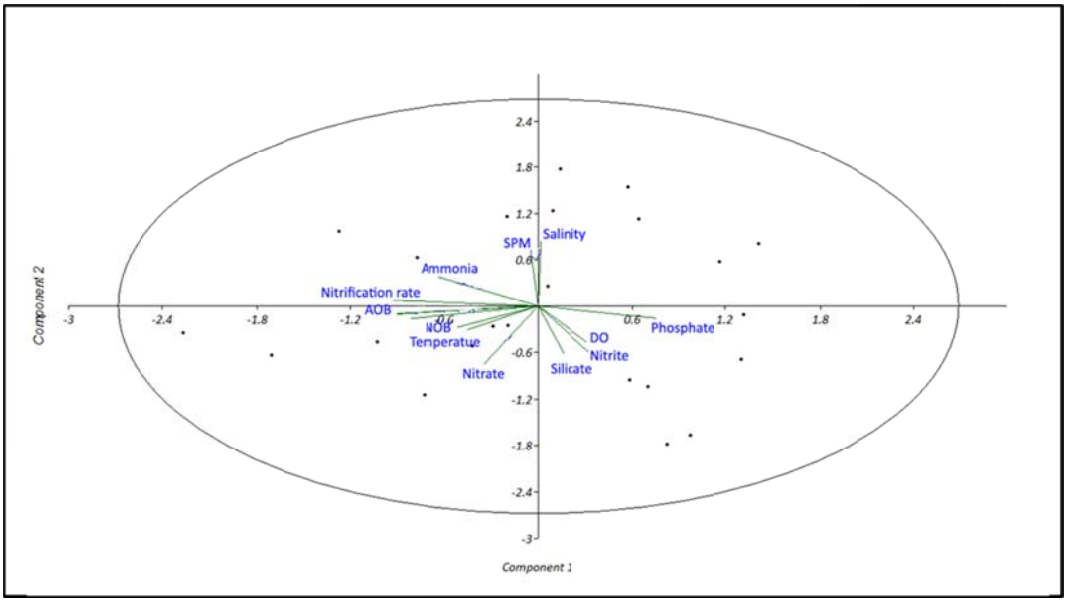


Figure 4.34. PCA analysis biplot displaying the ecological relationship between biotic and abiotic variables and nitrification rate. The analysis was done based on the correlation matrix and in the biplot the relative direction of the straight line of different variables indicates the correlation among them.

4.6 Contribution of AOA and AOB towards Ammonia Oxidation

The differential contribution of AOA and AOB to ammonia oxidation was studied using ammonia oxidation recovery rate assay. Ammonia oxidation activity of about 50 – 75 % could be recovered in the control bottles after removing acetylene gas, which confirms the active recovery of ammonia oxidation. It was observed that AOB mediated ammonia oxidation was higher than that of AOA in the CE (Figure 24).

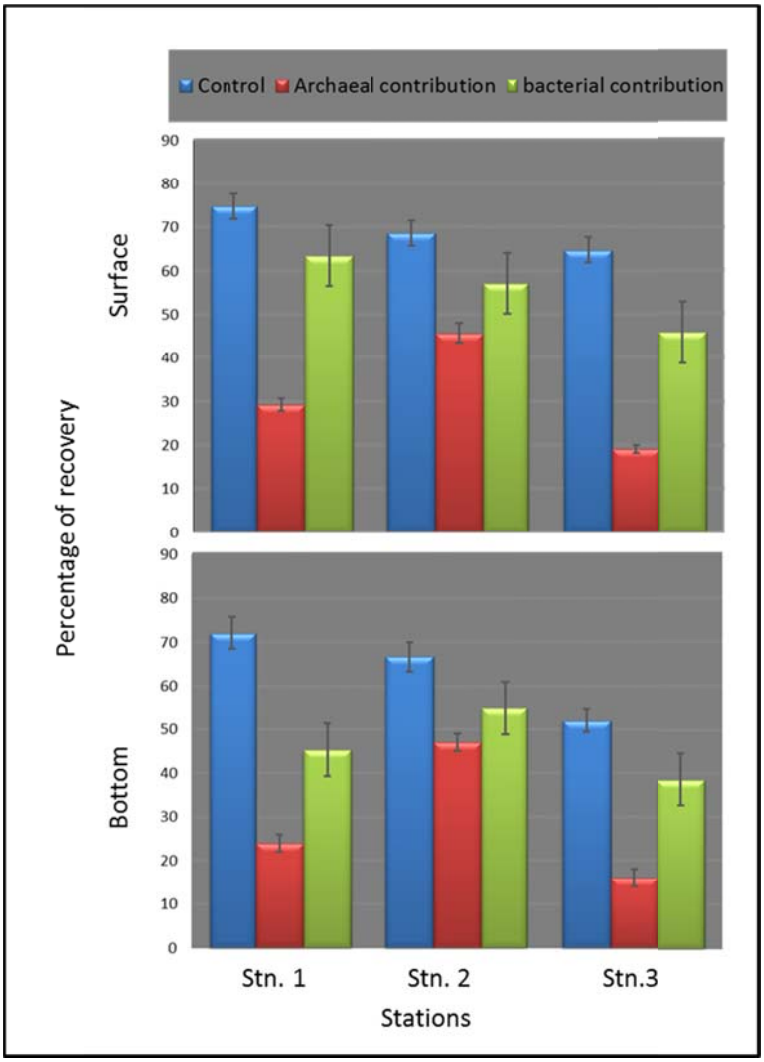


Figure 4.35 Comparison of ammonia oxidation recovery rate in surface and bottom waters of the CE.

It was observed that 40 -65% of ammonia oxidization activity could be recovered in the bottles supplemented with archaeal protein inhibitors (cycloheximide), which may be contributed by AOB. On the other hand, recovery of ammonia oxidation rate was reduced to 15 – 45% in the samples treated with bacterial protein synthesis inhibitors (kanamycin and gentamycin), which indicates that the contribution of AOA in ammonia oxidation in the CE was lower than bacterial contribution. Maximum recovery of AOA mediated ammonia oxidation was observed in the surface and bottom waters at station 2 (45 %), while it was minimum (< 19 %) at station 3. AOB mediated recovery of ammonia oxidation rate was > 50 % in the surface and bottom waters at stations 1 and 2, while it was 40 - 45 % at station 3. However, no significant difference was noticed in the relative contribution of AOA and AOB between surface and bottom waters.

Chapter - 5
DISCUSSION

Contents

Estuarine water column is subjected to wide fluctuations in various physical (tides, salinity, temperature and turbidity) (Shish and Ducklow 1994, Wikner et al. 1999, Cunha et al. 2000) and chemical properties (DO, Nutrents, pH etc) (Thingstad and Billen 1994, Amon and Benner 1996, Covert and Moran 2001). This system is persistently exposed to anthropogenic activities that cause negative impacts on their biodiversity and habitat suitability. It is well documented in temperate and sub-tropical estuaries that bacterial communities are heterogeneous in their abundance, physiological activity and diversity over trophic gradient (Hewson and Fuhrman 2004, Henriques et al. 2006). However, the biogeography and activity of the microbial assemblages in tropical estuarine environment has not been well understood (Dolan 2005). Nitrogen is considered as one of the major limiting factor in coastal waters (Howarth 1988). Estuaries are nutrient filters of coastal waters and thereby play a significant role in regulating the nutrients export from land to sea and making the nitrogen dynamics significant particularly in the estuaries. Human activities have considerably increased the availability of nitrogen in the biosphere (Vitousek et al. 1997), this excess nitrogen can leach from soils and enter to the natural aquatic system (Galloway et al. 2003) and finally to estuarine and coastal systems.

Estuaries may also serve as a significant nitrogen sink, owing to biotic removal by assimilation, denitrification or by burial processes, and also act as a source of nitrogen via direct discharge or degradation of organic matter. The major nitrogen pool in estuaries is total nitrogen (mostly organic nitrogen) and available inorganic nitrogen in water. The data presented by Downes (1988) suggested that nitrifiers play a major role in the dynamics of nitrous oxide in fresh water lakes. The data presented by (Downes 1988, Kim and Craig 1990) suggested that nitrifiers play a major role in the dynamics of nitrous oxide in fresh water lakes and nitrification is the origin of some nitrous oxide in deep ocean water respectively. However, the role of nitrifiers in the production of nitrous oxide in the oceans has been over estimated (Yoshida1988). In estuarine and coastal environment, nitrification is often coupled to denitrification (Jenkins and Kemp 1984, Sebilo et al. 2006). Hooper et al. (1997) also showed that some of the nitrous oxide may be produced through oxidation of ammonium. Nonetheless, recent studies generally consider nitrification as a major player in nitrous oxide production (Seitzinger and Kroeze 1998, Punshon and Moore 2004, Meyer et al. 2008).

Nitrification and nitrifiers in estuaries is modulated by the complex interplay among different microorganisms and between microorganisms and environmental variables, which in turn is dictated by various hydrodynamic characteristics like fresh water discharge and seawater influx. The highly dynamic system of tropical estuaries is an appropriate platform to study the influence of environmental parameters on the growth and activity of nitrifiers. Despite its importance, the studies on nitrifiers in tropical estuaries have eluded researchers and this area has lagged behind its pelagic counterpart.

5.1 Estuarine Characteristics

During the study period, the CE showed the characteristic of a typical micro tidal tropical estuary. The major hydrological variable in the CE was salinity which varied from limnetic to 35. This is similar to the situation encountered in any other Indian estuary where the salinity gradually declines from ~35 at the mouth to ~0.1 at the entry point of the rivers (Qasim 2003). The CE is influenced by two main factors viz. the short term changes induced by the tides and the long term

seasonal changes brought about by the monsoon system. The tides at the CE are mixed and semidiurnal in nature. Two high (flood) and two low (ebb) watermarks occur every day and these differ in amplitude (Srinivas et al. 2003). Though it is reported that the seawater through tidal intrusion reaches up to 60 kms towards the head of the estuary (Balachandran et al. 2008), the observed low salinity was mainly due to fresh water discharge from the six rivers that open into the estuary. The influence of fresh water became more obvious during the monsoon season because 1) the annual rain fall during the monsoon season along the southwest coast is close to 70% of the total and this voluminous fresh water finds its outlet into the estuary through the rivers (Srinivas et al. 2003) and 2) the high river discharge limits the saltwater intrusion through the two permanent openings from the Arabian Sea to a short distance of only up to 25 kms from the mouth into the estuary (Balachandran et al. 2008). The effect of the fresh water input during the monsoon is carried up to the coastal station (Stn. 4 which is about 20 kms away from the mouth of the estuary) where the salinity dropped from 35 to 14. Post-monsoon season is a transitional period, when the river discharges gradually decrease, the tidal influx gains momentum and the estuarine conditions turns to a partially mixed type thereby weakening the stratification (low salinity prevailing at the surface waters). High salinity in the CE during the pre-monsoon season (dry and stable period) is due to the low river input at the upstream, resulting in extended seawater intrusion into the estuary. Thus in the CE, fresh water mostly regulates the salinity gradient than the sea water intrusion. The water column temperature ranged from 24 to 32° C in the CE showing the typical nature of a tropical system. Water column was relatively cool during monsoon period (27.9 $\pm$ 1.6) and intense solar radiation increased the temperature relatively high (30 $\pm$ 1.6) during pre-monsoon and post monsoon seasons (29.3 $\pm$ 0.8).

The concentration of DO ranged from 2.16 to 7.47 mg L^{-1} in the CE. The oxygen concentration at the surface and near bottom water varied marginally between and within the stations. The average of observed DO in the water column (5.04 $\pm$ 1.4 mg L^{-1}) reflects healthy oxygenated water throughout the year except at few sampling occasions. This is mainly due to the mixing of water column by the tidal and fresh water influence. During few sampling occasions the DO decreased

drastically (down to 2.16 mg L^{-1}) may be because of stratification of the water column and the high oxygen consumption rates. Rates of microbial community respiration have been reported to decrease the DO concentrations below 0.8 mg L^{-1} in Chesapeake Bay waters (Sampou and Kemp 1994).

In the CE, the levels of dissolved inorganic nitrogen were high throughout the study period. Such high levels have been earlier reported from the CE, and this has been assigned mainly to industrial effluents and domestic sewage (Qasim 2003, Madhu et al. 2007). The concentration of inorganic nitrogen in estuarine stations ranged from 5.42 to 61.54 μM (during pre-monsoon), from 8.72 to 32.41 μM (during monsoon) and 10.23 to 57.73 μM (during post-monsoon) seasons. However, the concentrations were comparatively low in the coastal region during all the seasons. In this study, the post-monsoon values showed an increase in ammonium and nitrate as compared to the monsoon, but they were still lower than the pre-monsoon values. During the non-monsoon seasons, the physical conditions favour physiological and regenerative process of ammonium production and, as water movement is low, there is accumulation of nutrients in the water. Since the concentration of the nutrients viz. ammonium, nitrite and nitrate were comparable it appears that the prevailing conditions in the estuary were conducive for nitrification. In addition, in the present study, there was no significant correlation between ammonia, nitrite and nitrate, suggesting that these species of nitrogen had different controlling mechanisms. The utilization of nitrite by the autotrophs is also an important factor that could influence the nitrite pool significantly. Further it was observed that significant correlation was seen between nitrate and nitrification only during the pre-monsoon period collaborated to the fact that in the non-monsoon months, nitrate concentrations are controlled by microbial activity (nitrification) whereas, in the monsoon season it may have been influenced by transportation from watersheds. The dissolved inorganic nitrogen content observed in the CE was much higher than the reported values from other estuaries of India (Ram et al. 2003, Sarma et al. 2010, Sarma and Rao 2013). However high nutrient loading during the monsoon season has been earlier documented not only in the CE but also in other tropical estuaries such as the Mandovi-Zuari (Ram et al. 2003), Godavari (Sarma et al. 2009, Sarma et al. 2010), and Hoogly (Mukhopadhyay et al. 2006) around the

Indian peninsula. The nutrient concentration observed in the CE was comparable with that of major world estuaries like the Seine estuary in France (Garnier et al. 2006), Schelde estuary in Belgium (De Bie et al. 2002), and California estuary in USA (Boyle et al. 2004). Higher levels of N/P ratio was observed during pre-monsoon (av. 95.52 ± 120.72) and post-monsoon (av.37.16±26.6), which is significantly above the Redfield stoichiometry (16:1). Our observation corroborates the earlier observation on nutrient overloading in the CE, leading to eutrophication, which may further intensify in future if not regulate (Martin et al. 2011). Maximum terrestrial inputs enter in to the estuary during first onset of monsoon and it gradually reduces towards the peak monsoon season. Heavy rainfall and associated high fresh water influx for 4 to 5 months washes up the system and thus minimize the nutrient accumulation in the CE.

High SPM was observed during all the seasons (pre-monsoon average, 54.7 ± 53.5 mg L^{-1}, monsoon average 37.5 ± 36.4 mg L^{-1} and post monsoon average, 55.9 ± 41.4 mg L^{-1}) normally indicates the turbid condition of the estuary. Large quantity of suspended materials in the CE throughout the study period may be in the form of detritus (large fallout of plant and animal material) and sediments (terrestrial and riverine origin and from re-suspended from the bottom). This is primarily attributed to both autochthonous and allochthonous materials during the non-monsoon periods and more of allochthonous materials during monsoon (Madhu et al. 2007). It is also reported that phytoplankton biomass can be the major part of the SPM during pre-monsoon period in the CE (Madhu et al. 2007). The large quantity of SPM observed in the resultant water column turbidity limits light penetration in the euphotic zone (Qasim and Sankaranarayanan 1972) and since the primary production is light limited in the estuaries (Alpine and Cloern 1992), it significantly influence the entire productivity of the estuary. It can be concluded that in the CE most of the water quality parameters (temperature, salinity, DO, pH, SPM, nutrients etc), are primarily influenced (distribution in the estuary and concentration) by the tides, but the temporal changes in the hydrology is controlled by the prevailing SW monsoon.

5.2 Distribution of Eubacteria and Archaea

The total prokaryotic count (DAPI count) ranged from 7.53×10^5 to 1.99×10^6 cell ml^{-1}. This count represent the total prokaryotic load in the CE and was in agreement with earlier studies in Cochin estuary (Thottathil et al. 2008, Parvathi et al. 2014) and other Indian estuaries (Ram et al. 2007, Sarma et al. 2011). The population of Eubacteria and Archaea estimated based on FISH ranged from 3.3 to 6.91×10^5 and 1.93 to 5.48×10^5 cells ml^{-1} respectively. Eubacteria dominated the estuarine microbial community and accounted for 31 to 55% (average 46%) of the total prokaryotic population detected using DAPI counts, while Archaea accounted for 19 to 31% (average 27%). The planktonic Archaea in the CE, though make up for a small but significant percentage of the total microbial population. The eubacterial counts were comparable with other estuaries in the SW coast of India and also with other estuaries in the world. Whereas abundance of Archaea could not be compared with that of other Indian estuaries as the same is being reported for the first time, however it can compare with other world estuaries and coastal system. Quan et al. (2010) estimated the abundance of Eubacteria about 52 to 82% of total prokaryotic abundance in Daliao river water system and its estuary from NE China. However archaeal contribution (1 to 11.8%) in their study and was less than the present observation. Similar studies in two temperate estuaries namely Choptank and the Pocomoke River estuary (both sub-estuaries of the Chesapeake Bay) are also observed comparable results (Bouvier and del Giorgio 2002). In that studies Eubacteria enumerated was an average 32% of the total number of cells determined by DAPI direct counts and the probe targeting members of the Archaea detected on average less than 3% of DAPI counts along the estuaries. The archaeal abundance observed in this study was relatively higher than the previous reports from estuaries while it was in agreement with coastal and marine system as showing below.

The planktonic Archaea in the CE, though a small in number was a significant percentage of the total microbial population. Water column of the Eastern Mediterranean Sea also showed comparative results, where Eubacteria contributed between 24 and 72% to total prokaryotic abundance and Archaea

contribute up to 35% (La Ferla et al. 2010). But the contribution of bacteria to total prokaryotes did not differ significantly among the stations and did not show any depth related pattern (La Ferla et al. 2010). Dominance of bacteria over archaea was also evident in other marine environments. Ye et al. (2009) reported higher abundance of bacteria ranging from 5.8×10^6 to 3.3×10^8 gene copies g^{-1} sediment than archaeal abundance from 4.3×10^4 to 8.5×10^5 gene copies g^{-1} sediments using 16S RNA gene PCR method in the Mississippi Canyon. Teira et al. (2004) reported that in the deep waters of the North Atlantic Eubacteria were more abundant than Archaea (42% versus 32% of DAPI counts) and the percentage of bacteria decreased with depth, whereas archaeal abundance increased with depth (Teira et al. 2004). Similar results were also reported by Herndl et al. (2005) in Atlantic sea. Their study also indicated the importance of archaeal production in the deeper ocean and although its productivity is generally lesser than that of bacteria it can reach up to 84% of total prokaryotic production. However it has been suggested that Archaea are possibly outcompeted for resources by other microbial populations in less extreme environmental conditions, but they dominate in more extreme natural environments such as deep pelagic waters or cold Antarctic waters (Murray et al. 1998).

In this study it was found that depth did not have a significant effect on microbial abundances as surface and bottom waters did not show any significant variation in abundance of these two groups of microorganisms. This result is not in agreement with previous studies that reported archaeal densities increased with depth (Massana et al. 1997, Fuhrman and Ouverney 1998). This may be due to the mixing of water column and also could be because the study stations in the CE are shallow compare to the deeper waters. The spatio- temporal distribution of both groups showed similar pattern, with an increase in cell numbers during the pre-monsoon followed by a decrease during the monsoon season. So it can be suggested that Eubacteria and Archaea in the CE have similar response to the environmental factors that determining the microbial population. High water resident time during pre-monsoon and least water resident time in monsoon (Revichandran et al. 2012) may be the critical factor that regulated the microbial load in the CE. Based on the study in the turbidity maxima region of the Columbia estuary, Crump et al. (1999)

suggest that the free living bacteria may not develop into a uniquely adapted estuarine community due to a short residence time of water (during high rainfall period). However, during low rainfall periods the retention of water in the estuary was theoretically sufficient for development of a bacterial community.

As reported for other estuaries, (Bouvier and del Giorgio 2002, Kan et al. 2006), it is probable the environmental factors like residence time, salinity dissolved oxygen and nutrients composition and concentration are the major factors in determining the composition of microbial community in the CE. Inorganic and organic nutrient availability may profoundly influence not only microbial metabolism but its community composition (Rappé and Giovannoni 2003). Both Eubacteria and Archaea were found to be affected by water salinity ($r = -0.49$, $p<001$, $n = 48$; $r = -0.31$ $p<001$, $n = 48$, respectively) and cell numbers increased with decreasing salinity at all the stations. Maximum abundance of Eubacteria and Archaea were observed in the low saline station, and at the times of high nutrient concentration. However, the count was found decreasing during the time of peak monsoon. Such inverse significant correlation with salinity has been reported from other estuaries (Iriarte et al. 1997, Bouvier and del Giorgio 2002, Hewson and Fuhrman 2004). Nitrate concentration was the other factor that showed influence on eubacterial and archaeal distribution whereas ammonia concentration showed influence with archaeal abundance only. The distribution pattern of Eubacteria and Archaea in the CE and its response to varying environmental parameters suggests that salinity could be an important controlling factor, in addition to quantitative and qualitative changes in the nutrient composition. Sodium inhibition has previously been suggested to explain the higher occurrence of microbes in freshwater and low salinity coastal waters (Hiraishi et al. 1991, Rheinheimer 1997).

5.3 Distribution AOB and NOB

After the first reports on the successful isolation of chemo-lithoautotrophic ammonia oxidizers at the end of the 19th century (Winogradsky 1890), researchers continued to investigate the diversity of ammonium oxidizers in natural and engineered environments by applying enrichment and isolation techniques. However, low maximum growth rates and growth yields of ammonium

oxidizers render cultivation based analysis of their environmental diversity extremely time consuming and tedious. Furthermore, all culture techniques are potentially selective and thus bear the risk of incomplete coverage of the actually existing bacterial diversity (Amann et al. 1995, Wagner et al. 1995, Theron and Cloete 2000). Recently, a battery of molecular tools to infer the presence of ammonium oxidizing bacteria in the environment has been supplemented by PCR primers for specific amplification of the ammonia monooxygenase structural gene *amoA* (Holmes et al. 1995, Sinigalliano et al. 1995, Rotthauwe et al. 1997, Mendum et al. 1999). Quantitative information on ammonium oxidizing bacterial population structure and dynamics in the environment is obtainable via membrane or *in situ* hybridization techniques in combination with ammonium oxidizing bacteria specific oligonucleotide probes (Wagner et al. 1995, Mobarry et al. 1996, Wagner et al. 1996, Schramm et al. 1997, Juretschko et al. 1998, Logemann et al. 1998). The latter approach also allows one to directly relate community structure with the morphology and spatial distribution of the detected organisms. On the other hand, more sensitive quantitative PCR techniques also have limitations in that it measures the copy number of genes and not the number of organisms. For example AOB having an average of 2.5 gene copy number of ammonia monoxygenase gene per organism will give a count of approximately double the number of actual AOB present (Norton et al. 2002). The observed function diversity is not well supported by the taxonomic diversity of nitrifiers hitherto recorded.

The range of abundance of nitrifiers in this study was β AOB (3.15 to 9.3 $\times 10^4$ cells ml^{-1}) , *N. mobilis* (1.01 to 4 $\times 10^4$ cells ml^{-1}), *Nitrobacter* sp. (2.69 to 7.63 $\times$ 10^4 cells ml^{-1}) and *Nitrospira* sp. (2.51 to 6.17 $\times 10^4$ cells ml^{-1}) in the water column they showed a spatial and temporal variation. Nitrifiers counts in the order of 10^4 ml^{-1} may signal the possibility of novel groups of nitrifiers (inference drawn based of limited cultivable nitrifiers) though the probes used in this study are known to cover most of the nitrifiers. A direct comparison of the FISH results with water column of world estuaries is difficult as majority of the reports on the distribution of nitrifiers are restricted mostly to sediment samples, enumerated using MPN or quantitative PCR technique (Dai et al. 2008, Mosier and Francis 2008, Jin et al. 2011). The available references on FISH based enumeration of nitrifiers are widely

reported in sewage systems (Wagner et al. 1996) and anammox in oxygen minimum zone (Schmid et al. 2007). The observed abundance of AOB in the CE is less than in Elbe Estuary where AOB population was at a magnitude of 10^3 cells ml^{-1} (Stehr et al. 1995). The abundance of nitrifiers in the Pearl River estuary in China, measured using MPN methods, was reported to range from 2 to 4 × 10^3cells ml^{-1} (Dai et al. 2008), which is approximately one order less than the nitrifiers counted in the CE. It can be presumed that this difference may be due to the limitations of culture dependent techniques, which permits the growth of only actively growing organisms. When looking to the qPCR based abundance estimation of nitrifiers, range of 10^4 to 10^7 copy numbers of β-AOB amoA gene have been reported from a millilitre of water sample from temperate environment (Laanbroek 2013) and estuarine sediment. For example Mosier and Francis (2008) has reported the abundance of β-AOB amoA ranging from 3.1 × 10^4 to 5.3 × 10^7 copies g^{-1} in the sediment of San Fransisco Bay Estuary. If the limitations of both MPN and qPCR techniques are accommodated and extrapolating the numbers with reference to sediment, the observed the abundance of AOB and NOB in the CE is not under estimated.

Previous studies have reported that *Nitrobacter* sp. and *Nitrospira* sp. are the major nitrite oxidizing organisms in the nutrient rich estuaries (Cébron et al. 2003). Abundance of *Nitrobacter* sp. and *Nitrospira* sp. in the CE were in the order of 10^4 and these results are in agreement with those of other estuaries such as the Sein river estuary, where Cébron et al. (2003) reported an abundance of from 9.8 × 10^2 to 3.6 × 10^4 *Nitrobacter* sp. cells ml^{-1}. Their study reported sharp decrease and increase in abundance of *Nitrobacte*r sp. with changing salinity and other environmental parameters. Though there was a significant spatial and temporal variation in abundance of *Nitrobacter* sp. in the CE, such sharp changes in abundance was not observed and the abundance was in the order of 10^4. The possible reason for this contrast is that NOB community in the tropical region may be more adaptable towards the environmental variations.

The distribution pattern and seasonality of AOB and NOB in the CE suggest the coexistence of these organisms, which are responsible for modulating

the entire nitrification process in the estuary. AOB and NOB showed similar response to important physical and chemical characteristics of the environment. It has been reported that the coexistence of AOB and NOB may create a suitable micro niche that support the growth and activity of each other (Costa et al. 2006). For instance, the nitrite released by AOB could be utilized by NOB. A direct measurement of the abundance of AOA was not done in the present study due to the technical limitations of FISH to measure low copy number functional genes. Results showed the possibility of a cross feeding between ammonia oxidizing microorganisms (AOB, anammox, and AOA) and NOB. The cross-feeding between AOB and NOB has been reported earlier in biofilms, using confocal microscopy and microautoradiography fluorescent in situ hybridization (MAR-FISH) techniques (Okabe et al. 2004).

In estuaries, the environmental factors co-vary depending on the seawater influx or freshwater discharge and hence it is not a single parameter but the co-influence of different conditions that would determine the distribution and activities of microorganisms. Salinity (Caffrey et al. 2007) ammonia, (Jones and Hood 1980, Dang et al. 2008, Cao et al. 2012) and temperature (Iriarte et al. 1997) are considered as the predominant environmental factors influencing nitrifiers in estuaries, while influence of SPM, pH and other dissolved nutrients have also been discussed in literature (Allison and Prosser 1993, Cébron et al. 2003). Allochthonous ammonia reaches the CE through various routes including domestic and industrial wastes and land runoff. In the CE, ammonia was the nutrient which had a significant influence on the distribution of both AOB and NOB ($r = <2.8$, $p<0.01$, $n = 48$). Ammonia forms the first substrate for initiating the rate limiting step of nitrification; therefore it can influence AOB as a substrate and NOB as a source of substrate and such relations are obvious in estuarine and marine environments (Bouskill et al. 2012, Cao et al. 2012). In agriculturally impacted Elkhorn Slough Estuary in California, high AOB amoA gene abundance was recorded when the concentration of organic content and ammonia are high (Wankel et al. 2011). Although there was marked difference in the abundance of AOB and NOB between seasons in the CE, salinity could not be established as the critical factor controlling their abundance. This indicates that the seasonal variation in

abundance of AOB and NOB in the CE are being modulated by freshwater discharge rather than seawater influx. Further, reports from other estuaries suggest that intermediate salinity may be the preferred environment for nitrifiers (Rysgaard et al. 1999, Bernhard et al. 2007, Bernhard and Bollmann 2010). For example in the Plum Island Sound estuary, Bernhard et al. (2010) documented no linear relationship between AOB abundance and salinity, but observed that there was a pattern of high AOB abundance in intermediate salinity of ~ 20. Similarly pH also did not show much influence on the abundance of AOB and NOB in the CE, as the observed pH in the CE was near neutral or slight alkaline. The DO levels of the water column in the CE varied between seasons and was always well above the minimum concentration, i.e. 1 to 1.5 mg L^{-1} required for maintaining the growth and activity of both AOB and NOB (Garnier et al. 2007). Therefore no correlation was observed between the oxidizers and DO in the CE.

Estuaries not only act as a transition zone for fresh and marine waters but also for microorganisms from these two different environments, and in an active estuary these microorganisms may play a fundamental role in the ecological functioning of the system. The abundance and distribution of nitrifiers in the CE is controlled by a combined effect of river water discharge and flushing. Flushing activity would be inactive in the CE during pre-monsoon (Revichandran et al. 2012), while it experiences multiple flushing in addition to heavy rain fall during monsoon. In concurrence with this, the ammonia levels and abundance of AOB and NOB were found to be higher during pre-monsoon and lower during monsoon. In a seasonal perspective, in the CE the availability of ammonium is the most important factor governing the abundance of autotrophic nitrifiers during all the seasons. Interestingly, the CDA analysis showed that estuarine and coastal regions of the CE formed different clusters with respect to the abundance of nitrifiers (AOB and NOB) and environmental variables. It is clear that the estuarine region of the CE (Stns. 1-3) being more dynamic due to tidal influence and more anthropogenic activities than the coastal region (Stn. 4). Further, less stability was observed in the estuarine region as indicated by more factors influencing the abundance of AOB and NOB with less prediction efficiency (less VE) compared to coastal region.

5.4 Community Structure of AOB and AOA

Many Earth system processes, such as the biogeochemical cycles of carbon, nitrogen and sulphur (Falkowski et al. 2008, Fuhrman 2009) are driven mainly by marine microbial communities, in which prokaryotes play a fundamental role (Azam et al. 1983, Karl 2002). The current distributions of microorganisms are actually the result of contemporary selection and historical processes. The geographic distance effect should be relatively weak in habitats where dispersal is high, such as in coastal estuaries. However, at the same time, selective factors such as salinity and nutrients are often organized in a gradient in an estuary, tending to produce a distance effect on spatial variation in microbial composition. Tropical estuaries are highly productive and rich in biodiversity and the microbial community in the biodiversity is a central paradigm of the estuarine ecosystems (Venkataraman and Wafar 2005). Microbial communities in the estuaries actively involve and play important roles in a number of nutrient regeneration and biogeochemical cycles. At the same time, these are highly diverse communities which respond rapidly to changing environmental conditions. Hence changes in composition and community structure of nitrifiers can be used as a potential bio-indicator of environmental disturbance (Kowalchuk and Stephen 2001). The adaptability and susceptibility of microorganism may play significant role in nitrification in the nutrient rich and dynamic tropical estuaries (Mosier and Francis 2008). Therefore, knowing the microbial community is a pre-requisite for the systematic study of microbial biogeography and community assembly of nitrifiers in the nitrogen cycle. Spatio-temporal changes in community structure of ammonia oxidizers (AOB and AOA) was studied as these organisms are main players in the rate limiting step of nitrification. Numerous studies based on qPCR analysis of amoA genes have shown AOA to greatly outnumber AOB, in deep oceans and soil (Leininger et al. 2006, He et al. 2007, Mincer et al. 2007, Shen et al. 2008). However, mounting evidence from various estuarine and coastal studies suggests that AOB amoA gene abundance may actually be greater than AOA amoA gene abundance in certain regions of estuaries especially in nutrient rich environments.

For example, Wankel et al. (2011) reported substantially higher AOB amoA gene copy numbers than AOA in Elkhorn Slough estuary, where the AOA amoA gene copy numbers ranged from 4.9 10^3 to 1.2 10^5 copies μg^{-1} DNA and AOB amoA gene copy numbers, ranged from 1.2 10^4 to 4.8 10^6 copies μg^{-1} DNA. Similar observation on higher abundance of AOB than AOA by two order in nitrogen rich wetlands of China was recorded Wang et al. (2011).

PCR DGGE method was chosen for spatio-temporal studies over other techniques to analyse the diversity of AOB because of the large number of samples despite the possible limitations of this technique (Cilia et al. 1996, Kowalchuk et al. 1997). DGGE gel analysis of AOB showed 4 to 10 predominant phylotypes and AOA showed 7 to 26 phylotypes in the CE. Although there is no marked variation between surface and bottom samples in the band pattern, high number of phylotypes were observed generally in the bottom waters than in the surface waters, which may be due to the re-suspension of ammonia oxidisers from the bottom sediment. The community structure of ammonia oxidizers in the CE could not be compared with other Indian estuaries as it is the first study but it is available from estuarine mangrove ecosystems of India (Krishnan and Bharathi 2009, Das et al. 2013). Hence, the results have been discussed with the available literature from other estuaries in the world. In the clone library of sediment samples of Pearl River estuary, China, 36 OTU were observed for AOA as against 7 OTU for AOB (Jin et al. 2011). Similarly, in agriculturally impacted Elkhorn Slough Estuary, California from 6 to 12 bands was observed for AOB whereas 10 to 24 bands were observed for AOA (Wankel et al. 2011). AOB community in the CE did not show any seasonal variation in the DGGE pattern and 4 to 5 bands were present in the same position in the whole wells of a single gel suggesting the high adaptability of AOB community to varying estuarine conditions. Although microorganisms respond quickly to environmental changes and their community structure are determined by environment, certain level of adaptability towards particular changes is also seen among many microorganisms (Andersson et al. 2006). Community structure stability of AOB population has been reported from Seine estuary, France (Cébron et al. 2004). Unlike AOB, AOA population not only recorded high number of phylotype but also showed temporal variation in diversity in the CE. This temporal

variation of AOA may be due to the less adaptability to varying salinity and nutrient levels in estuary (Liu et al. 2013, Wang et al. 2014). Occurrence of higher DGGE band diversity and richness of AOA compared to AOB have also been reported from Plum Island Sound estuary in USA; Westerschelde estuary in the Netherlands and Bahi'adelTo'bari in Mexico (Beman and Francis 2006, Sahan and Muyzer 2008, Bernhard and Bollmann 2010).

5.5 Phylogeny of AOB

As the Rate recovery study demonstrated that AOB is the major ammonia oxidizers in the water column of the CE, the diversity of AOB was looked in to in the present study. Moreover, phylogeny of AOB is more clearly established than AOA as this organism discovered more than 100 years ago. While studies on the phylogeny of AOA started only after its discovery in 2005, but the classifications and the availability of sequence database are still growing in log phase. Comparative sequence analysis of 40 unique bands of AOB in the CE showed major affiliation of the sequence to uncultured β-proteobacterial AOB. Sequences related to *Nitrosomonas* sp. and *Nitrosospira* sp. were also obtained. Interestingly one band related to γ proteobacteria was also obtained in the sequence comparison. Previous studies have been documented that, among various groups of AOB *Nitrosomonas* sp. and *Nitrosospira* sp. are more predominant in estuarine and coastal environment (Cébron et al. 2004, Cebron and Garnier 2005, Freitag et al. 2005). It has been suggested that freshwater with low oxygen and a high ammonia condition is a possible environmental conditions for dominance AOB with in the *Nitrosomonas* Cluster (De Bie et al. 2001).

5.6 Nitrification Rate

Nitrification is a microbial mediated process that converts ammonium to nitrate via nitrite and occupies a central position within the global nitrogen cycle. Hence, the factors regulating this process are vital to eutrophication as well as to health concerns related to enhanced nitrate levels in aquatic ecosystems (Conley et al. 2009). The observed nitrification rate in the CE ranged from 0.05 to 10.22 μM day^{-1} and it showed a strong spatio-temporal variation. Nonetheless, the present

nitrification rate is much higher than the previous observation in 2005 in the CE (Miranda et al. 2008). They observed low nitrification rate from a non-detecteble level to 3.98 μM day^{-1} in the CE when the dissolved ammonia concentration was ca 20 μM, but during the present study period (2011) the ammonia concentration reached up 49 μM and the activity increased by three times. This clearly indicates that rise in anthropogenic nitrogen input in the estuary with time. It has been observed that the nitrification rate as well as nutrient input increased substantially in the CE during the past decade. However the nitrification rate (0.05 to 5.4 μM N day^{-1}) observed in the coastal station (Stn.4) is in close agreement with the recent report of nitrification rate (0.48 to 7.68 μM N day^{-1}) in the upwelling coastal waters of SW Arabian Sea (Fernandes et al. 2014).

The range of nitrification observed in the CE, was comparable with results obtained from various Indian estuaries like Mahanadi estuary (0.87 μM N^{-1} d^{-1}, when NH_4 = 1.5 μM), Narmada estuary (0.82 μM N^{-1} d^{-1}, when NH_4 = 4.0 μM) and Tapti (0.42 μM N^{-1} d^{-1}, when NH_4 = 13.0 μM) (Sarma and Rao 2013). However nitrification in these estuaries are lower than in the CE at the same time NH_4 concentration was also considerably low. Comparable range of nitrification were observed from other world estuaries like Rhone river plume (NW Mediterranean) (up to 2 μM N^{-1} d^{-1}, NH_4 ~ 2.0 μM) (Bianchi et al. 1994), Seine estuary in France (up to 16.8 μM N^{-1} d^{-1}, NH_4 ~ 180 μM (Brion et al. 2000), Providence River estuary U.S.A (up to 11.6 μM N^{-1} d^{-1}, NH_4 ~ 100) (Berounsky and Nixon 1993) and Pearl River Estuary in China (up to 33 μM N^{-1} d^{-1}, NH_4 ~ 350 μM) (Dai et al. 2008). However, comparatively higher nitrification rate than the CE, up to 45 μM day^{-1} has been reported from Schelde estuary in Belgium when the dissolved ammonia concentration up to 150 μM (Bie et al. 2002). More increased in nitrification rate of up to 80 μM day^{-1} was also recorded in the same estuary in 1984 when the ammonia concentration was 500 μM (Somville 1984). Waste loadings in the Schelde estuary were higher during the first study, which induced higher organic pollution and accompanying oxygen depletion. Rates in the Schelde estuary are still among the highest reported, despite the improved water quality of the estuary. However, in the CE the water quality is deteriorating due to increase anthropogenic inputs. Similar to the abundance of AOB and NOB, the nitrification

rate was also highest during the pre-monsoon and the lowest during monsoon in the CE. A 10 to 40 fold increases in nitrification rate during the pre-monsoon season compared to the monsoon season was observed. The nutrient level in the CE was less during monsoon season due to heavy inflow of rainwater. A previous study on flushing characteristics of the CE showed that the estuary flushes ~42 times a year, and would have freshened many times during monsoon (Revichandran et al. 2012). The increased flushing during the monsoon season along with heavy rain fall may result in dilution of nutrients, (in this case ammonia) and hence the abundance of nitrifiers and their activity would become low during monsoon. Furthermore, the lower residence time of the water in the estuary during peak monsoon play a role in the decreased activity. On the other hand, the discharge becomes inactive during pre-monsoon giving more residence time for nutrients and microorganisms to interact, which results in higher abundance of AOB and NOB and enhanced nitrification rate. Sarma et al. (2012) calculated the relationship between water resident time and nitrification rate from different Indian and world estuaries and found that, the mean flushing time for the Indian estuaries to be <10d, whereas it is < 40d for the estuaries from Europe and USA. Hence, microbes are not able to oxidize ammonium efficiently resulting in low nitrification rates in the Indian estuaries.

5.6.1 Contribution of AOA and AOB towards ammonia oxidation

Many reports are available on the abundance of AOB and AOA in marine and estuarine environments (Crump et al. 1999, Mosier and Francis 2008, Cao et al. 2011) but their relative contribution to ammonia oxidation is hitherto not studied in detail. Both AOB and AOA are present in the CE, the differential contribution of these two groups of organisms to ammonia oxidation is important. Recovered ammonia oxidation rate assay in the presence of specific antibiotics was used to understand the contribution of AOB and AOA in nitrification. Ammonia oxidation activity of about 50–75% could be recovered in the water sample after removing acetylene gas, which confirms the active recovery of ammonia oxidation. It was observed that 40–65% of ammonia oxidization activity was contributed by AOB from the water samples supplemented with archaeal protein inhibitors. On the

other hand, the contribution of AOA was considerably low in the CE as the recovered ammonia oxidation rate was reduced to 15–45 % in the water treated with bacterial protein synthesis inhibitors. No significant difference was noticed in the relative contribution of AOA between surface and bottom waters, whereas spatial differences were observed. Maximum recovery of AOA mediated ammonia oxidation was observed in the surface and bottom waters at station 2 (45 %) while it was minimum in the bottom water at station 3 (14%). AOB-mediated recovery of ammonia oxidation rate was < 50 % in the surface and bottom waters at station 1 and 2, while it was >50 % at station 3. Although both AOB and AOA harbour ammonia monooxygenase gene, the structure and mode of action of the respective enzymes are different. Archaeal ammonia monooxygenase gets triggered at lower concentrations of ammonia and switches off at higher concentrations, while that of bacterial gets triggered at higher concentration of ammonia (Bernhard et al. 2010). In the CE, concentrations of ammonia were high close to 50–65 % of the dissolved inorganic nitrogen which is conducive for AOB. However it may vary depending on the system. Our results are in agreement with the recent study in Colne Estuary, United Kingdom (Li et al. 2015). Similar observation has been reported from terrestrial ecosystems. Taylor et al. (2010) reported variation in the dominance of either bacterial or archaea or both in different soil system. Nitrification driven by bacteria and less contribution of Archaea was reported by (Di et al. 2009) in nitrogen-rich grassland soils. Similar results were also reported in Zinc contaminated soil system by (Mertens et al. 2009).

5.6.2 Inter parameter relationships

As for 'the environment selects' have shown a significant correlation between microbial composition and at least one measured environmental variable (availability of resources such as nutrients and dissolved organic carbon) or habitat feature F (physical parameters such as temperature and salinity) (Kamke et al. 2010, Agogué et al. 2011, Campbell et al. 2011). Nitrification rate in general, is categorizes under 'the environment selects' as it is regulated by many factors including salinity (Santoro and Enrich-Prast 2009), NH_4 (Triska et al. 1990, Jones Jr et al. 1995), pH (Sarathchandra 1978), temperature (Jones and Hood 1980), oxygen

concentration (Stenstrom and Poduska 1980, Triska et al. 1990), competition for NH_4 (Verhagen and Laanbroek 1991), and organic carbon availability (Verhagen and Laanbroek 1991). Nitrification also depends on NH_4 regeneration rates, which in turn is positively influenced by temperature (Nixon 1981). In the present study, simple correlation analysis and PCA analysis were employed to elucidate the factors governing the nitrification rate. The results of the regression analysis are given in Table 4.10. Seawater influx, i.e salinity, is considered as one of the major factors controlling nitrification process in many estuaries (Stehr et al. 1995, Rysgaard et al. 1999, Mosier and Francis 2008). However, differences in the optimum salinity for nitrification rate has been reported from many estuaries; for example low salinity (0 to 5) in Barataria Bay estuary in Mexico (Jones and Hood 1980) and high salinity (25 to 35) in Douro River estuary in Portugal (Magalhães et al. 2005). In the CE, although no significant correlation between salinity and nitrification rate was seen, high activity was observed at the intermediate salinity waters. The similar result has been reported in the CE previously by Miranda et al. (2008). This is also in agreement with reports from other estuaries like Scheldt estuary in Netherlands (Andersson et al. 2006) and Fjord estuary in Denmark (Rysgaard et al. 1999), where high nitrification was observed at intermediate salinity of 10 to 20. Temperature has been shown to be a major factor controlling the seasonal variations in pelagic nitrification (Berounsky and Nixon 1993). For example, the nitrification rate in Narragansett Bay ranges from near zero during winter to~1µM N L^{-1} d^{-1} during summer, with an apparent Q10~6.8 (Q10 represents the increase in the rate of a process at each 10°C increase in temperature (Berounsky and Nixon 1990). However in the present study no clear response of nitrification to the relatively small variation in temperature was detected.

Nitrification rate in the CE was largely controlled by ammonia levels ($r = 0.65$, $p < 0.01$, $n = 28$), which in turn is regulated through freshwater discharge (anthropogenic inputs) and flushing. This is in agreement with previous reports from Elbe estuary in Germany (Stehr et al. 1995), Seine estuary (Cébron et al. 2003) and Urdaibai estuary (Iriarte et al. 1997). The spatio-temporal variation in nitrification rate ($r<0.8$, $p<0.01$, $n = 24$ except *N. mobilis*), was also limited by AOB and NOB abundance. Majority of the earlier studies on nitrification rate did not

consider the role of the nitrifying organisms in the process (Somville 1984, Berounsky and Nixon 1993, Feliatra and Bianchi 1993, Bianchi et al. 1994, Brion et al. 2000), and the limited study on the nitrifiers abundance was based on culture dependant (Dai et al. 2008). Recently, a couple of ecological studies have dealt with nitrifiers abundance and phylogeny. However, interestingly majority of these studies did not studied nitrification rate or if studied it was only on the potential nitrification rate (Cao et al. 2011, Smith et al. 2015). This potential nitrification rate is not comparable with *in situ* nitrification as the rate estimation is carried out non-limiting substrate and oxygen concentration. Therefore the relationship between nitrifiers abundance and nitrification rate in the present study is compared with few estuaries and coastal waters. Significant positive correlation observed between nitrification rate and nitrifiers abundance in the CE was in agreement with these estuaries and coastal system (Beman and Francis 2006, Caffrey et al. 2007, Beman et al. 2008, Smith et al. 2014). The correlation between nitrification rate and DIN, nitrification rate and nitrifiers abundance and between DIN and nitrifiers abundance suggest that the dominant process affecting DIN dynamics in the CE is nitrification. Intense nitrification in estuaries of large rivers receiving important ammonia inputs is a general observation (Brion et al. 2000). High turbidity in the CE may also enhance the intense nitrification rate. This is mainly due to the close association between nitrifying organisms and particles (Helder and De Vries 1983, Owens 1986) and thereby providing optimal substrate concentrations and habitat for estuarine nitrifiers (Balls et al. 1996). Moreover, turbidity can also reduce the inhibitory effect of light (Merbt et al. 2012). In CE, significant statistical correlation between SPM and nitrification was not observed as SPM was high throughout and did not show any variability. Similarly, O_2 did not show any relationship to nitrification rate in the CE as the water column is well oxygenated and its much above the oxygen requirements of nitrifiers (Garnier et al. 2007).

The present study reports for the first time the spatial and temporal variation in the abundance and activity of nitrifiers from the CE, a monsoon driven nutrient rich tropical estuary along the southwest coast of India. The variability in temporal and seasonal patterns indicates a complex relationship between physico-chemical and biological controlling factors. It was observed that the levels of

ammonia in the water column have significant influence on the abundance of AOB, NOB and nitrification rate. Recovered ammonia oxidation rate experiment suggests that, though both AOB and AOA contributed in ammonia oxidation in the CE, AOB is the major player in nitrification. As AOB are more adapted to varying environmental conditions of the CE compared to AOA. From the study it could be concluded that in the CE, a monsoon driven estuary, the nitrification rate and microorganisms involved are greatly influenced by seasonal variation brought in by river water discharge and flushing. Though nitrification rate was found to be increasing with increased nutrient concentration in the CE, the anthropogenic inputs have to be controlled to prevent eutrophication and associated environmental changes.

Chapter - 6
SUMMARY AND CONCLUSION

Content

This thesis entitled "**Studies on Nitrifying Microorganisms in Cochin Estuary and Adjacent Coastal Waters"** reports for the first time the spatial and temporal variations in the abundance and activity of nitrifiers (Ammonia oxidizing bacteria-AOB; Nitrite oxidizing bacteria- NOB and Ammonia oxidizing archaea-AOA) from the Cochin Estuary (CE), a monsoon driven, nutrient rich tropical estuary along the southwest coast of India. It also form the first study of similar nature carried out from any estuary in the Indian region.

Estuaries are the transition zones between fresh- and marine-waters, and are greatly influenced by near shore and anthropogenic activities. Being ecologically sensitive, any change in this buffer zone will have severe impact on the biogeochemical cycles especially the complex biogeochemical cycle of nitrogen. Among the various processes of nitrogen cycle in the estuaries, nitrification has attracted considerable research interest among both chemical and biological oceanographers due to various reasons, from its environmental importance in eutrophication and nitrous oxide emission to the intricacies of chemical transformations and diversity of microorganisms involved. Nitrification is a microbiologically mediated two-step process involving the conversion or ammonia

to nitrate via nitrite. Bacteria were thought to be the only group of microorganism involved in the nitrification process until the identification of Archaea involved in this process from natural environment. Studies on distribution of nitrifying microorganisms and nitrification rates in the estuaries are very limited in comparison to ca 1200 estuaries in the world and are mostly from temperate and subtropical estuaries, while tropical estuaries especially those along the southeast Arabian Sea have not received enough attention. In the present scenario, the most important question to be addressed on nitrifiers is what is the contribution of AOB and AOA in the ammonia oxidation process in the estuaries and also to the nitrogen cycle? Hence, the aim of the present study was to understand the principle biotic and abiotic factors influencing the nitrification rates in the CE with the following objectives:

1. Distribution pattern of nitrifying bacteria, their response to environmental changes in the CE and adjacent coastal waters.
2. Molecular Investigation on the community structure of Ammonia oxidizers and its spatio- temporal variation.
3. Nitrification activity in the CE and adjacent coastal waters and the differential contribution of ammonia oxidizers towards the process.

To fulfil the above objectives, field observations were carried out for a period of one year (2011) in the CE. Surface (1 m below surface) and near-bottom water samples were collected from four locations (stations 1 to 3 in estuary and 4 in coastal region), covering pre-monsoon, monsoon and post-monsoon seasons. Station 1 is a low saline station (salinity range 0-10) with high freshwater influx while stations 2 and 3 are intermediately saline stations (salinity range 10-25). Station 4 is located ~20 km away from station 3 with least influence of fresh water and is considered as high saline (salinity range 25- 35) station. Ambient physico-chemical parameters like temperature, pH, salinity, dissolved oxygen (DO), ammonium, nitrite, nitrate, phosphate and silicate of surface and bottom waters were measured using standard techniques. Abundance of Eubacteria, total Archaea and ammonia and nitrite oxidizing bacteria (AOB and NOB) were quantified using Fluorescent *in situ* Hybridization (FISH) with oligonucleotide probes labeled with

Cy_3. Community structure of AOB and AOA was studied using PCR Denaturing Gradient Gel Electrophoresis (DGGE) technique. PCR products were cloned and sequenced to determine approximate phylogenetic affiliations. Nitrification rate in the water samples were analyzed using chemical $NaClO_3$ (inhibitor of nitrite oxidation), and ATU (inhibitor of ammonium oxidation). Contribution of AOA and AOB in ammonia oxidation process was measured based on the recovered ammonia oxidation rate. The contribution of AOB and AOA were analyzed after inhibiting the activities of AOB and AOA separately using specific protein inhibitors. To understand the factors influencing or controlling nitrification, various statistical tools were used viz. Karl Pearson's correlation (to find out the relationship between environmental parameters, bacterial abundance and activity), three-way ANOVA (to find out the significant variation between observations), Canonical Discriminant Analysis (CDA) (for the discrimination of stations based on observations), Multivariate statistics, Principal components analysis (PCA) and Step up multiple regression model (SMRM) (First order interaction effects were applied to determine the significantly contributing biological and environmental parameters to the numerical abundance of nitrifiers).

6.1 Salient Results

Environmental Parameters

- A marked gradient in salinity (0 to 35) was observed in the CE during the study period. The average salinity was 3.8 $\pm$ 4.3 in the low saline station, 15.5 $\pm$ 9.8 in the intermediate saline stations and 24.3 $\pm$ 8.1 in the high saline coastal station. Salinity decreased with the onset of monsoon, (the lowest observed in this study was in July), slowly increased during post-monsoon and reached the maximum during pre-monsoon at all the stations.

- Water column in the study area was well oxygenated throughout the year except for few sampling occasions (average 5.04 $\pm$ 1.4 mg L^{-1}). Low level of DO observed in the intermediate saline station. DO in the CE did not follow any seasonality.

- Suspended particulate matter (SPM) levels in the CE varied from 3.2 to 155 mg L^{-1}, with higher concentration during monsoon. High turbidity in the CE may enhance the nitrification rate.

- Seasonal and spatial variations of nutrient levels were observed at all the stations. Ammonia was the major component of dissolved inorganic nitrogen and was significantly lower during the peak monsoon period and maximum at the end of pre-monsoon. Ammonia varied from 5.65 to 47.72 μM in the low saline station, 1.32 to 49.0 μM in intermediate saline stations and 0.17 to 28.35 μM in the high saline station. Unlike ammonia, nitrite levels were relatively high during monsoon and low during the pre-monsoon period. While the nitrate concentration exhibited the vice versa. Nitrite concentration varied from 0.04 to 0.84 μM, whereas nitrate varied from 0.34 to 28.35 μM. Phosphate concentration ranged from 0.1 to 2.81 μM with higher values during monsoon season. The N / P ratio was higher during the pre-monsoon (average 72.0 ± 62.4) and post-monsoon (average 44.1 ± 32.5) seasons, but relatively lower (average 11.5 ± 8.7) during the monsoon season. Silicate concentration ranged from 0.01 to 83.53 μM with higher values during monsoon and lower values during the pre-monsoon months.

Microbiology Parameters

- Total microbial abundance estimated by DAPI staining ranged between 7.53 × 10^5 and 1.91 × 10^6, cells ml^{-1}. Eubacteria and Archaea enumerated using FISH ranged between 3.3 and 6.9 × 10^5 and 1.9 and 5.48 × 10^5 cells ml^{-1}, respectively. Maximum abundance of Eubacteria and Archaea was observed during the pre-monsoon season and minimum during the monsoon season with higher abundance at low and intermediate saline stations. Irrespective of the stations or seasons bottom waters recorded higher population of both Eubacteria and Archaea than the surface waters.

- Abundance of β AOB and *N. mobilis* ranged from 3.15 to 9.31 × 10^4 and 1.01 to 4 × 10^4 cells ml^{-1}, respectively. Among the NOB, *Nitrobacter* sp. and *Nitrospira* sp. abundance ranged from 2.69 to 7.63 ×10^4 and 2.51 to 6.17 ×

10^4 cells ml^{-1}, respectively. Higher abundance of nitrifiers were observed during the pre-monsoon months.

- Abundance of AOB and NOB showed heterogeneity between the sampling sites. Higher abundance of AOB and NOB was recorded during the pre-monsoon and the lowest during the monsoon, indicating significant seasonal variation ($p < 0.01$). Significant difference in the abundance was also observed between surface and bottom waters ($p < 0.05$).

- Diversity of AOA was higher than AOB. Community structure of AOB did not show **s**patial and temporal changes whereas AOA showed **s**patial and temporal changes. Phylogenetic analysis of DGGE bands showed major affiliation of AOB to β proteobacteria.

Nitrification

- Nitrification rate varied from 0.05 to 10.22 μM N day^{-1}in the CE with comparatively higher activity in estuarine stations than the coastal station. A 10 to 40 fold increase in the nitrification rate was observed during the pre-monsoon season compared to the monsoon season (0.05- 0.26 μM N day^{-1}).

- The recovered ammonia oxidation rate of AOB was in the range of 45-65%, whereas for AOA, it was 15-45 %, indicating that AOB were mostly responsible for the ammonia oxidation in the CE.

Inter-relationship

- The sampling stations in the CE evolved from a low ammonia; low AOB- low nitrification in the monsoon season to high ammonia; high AOB high nitrification rate in the pre-monsoon with post monsoon season as a transition period where the ratio gradually increased.

- Ammonia concentrations modulate the nitrification in the CE and intermediate salinity was the most preferred environmental condition.

- AOB were the major players in modulating ammonia oxidation compared to AOA.

- The overall assessment is that, though the CE was under the influence of high anthropogenic load, frequent eutrophication was not observed in these waters due to nitrification. Most importantly, it regulated the nutrient flux into coastal waters as the concentration of ammonia at the coastal station was much less than that observed in the estuarine stations.

- The study highlights the trophic nature of the nitrifiers prevailing in the CE waters, which will enable the efficient management of this estuary.

6.2 Conclusions

In the CE, nitrification is modulated by the complex interplay between different nitrifiers and environmental variables which in turn is dictated by various hydrodynamic characteristics like fresh water discharge and seawater influx brought in by river water discharge and flushing. AOB in the CE are more adapted to varying environmental conditions compared to AOA though the diversity of AOA is higher than AOB. The abundance and seasonality of AOB and NOB is influenced by the concentration of ammonia in the water column. AOB are the major players in modulating ammonia oxidation process in the water column of CE. The distribution pattern and seasonality of AOB and NOB in the CE suggest that these organisms coexist, and are responsible for modulating the entire nitrification process in the estuary. This process is fuelled by the cross feeding among different nitrifiers, which in turn is dictated by nutrient levels especially ammonia. Though nitrification modulates the increasing anthropogenic ammonia concentration the anthropogenic inputs have to be controlled to prevent eutrophication and associated environmental changes.

6.3 Future research plan

In this study, I could demonstrate the presence of AOA and its contribution to nitrification. There are still lots of questions to be answered while considering AOAs' ecology. The main reason for our poor knowledge on physiology of AOA is the fastidious nature which makes it difficult to culture these organisms in the laboratory. Another ammonia oxidizing pathway that has recently been established is Anammox, it is anaerobic oxidation of ammonia using nitrite as

the electron accepter and nitrogen gas as the end product. It occurs mostly in suboxic regions and is the final stage of nitrogen removal in the estuary especially in the sediments. My future plan of work will be to understand the diversity, physiology, and underlying biochemistry of estuarine AOA and Anammox and the contribution of Anammox in the maintenance of homeostatic of the estuary.

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Appendix I

Fluorescent *in situ* Hybridization

Hybridization buffer (2 ml in a microfuge tube) composition

Stock reagent	Volume	Final concentration in hybridization buffer
5M NaCl	360 µ	l 900 mM
1 M Tris / HCl	40 µl	20 mM
Formamide %	depending on probe	
Distilled H2O	add to 2 ml	
10% SDS (added last to avoid precipitation)	2 µl	0.01%

Wash buffer (in a polyethylene tube) composition

Stock reagent	Volume	final concentration in hybridization buffer
5M NaCl	depending on % formamide in hybridization buffer	
1 M Tris / HCl	1 ml	20 mM
0.5 M EDTA	500 µl depending on probe	5 mM
Distilled H2O	add to50 ml	
10% SDS (added last to avoid precipitation)	50 µl	0.01%

Concentrations of NaCl in washing buffer (48°C) at different concentrations of formamide in hybridization buffer (46°C).

% formamide in hybridization buffer	mMNaCl in washing buffer
0	900
5	636
10	450
15	318
20	225
25	159
30	112
35	80
40	56
45	40
50	28
55	20
60	14
65	10
70	7
75	5
80	3.5

Appendix II

DNA extraction from water sample and PCR amplification

Reagents for DNA extraction

1. Lysis buffer (pH 8)

	Chemicals	Concentration	Weight in gram for 100ml
	1.NaCl	400mM	2.337
	2. TrisHCl	50mM	0.605
	3. EDTA	20mM	0.744
	4. Sucrose	250mM	25.668
2.	Proteinase K	20 mg ml^{-1}	
3.	20% SDS	100 ml	
4.	Lysozyme	20mg ml^{-1}	

TE buffer (pH 8)

Chemicals	Concentration	Weight in gram for 100ml
Tris CL	10mM	0.121
EDTA	1mM	0.372

TAE buffer(pH 8)

Chemicals	Concentration	Quantiy for 1000 ml
Tris Base	2.0 M	242.2 g
Glacial Acetic Acid	1.0 M	57.1 ml
0.5 M EDTA Solution		100ml

PUBLICATIONS

Publications from the Thesis

1. ***Vipindas P.V****, Anas Abdulaziz, Jasmin C, Lallu K.R, Fausia K.H, Balachandran K.K, Muraleedharan K.R, Shanta Nair. 2014.* **Bacterial domination over Archaea in ammonia oxidation in a monsoon driven Tropical estuary.** Microbial Ecology. Doi 10.1007s00248-014-0519 (**IF 3.11**).

2. ***Vipindas P V****, Anas, Abdulaziz Jayalaksmy K V, Lallu K R, Benny PY, Shanta Nair.* **Influence of nutrient inputs on the distribution and activity of nitrifying bacteria in the water column of Cochin Estuary, Southwest coast of India** (Under review Limnology and Ocanography) (**IF 3.4**).

3. ***Vipindas P V****, Anas Abdulaziz, Shanta Nair.* **Community structure of ammonia oxidizing archaea in the water column of Cochin Estuary.** (Manuscript under preparation).

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ENVIRONMENTAL MICROBIOLOGY

Bacterial Domination Over Archaea in Ammonia Oxidation in a Monsoon-Driven Tropical Estuary

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Abstract Autotrophic ammonia oxidizing microorganisms, which are responsible for the rate-limiting step of nitrification in most aquatic systems, have not been studied in tropical estuaries. Cochin estuary (CE) is one of the largest, productive, and monsoon-driven estuary in India opening into the southeast Arabian Sea. CE receives surplus quantities of ammonia through industrial and domestic discharges. The distribution of ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), and anaerobic ammonia-oxidizing bacteria (anammox) were studied using fluorescence in situ hybridization (FISH) and their relative contribution to the process as well as the governing factors were examined and reported for the first time from CE. The order of occurrence of these assemblages was β-proteobacteria (0.79 to 2×10^5 cells ml^{-1})>γ-proteobacteria (0.9 to 4.6×10^4 cells ml^{-1})> anammox (0.49 to 1.9×10^4 cells ml^{-1})>AOA (0.56 to 6.3×10^3 cells ml^{-1}). Phylogenetic analysis of DGGE bands showed major affiliation of AOB to β-proteobacteria, while AOA was affiliated to Crenarchaeota. The abundance of AOB was mostly influenced by ammonia concentrations. The recovered ammonia oxidation rate of AOB was in the range of 45–65 %, whereas for AOA, it was 15–45 %, indicating that AOB were mostly responsible for the ammonia oxidation in CE during the study period. Overall, the present study provides an insight into the relevance and contribution of different groups of ammonia oxidizing bacteria in CE and emphasizes the need for further in depth studies across space and on season scale.

Keywords Ammonia oxidation · Tropical · Bacteria · Archaea · Anammox · Cochin estuary

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Introduction

Nitrogen (N) cycle involves the transformation of the element into various oxidation states that can affect key ecosystem processes such as primary production and decomposition. Ammonia oxidation is a critical process in linking biological N fixation, anaerobic N losses, and decomposition of nitrogenous organic matter, thereby controlling the productivity of coastal and estuarine environments. Our understanding about the microorganisms involved in ammonia oxidation has evolved substantially in the last two decades with the identification of anaerobic ammonia-oxidizing bacteria (anammox) [1] and ammonia-oxidizing archaea (AOA) [2]. Most of the earlier studies from the temperate and subtropical estuaries were on the community structure and abundance of each rate-limiting process, i.e., either ammonia-oxidizing bacteria (AOB) coupled or/with anammox or nitrite-oxidizing bacteria (NOB) and the responses to environmental changes [3–6]. AOA were initially considered as an inhabitant of only the open ocean and extreme environments, but their presence in coastal oceans, estuaries, and bays has been recently recognized [7–9]. Though a few reports on the relative abundance of AOB and AOA are available from marine and estuarine environments [10], information on their relative contribution to ammonia oxidation is very much limited, particularly from the tropical estuaries.

Cochin estuary (CE) is the second largest wetland ecosystem opening into the southeast Arabian Sea. The physiochemical conditions of CE are influenced by anthropogenic and terrestrial inputs from six rivers, seawater influx from two barmouths (major opening at Fort Cochin (450 m wide) and minor opening at Munambam (250 m wide)) and the prolonged southwest monsoon. Ammonia in CE accounts for 50–65 % of the dissolved inorganic nitrogen [11], and it experiences high nitrification rate of 166 nmol N l^{-1} h^{-1} in moderate salinities [12]. Despite these reports, no information is available on the microbial community participating in ammonia oxidation in this estuary. In this study, we used a combination of fluorescence in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and ammonia oxidation rate experiments to understand the distribution of different ammonia-oxidizing microorganisms, the factors that are influencing their abundance and their relative contribution to ammonia oxidation.

Material and Methods

Study Area and Sampling

CE is a monsoon-driven tropical estuary, situated (9° 30′–10° 12′ N and 76°10′–76° 29′ E) on the southwest coast of the Indian peninsula. It covers an area of ~25,600 ha. It receives fresh water from six rivers and opens in to the Arabian Sea through a major opening at Fort Cochin (450 m wide) and a minor one at Munambam (250 m wide). Surface and near-bottom samples were collected using a Niskin water sampler (10 l capacity) from five stations (Fig. 1) across the estuary, viz. Nedungadu (station 1), Varappuzha (station 2), Fort Cochin (station 3), Arookkuty (station 4), and Thaneermukkam (station 5). To study the spatial variability in the distribution of nitrifiers, sampling was carried out during the southwest monsoon when the estuarine condition was limnitic to reduce the influence of discreet salinity gradient.

Analysis of Environmental Variables

Environmental variables were measured following standard protocols. Dissolved oxygen (DO) content in the ambient water was determined following Winkler's titration method [13]. Samples for nutrients (ammonia, nitrite, nitrate, phosphate, and silicate) were filtered and estimated spectrophotometrically within 6 h of sampling [13].

Microbial Abundance

Abundance of total eubacteria, total archaea, AOB, anammox, AOA, and nitrite-oxidizing bacteria (AOB) were quantified using FISH following the protocol of Glockner et al. [14] with oligonucleotide probes labeled with Cy_3 (Table 1). Briefly, paraformaldehyde (final concentration 1 % *v/v*) preserved water samples were passed through 0.2 μm (Millipore GTTP2500) and 0.1 μm (Millipore VCTP02500) white polycarbonate membrane filters for bacteria and archaea, respectively. The filters were hybridized for 90–120 min in a hybridization solution (0.9 M NaCl, 20 mM Tris-HCl (pH 7.4) and 0.01 % sodium dodecyl sulfate (SDS)), containing 50 ng nucleotide probes and 40–60 % formamide (depending on the probe sequence, Table 1). The cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to localize the nuclei and distinguish nonspecific bindings. Fluorescent signals from labeled cells were counted after exciting under an epifluorescence microscope, equipped with a 100-W Hg lamp and filter sets specific for DAPI and Cy_3. The interrelationships between the different groups of nitrifiers and environmental variables were assessed using Statistica version 6 (StatSoft Inc., USA). The bacterial data were tested for its normal distribution and normalized before statistical analyses.

Community Structure of Ammonia-Oxidizing Bacteria and Archaea

Extraction of DNA from Water Samples

Genomic DNA from water samples were extracted following Boström et al. [15] with slight modification. Briefly, 1 l of water sample was passed through 0.2 μm polycarbonate membrane filter (Millipore; GTTP2500), followed by incubation at 37 °C for 1 h in a lysis buffer (NaCl 400 mM, sucrose 750 mM, EDTA 20 mM, and Tris-HCl 50 mM) containing 1 mg ml^{-1} lysozyme. Subsequently, SDS (1 %) and proteinase K (100 μg ml^{-1}) were added to the solution and continued incubation for 5 h at 55 °C. Further, 0.6 volume of isopropanol was added and DNA was precipitated by keeping at −20 °C for 60 min. DNA pellet was washed copiously with 70 % ethanol, dissolved in TE buffer and stored at −20 °C until used.

Denaturing Gradient Gel Electrophoresis

Community structure of AOB and AOA was studied using DGGE technique. Briefly, 5 μl DNA sample was used as template for 50 μl PCR reaction mixture containing 2 μl each of primers (10 pmol μl^{-1}), 5 μl 10× Taq polymerase buffer (NEB, Canada), 1 U Taq DNA polymerase (NEB, Canada), and 200 μM each dNTPs (NEB, Canada). 16S rRNA gene of ammonia oxidizing β-proteobacteria were amplified with an equimolar concentration of three forward primers (CTO189fA -GC and CTO189fB -GC, and CTO189fC-GC), each with a

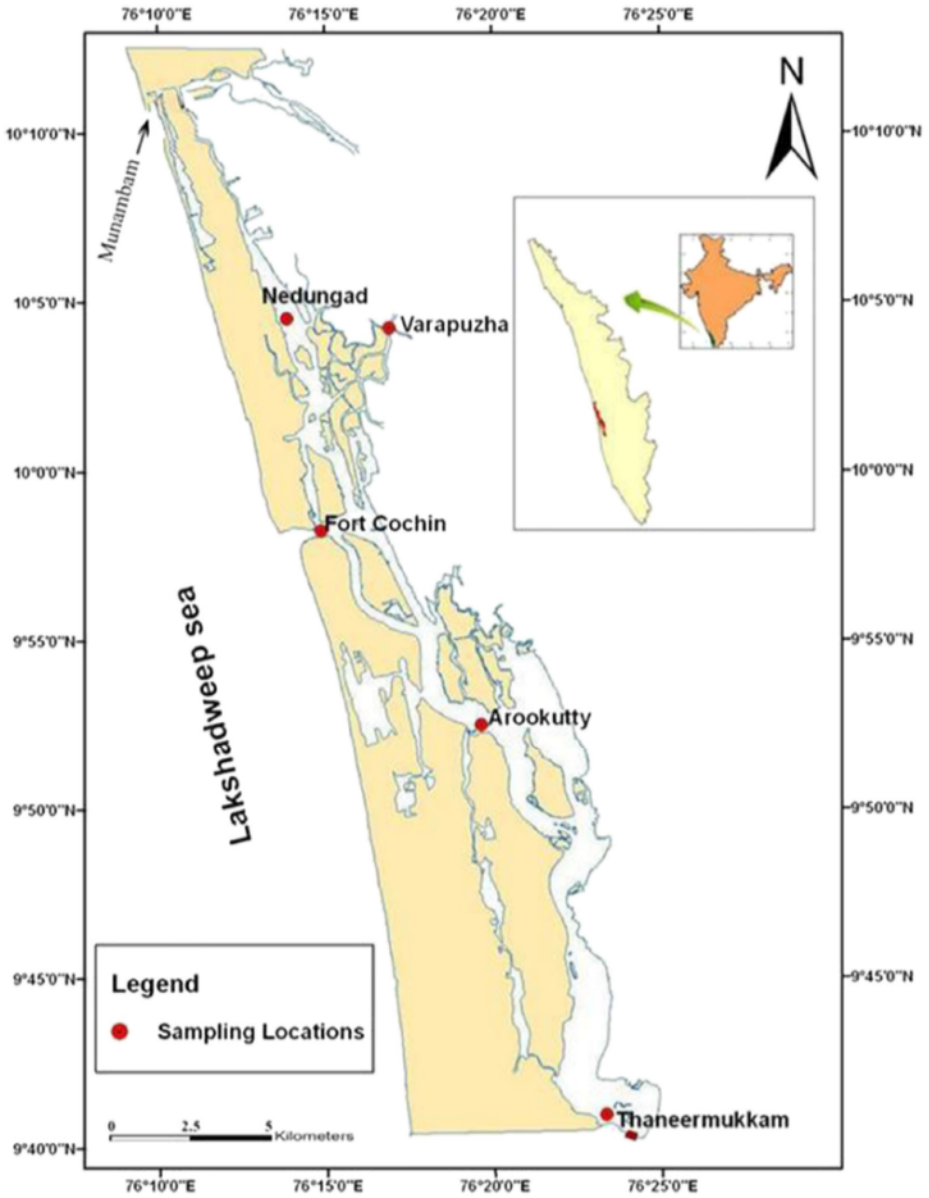


Fig. 1 Map showing the sampling stations. Station numbers in *parenthesis*

GC clamp at 5′ end and a reverse primer (CTO654r) containing a single ambiguous base [16]. The forward primers CTO189fA and CTo189fB (GGAGRAAAGCAGGGGATCG) and CTO189fC (GAGGAAAGTAGGGGATCG) were synthesized separately and collectively referred to as CTO189f-GC. The sequence CTO654r (CTAGCYTTGTAGTTTCAAACGC) was used as reverse primer. The cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 60 s, annealing at 55 °C for 60 s, extension at 72 °C for 60 s, and a final extension for 10 min at 72 °C. For amplification of archaeal ammonia monooxygenase gene (amoA) of AOA,

Table 1 FISH probes used in the study

Group	Probe	Sequence	Target site	Target organism(s)	Formamide (%)	Reference
	EUB 338	GCTGCCTCCCGTAGGAGT	16S (338–355)	Domain bacteria	55	[46]
AOB	Nso190	CGATCCCCTGCTTTTCTCC	16S (190–208)	Ammonia-oxidizing β-proteobacteria	55	[47]
	NmV	TCCTCAGAGACTACGCGG	16S (174–191)	*Nitrosococcus Sp.*	40	[48]
NOB	NIT	CCTGTGCTCCATGCTCCG	16S (1035–1048)	*Nitrobacter* spp.	45	[49]
	Ntspa 712	CGCCTTCGCCACCGGCCTTCC	16S (712–732)	Phylum Nitrospirae	40	[50]
Archaea	ARCH 915	GTGCTCCCCCGCCAATTCCT	16S (934–915)	Archaea	40	[51]
	Arch-amo AFA	ACACCAGTTTGGYTAC CWTCDGC	amoA gene	Archaeal ammonia-oxidizing gene	40	[52]
Anammox	BS820	TAATTCCCTCTACTTAGTGCCC	16S (820–840)	Annamox16S	35	[53]

we used a combination of GC clamp attached forward primer amoAf (CTGAYTGGGCYTGGACATC) and reverse primer amoAr (TTCTTCTTTGTTGCCCAGTA). The GC clamp was attached to the 5′ end of forward primer. The cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 45 s, and a final extension for 10 min at 72 °C.

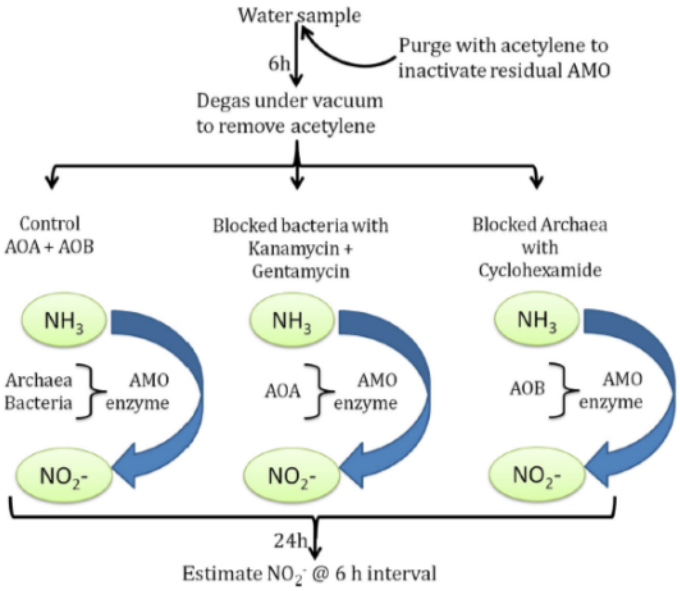
The PCR products of AOB (480 bp) and AOA (256 bp) were run on an 8 % polyacrylamide denaturing gradient gel prepared with 35–50 % (for AOB) and 30–55 % (for AOA) formamide for 17.5 h at 75 V. The bands separated were stained with SYBR green and observed in a gel documentation system (BioRAD, USA). The bands were picked, incubated overnight in a 50 µl TE, and re-amplified with respective primers without GC clamps. The PCR products were purified using Nucleo-pore Genetix Brand Sure Extract PCR clean up/gel extraction kit (Genetix Biotech, India) and cloned into TOPO vector (Invitrogen, USA), following supplier's manual, and the plasmids were transformed into chemically competent *Escherichia coli* DH5α by heat shock method. The recombinant colonies were picked and grown at 37 °C for overnight in LB broth, an aliquote of which was preserved in glycerol at −80 °C, and the remaining was used for plasmid preparation. Recombinant plasmids were purified using Nucleo-pore Genetix Brand SureSpin plasmid mini prep kit (Genetix Biotech, India) and used as a template for sequencing PCR reactions in combination with vector-specific primers, T7 and SP6, on an ABI sequencer. Sequence data obtained were analyzed and edited using Sequencher V4.10.1 (GeneCodes). Subsequently, the sequences were compared with those in the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm to determine approximate phylogenetic affiliations. The nucleic acid sequences showing the closest similarities were used as reference sequences while constructing neighbor-joining tree using the software MEGA (version 5.0). DGGE approach was followed to get some insight into the overall distribution of AOB and AOA in CE. The sequences were submitted to NCBI GenBank (accession numbers KM386955 to KM386977 and KM404171 to KM404173).

Contribution of AOB and AOA in Nitrification Rate

Contribution of AOB and AOA in ammonia oxidation rate were measured based on recovered ammonia oxidation [17]. All samples were pretreated with acetylene to irreversibly inactivate ammonia monooxygenase, and upon the removal of acetylene, the recovery of ammonia oxidation rate was monitored in the presence and absence of bacterial or archaeal protein synthesis inhibitors [17]. The schematic flow chart is given in Scheme 1. Water samples were amended with sodium chlorate (20 ppm) to inhibit the conversion of nitrite to nitrate. Experiments were carried out (in triplicate) in the dark at room temperature. Briefly, samples were purged with acetylene gas to inactivate residual ammonia monooxygenase. After 6 h of inactivation, the residual acetylene gas was removed completely by degassing under vacuum for 10 min. In one set of experimental bottles, a cocktail of protein synthesis inhibiting class of antibiotics, kanamycin (750 µg ml^{-1}) and gentamycin (750 µg ml^{-1}), were added to prevent the contribution of AOB. Another set of bottles was incubated in the presence of cyclohexamide (650 µg ml^{-1}) to prevent AOA. A third set of bottles without any antibiotics was kept as control. Nitrite accumulation was monitored at 6 h interval for 24 h following standard spectrophotometric technique. The suitability of this method was also tested by measuring ammonia oxidation rate of the water sample without acetylene treatment.

Results and Discussion

Low salinity (0 to 2.29) was recorded at all stations in CE except in the bottom waters of station 3 (Table 2), where the stratification of water body during the high tide was observed with fresh water at the surface (0–5 m depth) and seawater at



Scheme 1 Schematic representation of experimental approach used for measuring differential contribution of AOB and AOA in ammonia oxidation rate

the bottom (5 m depth) (Fig. 2). High nutrient loading was observed during the study period. The concentrations of ammonia (2.3–9.9 μM), nitrite (0.3–1.0 μM), and nitrate (15.1–36.1 μM) were high due to increased discharge of nutrient-rich industrial effluents [18] and formation of perennially undulating water bodies or null zones due to its geomorphology (oxbow shaped) and meandering flow [19–21]. High nutrient loading during monsoon season has been earlier documented not only in CE [11] but also in other tropical estuaries such as Mandovi-Zuari [22], Godavari [23], and Hoogly [24] around the Indian peninsula.

Abundance of eubacteria, archaea, AOB, anammox, and AOA in CE was enumerated using FISH technique and representative images are given in Supplementary Figure 1. In CE, we observed eubacterial dominance (1.8 to 3.5 × 10^5 cells ml^{-1}) by an order of magnitude higher than archaea (1.7 to 2.7 × 10^4 cells ml^{-1}) at all the stations (Fig. 3). The abundance of eubacteria was consistent with the earlier reports from other Indian estuaries [25]. No significant variation in eubacterial abundance was noticed with stations. Abundance of archaea could not be compared with that of other Indian estuaries as the same is being reported for the first time. Nevertheless, our results corroborate with the earlier findings on the dominance of eubacteria over archaea in Chesapeake Bay [26] and Columbia River estuary [27].

Among the two classes of AOB, β-proteobacteria (0.79 to 2.0 × 10^5 cells ml^{-1}) showed significant dominance, which was approximately 1 order of magnitude higher than γ- proteobacteria (*Nitrosococcus* (0.9 to 4.6 × 10^4 cells ml^{-1})) (Fig. 4). Their abundance was higher in the

Table 2 Environmental parameters of surface (S) and bottom (B) waters in Cochin estuary stations

Parameters	Stations									
	1		2		3		4		5	
	S	B	S	B	S	B	S	B	S	B
pH	7.2	7.3	6.7	6.7	6.8	7.7	6.6	6.6	7.0	7.0
Salinity	0.84	1.09	00	00	2.29	20.6	00	00	00	00
DO (mg l^{-1})	7.2	6.0	6.9	6.7	6.5	3.4	6.8	6.8	7.9	7.0
NH_4-N (μM)	3.5	5.6	6.9	5.9	9.9	7.7	8.8	7.7	5.1	2.3
NO_2-N (μM)	1.0	0.7	0.4	0.3	0.6	1.5	0.8	0.4	0.3	0.4
NO_3-N (μM)	15.9	17.6	36.1	21.1	18.2	19.8	15.1	17.2	22.4	22.6
PO_4-P (μM)	2.6	3.1	0.8	0.7	2.1	1.8	2.2	1.4	0.5	0.6

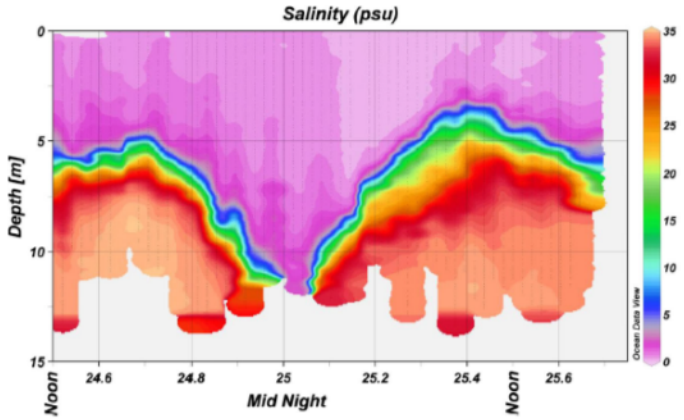


Fig. 2 Salinity variation at station 3 showing stratification

surface waters compared to the bottom. Phylogenetic analysis of the DGGE bands showed major affiliation of AOB to β-proteobacteria, while one band showed similarity with γ-proteobacteria *Nitrosococcus* sp. (Fig. 5 and Supplementary Fig. 2a). The nutritional gradient influences the community structure and nitrification efficiencies of ammonia-oxidizing microorganisms [28]. It was observed that ammonia concentrations in the water column had positive influence on the abundance of β-proteobacteria (r=0.639, p<0.05) and *Nitrosococcus* (r=0.814, p<0.01) (Supplementary Table 1). Our result is in agreement with the earlier findings that the available ammonium limits the abundance of AOB [5, 29]. Other critical environmental variables which are known to be influencing the ammonia oxidation in the estuaries, such as salinity, temperature, and dissolved oxygen [30, 31], had little effect on the distribution of AOB in CE. Since CE was limnitic during the study period, the effect of salinity on abundance of ammonia oxidizing microorganisms was not

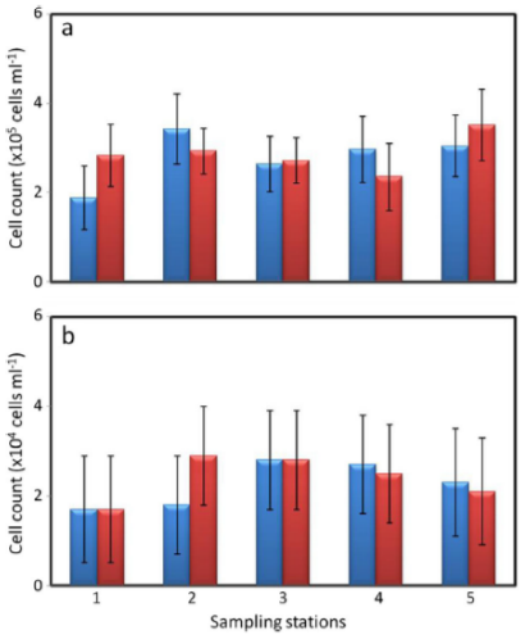


Fig. 3 Abundance of Eubacteria (a) and Archaea (b) in the surface (*blue bars*) and bottom (*red bars*) waters from five sampling stations in Cochin estuary

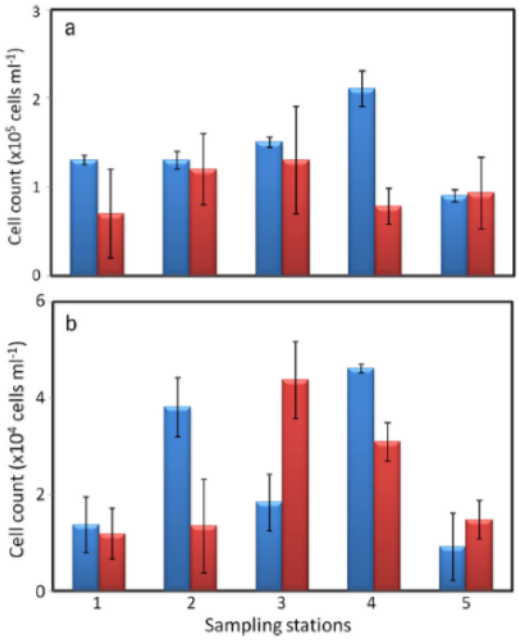


Fig. 4 Abundance of ammonia oxidizing β-proteobacteria (a) and Nitrosococcus (b) in the surface (*blue bars*) and bottom (*red bars*) waters from five sampling stations in Cochin estuary

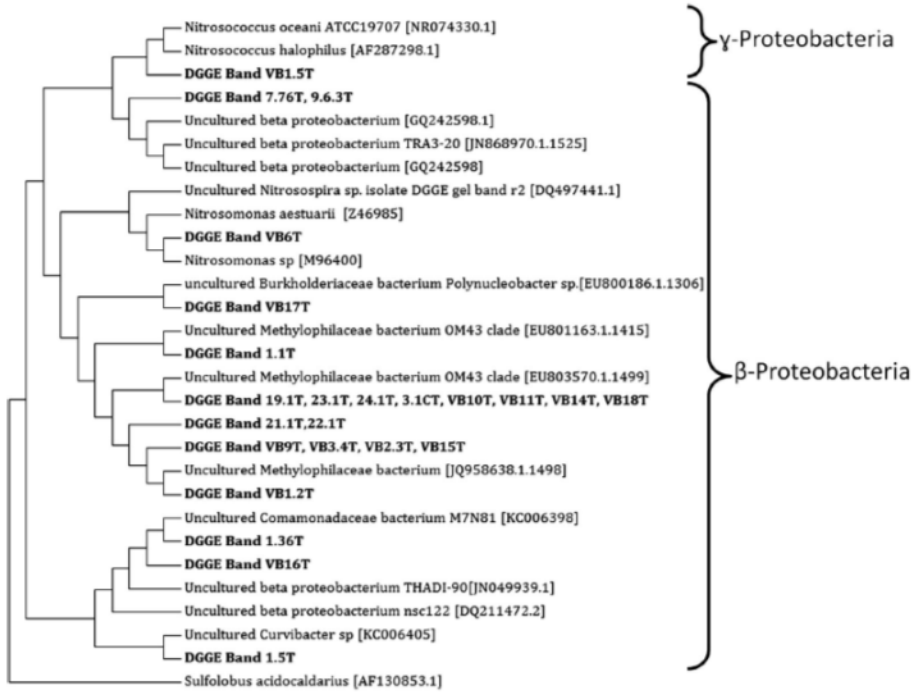


Fig. 5 Rooted neighbor-joining phylogenetic tree based on 16S rRNA gene of ammonia oxidizing β-proteobacteria sequences retrieved from DGGE bands

evident. Moreover, AOB is also known to exhibit a broad range of salinity tolerance [6].

Anammox and AOA were the other ammonia-oxidizing microorganisms recorded in CE (Figs. 6 and 7). The ubiquitous presence of anammox has been reported from sediments of estuaries and fresh water and also from hypoxic region of ocean [7, 32, 33]. In CE anammox was observed in the water column and its abundance ranged from 0.49 to 1.9 × 10^4 cells ml^{-1}, with minimum in the surface waters at station 5 and maximum in the bottom waters at station 3 (Fig. 6). The anammox present in the water column may be resuspended from sediments, due to excessive mixing of water column and continuous dredging operations in CE. Anammox-mediated oxidation of ammonia to nitrogen gets inhibited at higher concentration of DO (more than 2 μM) [34]. Therefore, we presume that the contribution of anammox may be negligible compared to the other groups of microorganisms in CE as the

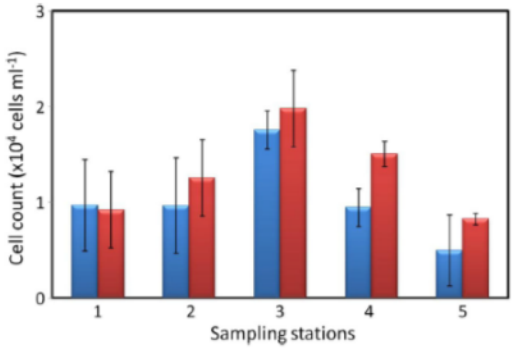


Fig. 6 Abundance of anammox in the surface (*blue bars*) and bottom (*red bars*) waters from five sampling stations in Cochin estuary

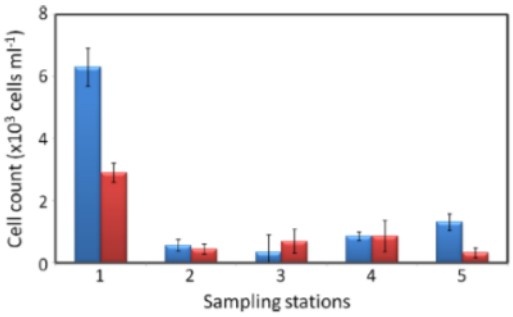


Fig. 7 Abundance of archaeal ammonia oxidation gene (amoA) in the surface (*blue bars*) and bottom (*red bars*) waters from five sampling stations in Cochin estuary

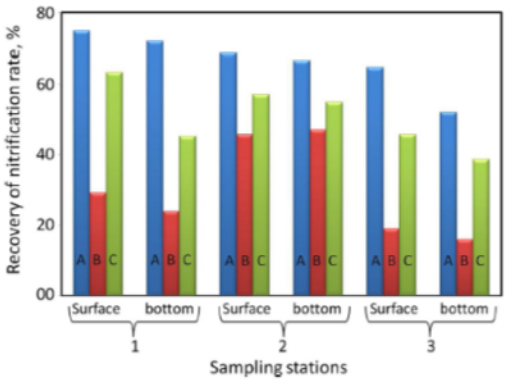


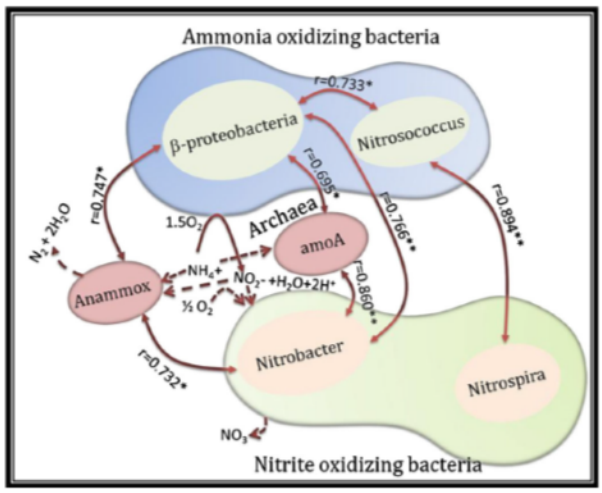
Fig. 8 Comparison of ammonia oxidation recovery rate of control (a), AOA (b), and AOB (c) in surface and bottom waters of sampling stations 1, 2, and 3

level of oxygen was ca. 3.9 to 7.9 mg l^{-1} in the water column during this period.

We observed the presence of AOA in the surface (0.56 to 6.3×10^3 cells ml^{-1}) and bottom (0.32 to 2.9×10^3 cells ml^{-1}) waters of CE, which was less than AOB (Fig. 7). The diversity of AOA was monitored using PCR DGGE analysis of ammonia monooxygenase gene (amoA) (Supplementary Fig. 2b). Phylogenetic analysis of the DGGE bands showed major affiliation of AOA to Crenarchaeota. Similar results have been reported from Elkhorn Slough estuary in California where the organic load due to agricultural impact was higher [35]. Ammonia monooxygenase of archaea is tuned to work more efficiently in oligotrophic conditions [36], and hence AOA outnumber AOB in the open ocean. Recently, AOA dominance has been reported in sediments from Plum Island Sound estuary, Massachusetts [37] and Pearl River estuary, China [38].

Although both AOB and AOA are present in CE, it is important to understand the differential contribution of these two groups of organisms to ammonia oxidation. The ammonia oxidation recovery rate assays showed that AOB-mediated ammonia oxidation was higher than that of AOA in CE (Fig. 8). Ammonia oxidation activity of about 50–75 % could be recovered in control bottles after removing acetylene gas, which confirms the active recovery of ammonia oxidation. The protein synthesis of bacteria was inhibited by a combination of kanamycin and gentamycin while archaeal protein synthesis was inhibited by cyclohexamide. It was observed that 40–65 % of ammonia oxidization activity could be recovered in the bottles supplemented with archaeal protein inhibitors, which may be contributed by AOB. On the other hand, recovery of ammonia oxidation rate was reduced to 15–45 % in the samples treated with bacterial protein synthesis inhibitors, which indicates that the contribution of AOA in CE was considerably low during the period of sampling. While no significant difference was noticed in the relative contribution of AOA between surface and bottom waters, spatial differences were observed. Maximum recovery of AOA-mediated ammonia oxidation was observed in the surface and bottom waters at station 2 (45 %) while it was <19 % at station 3. AOB-mediated recovery of ammonia oxidation rate was >50 % in the surface and bottom waters at stations 1 and 2, while it was 40–45 % at station 3. Many reports are

Fig. 9 Schematic representation of interactions among nitrifiers in Cochin estuary (*p<0.05; **p<0.01)



* p<0.05, **p<0.01

available on the abundance of AOB and AOA in marine and estuarine environments, but their relative contribution to ammonia oxidation is hitherto not studied [4, 17, 39, 40]. Although both AOB and AOA harbor ammonia monooxygenase gene, the structure and mode of action of the respective enzymes are different [36]. Archaeal ammonia monooxygenase gets triggered at lower concentrations of ammonia and switches off at higher concentrations [36], while that of bacterial gets triggered at higher concentration of ammonia. CE contains high concentrations of ammonia, i.e., 50–65 % of the dissolved inorganic nitrogen [11], and therefore the AOB's contribution in ammonia oxidation could be higher.

NOB, such as *Nitrospira* and *Nitrobacter*, were observed in the range of 1.8 to 6.9×10^4 and 2.5 to 7.4×10^4 cells ml^{-1}, respectively (Supplementary Fig. S3), which were less than the ammonia-oxidizing β-proteobacteria of the respective stations. Interestingly, we observed a positive correlation of anammox with β-proteobacteria ($r=0.747$, $p<0.05$) and *Nitrobacter* ($r=0.732$, $p<0.05$). Similarly, positive correlations of AOA with β-proteobacteria ($r=0.695$, $p<0.05$) and *Nitrobacter* ($r=0.860$, $p<0.05$) were also observed. The γ-proteobacteria (*Nitrosococcus*) showed strong positive correlation with *Nitrospira* ($r=0.894$, $p<0.01$), whereas β-proteobacteria showed correlation with *Nitrobacter* ($r=0.766$, $p<0.01$). Our results show the possibility of a cross-feeding between ammonia oxidizing microorganisms (AOB, anammox, and AOA) and NOB (Fig. 9). The cross-feeding between AOB and NOB has been reported earlier in biofilms, using confocal microscopy and microautoradiography-fluorescent in situ hybridization (MAR-FISH) techniques [41–44]. Microscopic observations often showed the presence of NOB in the close proximity of AOB, which not only indicates the metabolic association between these two groups, but also confirms that their association is not mutually toxic [41–44]. It has been established that in controlled environments, the interactions between these microorganisms vary with the level of ammonia and dissolved oxygen present [29, 45], which is equally applicable in CE also. During the study period, DO was maintained at higher levels in CE except in the bottom waters of Fort Cochin, which incidentally accounted for higher numbers of AOB and NOB.

Our results showed the dominant role of AOB in modulating the ammonia oxidation in a monsoon-driven nutrient-rich tropical estuary. The process is fuelled by cross-feeding among the organisms in the proximity, which in turn is dictated mainly by ammonia and dissolved oxygen.

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Limnology and Oceanography

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Influence of nutrient inputs on the distribution and activity of nitrifying bacteria in the water column of Cochin Estuary, southwest coast of India

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Keywords:	Nitrification, Cochin estuary, bacteria
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Limnology and Oceanography

Influence of nutrient inputs on the distribution and activity of nitrifying bacteria in the water column of Cochin Estuary, southwest coast of India

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Running head: Distribution and activity of nitrifiers in CE

Abstract

Spatial and temporal variations in the distribution, community structure and activity of nitrifying bacteria [ammonia (AOB) and Nitrite (NOB) oxidizing bacteria] and the community structure of ammonia oxidizing archaea (AOA) along a salinity gradient in the Cochin estuary (CE), a monsoon driven tropical estuary located along the southwest coast of India were studied for one year. AOB and NOB were enumerated using fluorescent *in situ* hybridization (FISH) which showed marked seasonality while maintaining the abundance within an order of 10^4 cells ml^{-1}. DGGE analysis of AOB exhibited spatio-temporal adaptability without much variation, while AOA showed minimum seasonal variations at intermediate and low saline stations. Nitrification rate in the CE ranged from 0.05 to 10.2 µmol day^{-1} and showed 10 to 40 fold increase in activity during the pre-monsoon season compared to the monsoon season, indicating that the seasonal variation was stronger than spatial variations. It could be deduced from the study that the activity and distribution of nitrifiers in the CE is controlled by nutrient inputs which in turn are modulated by seasonal variation in river water discharge and flushing.

Key words: *Nitrification, bacteria, archaea, nutrient loading, Cochin estuary, southwest coast of India*

Introduction

Estuaries are the transition zones connecting fresh- and marine-waters, and are greatly influenced by near shore activities. Environmental gradients originating from mixing of turbid and nutrient rich fresh water with oligotrophic seawater is the major characteristic of these systems, which establishes apparently distinct zones in estuaries worldwide (Mosier and Francis 2008). Among the various nutrients in estuarine systems, nitrogen gained particular attention because of its increased environmental concerns and the complex processes involved in its transformations (Conley et al. 2009). It is estimated that up to 50 % of the dissolved nitrogen of anthropogenic origin in estuaries are recycled through coupled nitrification and denitrification (Seitzinger et al. 2006). Nitrification has attracted the research interest of both chemical and biological oceanographers due to various reasons, particularly its environmental importance in eutrophication, nitrous oxide emission, the intricacies of chemical transformations and diversity of microorganisms involved. Bacteria were thought to be the sole group of microorganisms involved in nitrification until the identification of archaea that were also involved in the process from natural environment (De La Torre et al. 2008; Taylor et al. 2003; Treusch et al. 2005). It has been reported that archaea are mainly responsible for nitrification in oligotrophic environments (Alves et al. 2013), while bacteria play important roles in the nitrogen cycle in nutrient rich estuaries (Vipindas et al. 2014). While considering the total number of about 1200 estuaries the world over, studies on the distribution of nitrifying microorganisms and the nitrification rates are very limited and most of the studies were focused on sediments (Bernhard and Bollmann 2010). Influence of environmental factors, such as salinity, temperature, pH and nutrients on nitrification in the estuarine and marine environments has been elaborated in various reports (Allison and Prosser 1993; Caffrey et al. 2007; Cébron et al. 2003; Jones and Hood 1980;

Rysgaard et al. 1999). However, studies on the influence of these factors on the distribution and dynamics of nitrifiers may be complex because different microorganisms compete for common substrate and niche. Previous reports on nitrifying organisms are mostly from the temperate and subtropical estuaries (Bernhard and Bollmann 2010), while tropical estuaries especially those along the southeast Arabian Sea have not received enough attention.

The Cochin estuary (CE) is the second largest wetland ecosystem along the southwest coast of India, covering an area of ~25600 ha, extending from 9° 30' - 10° 12' N to 76° 10' - 76° 29' E. The south-west monsoon (SW monsoon) is the main cause for the seasonal variation in the CE, and the seasons can be discerned as pre-monsoon (February–May), monsoon (June–September) and post-monsoon (October– January). A considerable amount of freshwater is added to the CE during the SW monsoon from precipitation and land runoff. The nutrient composition of the estuary is greatly influenced by anthropogenic and terrestrial inputs from six rivers, seawater influx from two bar mouths and the prolonged monsoon (Menon et al. 2000). The CE also receives high concentrations of industrial effluents (104 x 10^3 m^3 day^{-1}) and untreated domestic wastes (260 m^3 day^{-1}). The reported dissolved nutrient concentration in the CE is high and has increased substantially in the past five decades (Martin et al. 2010). A recent study has classified the CE as a sink of nutrients based on the calculation that only 50 % of the inorganic nitrogen received is being exported to the coastal waters (Martin et al. 2011). It is possible that a considerable fraction of the remaining 50 % is being processed within the estuary by microorganisms. While high nutrient load has been recorded in the CE, the studies related to nitrification are limited to the nitrification rate estimation by Miranda et al (2008) and a recent report on the bacterial domination over archaea in ammonia oxidation by Vipindas et al. (2014). Since the CE is highly dynamic, it is important to understand the influence of environmental

90 factors on the distribution, community structure and activities of nitrifiers and hence the present
91 study was undertaken for a period of one year across a salinity gradient in the water column
92 covering three seasons. We hypothesize that environmental factors have significant but
93 differential role in determining the distribution and activity of nitrifying organisms. This study
94 forms the first report on the distribution and seasonality of nitrifying microorganisms from an
95 Indian estuary.

96 ## Methods

97 *Sample collection*

98 Subsurface (1m below surface) and near-bottom water samples were collected from four
99 stations across a salinity gradient in the CE (Fig. 1), using 5 L capacity Niskin sampler, once in
100 two months for a period of one year from January to December 2011. Stations 1, 2 and 3 are
101 located in the estuary and station 4 is located in the coastal region. Station 1 is low saline
102 (salinity range 0-10) with high freshwater influx while stations 2 and 3 are intermediately saline
103 (salinity range 10 -25). Station 4 is located 19 km away from station 3 with least influence of
104 fresh water and is considered as high saline (salinity range 25- 35) station.
105
106 *Analysis of Environmental parameters*

107 Environmental variables were measured following standard protocols. Salinity was
108 determined using a Digi Auto Salinometer (Model TSK, accuracy ±0.001) and pH using an
109 ELICO LI 610 pH meter. Dissolved oxygen (DO) content was determined following Winkler's
110 titration method (Grasshoff et al. 1983). Samples for nutrients (Ammonia, nitrite, nitrate,
111 phosphate and silicate) were filtered through Whatman No 1 filter paper and estimated
112 spectrophotometrically within six hours of sampling (Grasshoff, et al. 1983). Ammonia was

113 determined following standard indophenols blue method and absorbance was measured at 630
114 nm. Nitrite was determined as the formation of highly colored azo dye (Abs 543 nm) in a
115 reaction mixture containing N-(1-naphthyl)-ethylenediamine and a diazo compound formed
116 through the reaction of nitrite in water samples with sulphanilamide in acidic condition. Nitrate
117 in the water samples was measured after reducing it to nitrite by passing through cadmium-
118 copper column. Phosphate was measured spectrophotometrically (Abs 882 nm) following the
119 reduction of phophomolybdic complex, formed through the reaction of phosphate in water
120 sample with ammonium molybdate, with ascorbic acid. Silicate also measured in the same way,
121 where silicomolybdous complex was reduced with oxalic acid, and the optical density was
122 measured at 810 nm. Suspended particulate matter (SPM) was collected on a pre-combusted 0.45
123 µ GF/F filter paper (Whatman, USA) and measured gravimetrically after achieving constant
124 weight at 70 °C.
125
126 *Abundance of eubacteria, archaea and nitrifiers assemblage*
127 Abundance of eubacteria, total archaea and ammonia- (AOB) and nitrite (NOB) -
128 oxidizing bacteria were quantified using Fluorescent *in situ* hybridization (FISH) following the
129 protocol of Glöckner et al. (1999) with oligonucleotide probes labeled with Cy_3 (Table I).
130 Briefly, paraformaldehyde (final concentration 4 % v/v) preserved water samples were passed
131 through 0.2 µ white polycarbonate membrane filter (Millipore GTTP2500) for bacteria and
132 through 0.1 µ white polycarbonate membrane filter (Millipore VCTP02500) for archaea. The
133 membrane filters were hybridized for 90- 120 min in a hybridization solution (0.9 M NaCl, 20
134 mM Tris HCl (pH 7.4) and 0.01 % SDS) containing 50 ng nucleotide probes and 40 – 60 %
135 formamide (depending on the probe sequence). The cells were counterstained with DAPI to

localize the nuclei and distinguish nonspecific bindings. Fluorescent signals from labeled cells were counted after exciting under an epifluorescence microscope equipped with a 100 W Hg lamp and filter sets specific for DAPI and Cy_3.

Community Structure of AOB and AOA

Extraction of DNA from water samples

Genomic DNA from water samples were extracted following Boström et al. (2004) with slight modification. Briefly, 1 to 2 L of water sample was passed through 0.2 µ polycarobonate membrane filter (Milliipore; GTTP2500), followed by incubation at 37 ^{O}C for 1 hr in lysis buffer (NaCl 400 mM, Sucrose 750 mM, EDTA 20 mM and Tris HCl 50 mM), containing 1 mg ml^{-1} lysozyme. Subsequently, SDS (1 %) and proteinase K (100 µg ml^{-1}) were added to the solution and continued incubation for 5 hr at 55 ^{O}C. Further 0.6 volume of isopropanol was added and DNA was precipitated by keeping at -20 ^{O}C for 60 min. DNA pellet was washed copiously with 70 % ethanol, dissolved in TE buffer and stored at -20 ^{O}C until used.

PCR and denaturing gradient gel electrophoresis (DGGE)

Community structure of AOB and AOA was studied using PCR - DGGE technique. DNA extracted from all water samples collected were subjected to PCR reaction with gene specific primers for AOB and AOA. Briefly, 5 µl DNA sample was used as template for 50 µl PCR reaction mixture containing 2 µl each of primers (10 picomoles μl^{-1}), 5 µl 10X Taq polymerase buffer (NEB, Canada), 1 U Taq DNA polymerase (NEB, Canada) and 200 µM each dNTPs (NEB, Canada). 16S rRNA gene of ammonia oxidizing β-proteobacteria were amplified with an equimolar concentration of three forward primers (CTO189fA -GC and CTO189fB -GC and CTO189fC-GC), each with a GC clamp at 5' end and a reverse primer (CTO654r), containing a

single ambiguous base (Kowalchuk et al. 1997). The forward primers CTO189fA and CTO189fB (GGAGRAAAGCAGGGGATCG) and CTO189fC (GAGGAAAGTAGGGGATCG) were synthesized separately and are collectively referred to as CTO189f-GC. The sequence CTO654r (CTAGCYTTGTAGTTTCAAACGC) was used as reverse primer. The cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 60 sec, annealing at 55 °C for 60 sec, extension at 72 °C for 60 sec and a final extension for 10 min at 72 °C. For amplification of ammonia monoxygenase gene (amoA) of AOA, we used a combination of GC clamp attached forward primer amoAf (CTGAYTGGGCYTGGACATC) and reverse primer amoAr (TTCTTCTTTGTTGCCCAGTA). The GC clamp was attached to the 5' end of forward primer. The cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 45 sec and a final extension for 10 min at 72 °C.

DGGE of amplified PCR products of AOB (465 bp) and AOA (256 bp) were performed with the D-Code universal mutation detection system (Bio-Rad) as per the manufacturer's instructions. DNA concentration in the PCR products was quantified using Nano-drop (Thermo-Fisher) and an equal concentration of DNA per sample (1250 ng) was loaded on the DGGE gel. The PCR products were run on an 8 % and 10 % polyacrylamide denaturing gradient for AOB and AOA gel, respectively prepared with 35 – 50 % (for AOB) and 30 – 55 % (for AOA) denaturing gradient of urea and formamide for 17.5 hr at constant voltage of 75 V in 1× TAE (40 mM tris-HCl, 20 mM acetic acid, 1 mM EDTA). The bands separated were stained with ethidium bromide and observed in a gel documentation system (BioRAD, USA).

Analysis of DGGE band profiles

DGGE gel images were analyzed with Bionumerics software version 4.6 (Applied Maths USA). The software carries out a density profile analysis, detects the bands from each lane and calculates the relative contribution of each band to the total lane intensity. Numbers of operational taxonomic units (OTUs) in each sample were counted as number of DGGE bands. Gels were cross-checked visually as well as for number of bands per lane. An intensity matrix was constructed based on the relative contribution of the band to the total intensity of the lane. The relative intensity of each band was used to calculate the Shannon Wiener diversity index (H'). Cluster analysis of DGGE bands based on square root transformed community data matrix through Bray-Curtis similarity were performed with PRIMER v.6 software package (Ply- mouth Marine Laboratory).

Nitrification Rate

Nitrification rate in water samples were analyzed following the chemical inhibitor method (Bianchi et al. 1994). All the analyses were performed in triplicate. Five hundred millilitre samples were dispensed into nine (1 L capacity) bottles, and divided into three sets. First set was designated as control, while second set received 100 mgL^{-1} of allylthiourea (ATU) an inhibitor of ammonium oxidation and the third set of bottles received $NaClO_3$ (10 mgL^{-1}), an inhibitor of nitrite oxidation. Preliminary experiments were carried out to identify the minimum concentration of inhibitors required to block the respective reactions. All samples were incubated under dark for 36 hr; subsamples (25 ml) were taken at 4 hr interval and analyzed for nitrite concentration following spectrophotometric technique. When ATU was added in to the sample, NH_4^+ stayed relatively constant because of inhibition of NH_4^+ oxidation by ATU while it showed

a linear decrease in NO_2^- with an increase in NO_3^- due to nitrite oxidation. In contrast, there was a linear NO_2^- increase, NH_4^+ decrease and with no change of NO_3^- in the sample when $NaClO_3$ was added, suggesting the effective inhibition of nitrite oxidation by $NaClO_3$. Nitrification rate was calculated based on the accumulation of nitrite concentration in the bottles treated with $NaClO_3$, and the nitrite utilization in bottles supplemented with ATU. Rates were estimated during the exponential phase of the nitrite accumulation or utilization and the results are expressed as μmol N day^{-1}.

Statistical analysis

The environmental and bacterial abundance data were subjected to statistical analysis for significant variation across the sampling period, between the study stations and between subsurface and near-bottom along with their first order interactions using three-way ANOVA and thereafter with student's t test for paired comparison between stations and between seasons to estimate the significance of the spatial and temporal variations of these parameters along with biological parameter (SPSS, V13, Jayalakshmy,1998). Karl Pearson's correlation was used for understanding the relationship between water quality, bacterial abundance and activity. Principal components analysis (PCA), was carried out for understanding the influence of environmental variables on AOB and NOB distribution and nitrification rate. All the variables were normalized and analysis was done based on the correlation matrix using the statistical program PAST version 2.02. The biplot was drawn according to the correlation biplot of Legendre (Legendre and Legendre 1998). Canonical Discriminant Analysis (CDA) was performed for the discriminating stations based on environmental parameters and abundance of microorganisms together and nitrification rate separately. The classification success of the discriminant analysis

228 was checked using jackknifed cross-validation (www.spss.com) technique. To visualize the
229 station differences, first and second factors discriminant scores were plotted. The significance of
230 the environmental and biological parameters in influencing the bacterial abundance was also
231 determined with step up multiple regression model with interaction effects (Jayalakshmy 1998)
232 after normality testing and applying the appropriate transformation using Tukey's test of
233 additivity (Federer 1967).
234
235 ## Results
236 *Environmental characteristics of CE*
237 A marked gradient in salinity (0 to 35) was observed in the CE during the study
238 period (Fig. 2). Average salinity in low saline station was 3.8± 4.3, while it was 15.5± 9.8 in
239 intermediate saline stations and 24.3 ± 8.1 in high saline station. Salinity decreased with the
240 onset of monsoon, reached the minimum during July, slowly increased during post-monsoon and
241 reached the maximum during pre-monsoon at all the stations. DO concentration ranged from 2.2
242 to 7.5 mgl^{-1}. Low levels of DO were recorded in the intermediate saline stations. DO in the CE
243 was higher during the monsoon and lower during the post-monsoon season. Variation in pH was
244 between 6.9 and 8.5. SPM levels varied from 3.2 to 200 mgL^{-1}, with higher concentration during
245 the monsoon. Seasonal and spatial variations of nutrient levels were observed at all the stations.
246 Figure 3 shows the seasonal variation in dissolved inorganic nitrogen (ammonia, nitrite and
247 nitrate). Ammonia was the major component of dissolved inorganic nitrogen and was
248 significantly lower during the peak monsoon period and maximum at the end of pre-monsoon.
249 Ammonia varied from 5.7 to 47.7 µM in the low saline station, 1.3 to 49.0 µM in intermediate
250 saline stations and 0.2 to 28.4 µM in the high saline station. Nitrate and nitrite levels were

relatively higher during the monsoon and lower during the pre-monsoon periods. Nitrate concentration varied between 0.3 and 28.4 µM, while nitrite varied from 0.04 to 0.8 µM. Phosphate concentration ranged from 0.1 to 2.8 with higher values during the monsoon season. The N: P ratio was higher during the pre-monsoon (average 71.9± 62.4) and post-monsoon (average 44.1±32.5) seasons, but relatively lower (11.5±8.7) during the monsoon season. Silicate concentration ranged from 0.01 to 83.5 µM with higher values during the monsoon and lower values during the pre-monsoon period.

Distribution of microorganisms

Eubacteria and archaea

Total microbial abundance estimated by DAPI staining ranged between 7.5 x 10^5 and 1.9 x 10^6, cells ml^{-1}. Spatial and temporal variations of eubacteria and archaea (Fig. 4), enumerated using FISH ranged between 3.3 and 6.9 x10^5 and 2.7 and 5.5 X10^5 cells ml^{-1}, respectively. Maximum abundance of eubacteria and archaea were observed during the pre-monsoon season and minimum during the monsoon season with higher abundance at low and intermediate saline stations.

AOB and NOB

Figure 5 shows spatial and temporal variation in the distribution of AOB (β AOB and *Nitrosococcus mobilis* and NOB (*Nitrospira* and *Nitrobacter*) in the CE. The abundance of β AOB and *N. mobilis* ranged from 3.2 to 9.3 X10^4 and 1 to 4X10^4 cells ml^{-1}, respectively. Spatio-temporal variations in the distribution of NOB were also evident in CE ($P<0.01$, Table II). *Nitrobacter* and *Nitrospira* abundance ranged from 2.7 to 7.6 X10^4 and 2.5 to 6.2 X10^4 cells ml

274 [1], respectively. Higher abundance of AOB and NOB were recorded during pre-monsoon and the
275 lowest during monsoon, indicating significant seasonal variation (P<0.01, Table II). The
276 abundance of AOB and NOB showed heterogeneity between the sampling sites, where spatial
277 and temporal variations were statistically significant (P < 0.01). Significant difference in the
278 abundance was also observed between surface and bottom (P < 0.05) for all nirtrifiers except *N.*
279 *mobilis*. Low and intermediate saline stations showed relatively higher abundance of AOB and
280 NOB compared to high saline station.
281
282 *Influence of environmental variables on distribution of AOB and NOB*
283 AOB and NOB showed highly significant positive correlation with dissolved ammonia
284 (P< 0.01, n = 48) (Table III), while β-AOB showed significant correlation with nitrate also (P<
285 0.01, n = 48). *N. mobilis* and groups of NOB showed significant negative correlation with DO
286 (P< 0.01, n = 48). AOB and NOB distribution did not show any correlation with other nutrients
287 and salinity (P>0.05, n=48). Canonical discriminate analysis (CDA) was carried out to delineate
288 the factors which significantly contributed to discriminate between the stations regardless of the
289 differences observed between surface and bottom and between the seasons. It was observed that
290 all the biological parameters together with all the environmental parameters contributed
291 significantly to discriminate the stations (Fig. 6A). Based on CDA, the sampling stations were
292 uniquely demarcated as coastal (St. 4) and estuarine (St. 1-3) (Wilks lamda= 0.01., calculated $\chi 2$
293 = 173.55) with 48 degrees of freedom. The two eigen values, λ_1 (13.12) and λ_2 (2.87) together
294 explained 94.1% of the variation in the distribution of the various biological and environmental
295 parameters. CDA could classify about 89.6% of the grouped cases and 83.3% of the cross
296 validated grouped cases correctly.

297 Step up multiple regression model (SMRM) with first order interaction effects was
298 applied to determine the biological and environmental parameters that were significantly
299 contributing to the numerical abundance of archaea, eubacteria, AOB and NOB. According to
300 this model, in the estuarine region, the corresponding influencing factors were nitrate, nitrite,
301 ammonia, SPM, silicate and phosphate (72.6% variability explained (VE) for eubacteria; nitrate,
302 nitrite, salinity, DO, and silicate (83.5% VE) for archaea; nitrate, ammonia, salinity, and SPM
303 (90.4%VE) for β-AOB; nitrate, ammonia, salinity, DO, and phosphate (65.9% VE) for *N.*
304 *mobilis*; ammonia, DO, SPM and phosphate (73.5% VE) for *Nitrospira*; and nitrate, nitrite,
305 ammonia, pH, salinity and SPM (86.3% VE) for *Nitrobacter*. In the coastal region, the
306 distribution of eubacteria was mainly controlled by salinity, DO, SPM and silicate (94.2 % VE)
307 while archaea was controlled by salinity, and SPM (86.3 % VE). Among AOB in the coastal
308 region, β-AOB was controlled by salinity, SPM, and silicate (96.44 % VE), while that of *N.*
309 *mobilis* by DO, silicate and PO4 (96.6% VE). Among NOB in the coastal region, the
310 distribution of *Nitrospira* varied depending on nitrite, DO, and SPM (95.5 %VE) while that of
311 *Nitrobacter* by ammonia, DO, and silicate (91% VE).
312
313 *Community Structure of AOB and AOA*
314 Spatial and temporal changes in the DGGE banding pattern and Brey-Curtis similarity
315 cluster for AOA and AOB are shown in Figures 7 and 8, respectively. About 10 to 21 bands were
316 observed in the DGGE gel of AOA with maximum during monsoon. Temporal variation in
317 DGGE banding pattern of AOA was observed in low (Brey-Curtis similarity index up to 60 %)
318 and intermediate (Brey-Curtis similarity index up to 75 %) saline stations, while it was not
319 visible in high saline stations. In the estuarine region, the AOA communities in the monsoon

samples formed a distinct cluster, while such a clustering was not evident in the coastal samples. Five to ten bands were observed in the DGGE of AOB, which did not show significant temporal variation. Shannon Wiener diversity index for AOA was also consistently higher (2.4 to 3.1) than that for AOB (1.2 to 2.2).

Nitrification rate

Nitrification rate was calculated from the accumulation of nitrite in NaClO treated bottles. The observed nitrification rate varied from 0.05 to 10.2 µmol N day^{-1}. Nitrification rate was comparatively higher in the low and intermediate saline stations, which ranged from 0.2 to 8.3 and 0.1 to 10.2 µmol N day^{-1}, respectively, while it was 0.05 to 5.4 µmolN day^{-1} in the high saline station (Fig. 9). Nitrification rates were significantly low during the monsoon at all the stations (0.05 to 0.3 µmol N day^{-1}), which increased during the post- monsoon (0.6 to 4.7 µmol Nday^{-1}) and reached the plateau during the pre-monsoon (5.6 to 10.2 µmol N day^{-1}). Nitrification rate in the CE was positively correlated with abundance of both AOB and NOB ($P< 0.01$, $n = 24$) and also with dissolved ammonia concentration ($P< 0.01$, $n = 24$). Salinity and other environmental factors didn't show any significant correlation with the nitrification rate. CDA analysis performed on the nitrification rates (Fig. 6B) showed no unique station-wise discrimination, while seasonal clusters were visible (Wilks lamda= 0.01., calculated $\chi 2 = 81.64$ with 24 degrees of freedom). The two eigen values, λ_1 (41.76) and λ_2 (3.53) together explained 100 % of the variation in the distribution of various biological and environmental parameters. CDA could classify 100 % of the grouped cases correctly whereas only 91.7 % of the cross validated grouped cases were correctly classified.

Discussion

Nitrification, which is the stepwise conversion of ammonia to nitrite and then to nitrate, is the crucial step in the nitrogen cycle mediated exclusively by microorganisms. In estuaries it is modulated by the complex interplay between different microorganisms and environmental variables which in turn is dictated by various hydrodynamic characteristics like fresh water discharge and seawater influx (Vipindas et al. 2014). The seasonally changing salinity gradient in the CE indicates seawater influx during pre-monsoon and fresh water discharge during monsoon in to the estuary. Large input of nutrients irrespective of seasons has been reported in the CE, and this has been assigned mainly to industrial effluents and domestic sewage (Madhu et al. 2007; Qasim 2003). We also observed higher levels of dissolved inorganic nitrogen in the CE during pre-monsoon (5.4 to 61.5µM) monsoon (8.7 to 32.4 µM) and post-monsoon (10.2 to 57.7 µM) periods. The values were comparatively low in the coastal region (pre-monsoon -3.6 to 28.8 µM, monsoon- 5.9 to 10.2 µM and post-monsoon-7.7 to 33.5 µM). Higher levels of N:P ratio were observed during pre-monsoon (av. 95.5± 120.7) and post-monsoon (av. 37.2 ±26.6), which were significantly above the Redfield stochiometry (16:1). Our observation corroborates the earlier report on nutrient overloading in the CE, leading to eutrophication, which may further intensify in future (Martin et al. 2011). Although the dissolved inorganic nitrogen content observed in the CE was much higher than the reported values from other estuaries of India (Rao and Sarma 2012; Sarma et al. 2010), it was comparable with that of major world estuaries like the Seine estuary in France (Garnier et al. 2006), Schelde estuary in Belgium (De Bie et al. 2002) and California estuary in USA (Boyle et al. 2004).

Distribution of AOB and NOB

366 Estuaries not only act as a transition zone for fresh and marine waters but also for
367 microorganisms from these two different environments, and hence changes in composition and
368 community structure of nitrifiers can be used as a potential bio-indicator of environmental
369 disturbance (Kowalchuk and Stephen 2001). We observed a seasonal and temporal variation in
370 the abundance of AOB and NOB in the CE. The majority of reports on the distribution of
371 nitrifiers from world estuaries are restricted mostly to sediment samples, enumerated using MPN
372 or quantitative PCR technique (Dai et al. 2008; Jin et al. 2011; Mosier and Francis 2008). Hence
373 a direct comparison of our results with water column of world estuaries is difficult. FISH
374 technique used in the present study is widely reported for the enumeration of nitrifiers in sewage
375 systems (Wagner et al. 1996) and anammox in oxygen minimum zone (Schmid et al. 2007). The
376 abundance of nitrifiers in the Pearl River estuary in China, measured using MPN method, has
377 been reported to range from 2 – 4000 cells ml^{-1} (Dai et al. 2008), which is approximately one
378 order less than the nitrifiers counted in CE. We presume that this difference may be due to the
379 limitations of culture dependent techniques, which permits the growth of only actively growing
380 organisms. On the other hand, more sensitive quantitative PCR techniques also have limitations
381 in that it measures the copy number of genes and not the number of organisms. For example
382 AOB having an average of 2.5 gene copy number of amoA gene per organism will give a count
383 of approximately double the number of actual AOB present (Norton et al. 2002). On average, 10^4
384 to 10^7 copy numbers of amoA gene have been reported from a millilitre of water sample from
385 temperate environment (Laanbroek 2013). If the limitations of both MPN and qPCR techniques
386 are accommodated, the abundance of AOB and NOB in the CE matches with previous studies.
387 The distribution pattern and seasonality of AOB and NOB in the CE suggest the coexistence of
388 these organisms, which modulates the entire nitrification process in the estuary. AOB and NOB

showed similar response to important physical and chemical characteristics of the environment. It has been reported that the coexistence of AOB and NOB may create a suitable micro niche that support the growth and activity of each other (Costa et al. 2006). For instance, the nitrite released by AOB could be utilized by NOB. A direct measurement of the abundance of AOA was not done in the present study due to the technical limitations of FISH to measure low copy number functional genes.

In estuaries, the environmental factors co-vary depending on the seawater influx or freshwater discharge and hence it is not a single parameter but the co-influence of different conditions that would determine the distribution or activities of microorganisms. Salinity (Caffrey et al. 2007), ammonia (Cao et al. 2012; Dang et al. 2008; Jones and Hood 1980) and temperature (Iriarte et al. 1997) have been considered as the predominant environmental factors influencing nitrifiers in estuaries, while influence of SPM, pH and other dissolved nutrients have also been discussed in literature (Allison and Prosser 1993; Cébron et al. 2003). Statistical analyses showed that ammonia is the nutrient which has a positive influence on both AOB and NOB in the CE. Ammonia forms the first substrate for initiating the rate limiting step of nitrification; therefore it can influence AOB as a substrate and NOB as a source of substrate and such relations are obvious in estuarine and marine environments (Bouskill et al. 2012; Cao et al. 2012). Ammonia from the watershed reaches the CE through various routes including domestic and industrial wastes and land runoff. The fate of this nutrient in the CE is controlled by a combined effect of river water discharge and flushing. Flushing activity would be inactive in CE during pre-monsoon (Revichandran et al. 2012), while it experiences multiple flushing in addition to heavy rain fall during monsoon. In concurrence with this, the ammonia levels and abundance of AOB and NOB were found to be higher during pre-monsoon and lower during

434 Ammonia oxidation is the rate limiting step in nitrification, and hence we studied the
435 community structure of AOB and AOA. AOB did not show any seasonal variation in DGGE
436 band pattern, which may be because of the higher adaptability of this group of bacteria to
437 varying environmental conditions (Andersson et al. 2006). Although microorganisms respond
438 quickly to environmental changes and their community structure are determined by the
439 environment, certain level of adaptability towards particular changes is also seen among many
440 microorganisms. Temporal stability in the community structure of AOB population has been
441 reported from the Seine estuary, France (Cébron et al. 2004). Interestingly, the AOA population
442 in the CE showed temporal variation in diversity and was higher compared to the DGGE band
443 pattern of AOB. AOA are less adaptable to varying salinity and nutrient levels in estuaries (Liu
444 et al. 2013; Xie et al. 2014), which could be the reason for such seasonal difference in band
445 pattern. The DGGE band diversity and richness of AOA (H' index 2.4 to 3.1) were consistently
446 higher in the CE compared to that of AOB (H' index 1.2 to 2.2). Similar results have been
447 observed in clone library analysis of sediment samples of the Perl river estuary, China (Jin et al.
448 2011), where 36 OTUs were observed for AOA as against 7 OTU for AOB. Other estuaries
449 where similar results have been observed are the Plum Island Sound estuary in USA; the
450 Westerschelde estuary in the Netherlands and the Bahi'a del To'bari in Mexico (Beman and
451 Francis 2006; Bernhard et al. 2010; Sahan and Muyzer 2008). The adaptability and susceptibility
452 of microorganism may play a significant role in nitrification in the nutrient rich and dynamic
453 tropical estuaries (Mosier and Francis 2008).
454
455 *Nitrification rate*

456 The observed nitrification rate of 0.05 to 10.2 μmolday^{-1} in the CE, is comparable with
457 results obtained from various estuaries like the Rhone and Seine estuary in France and the Pearl
458 river estuary in China (Bianchi et al. 1994; Brion et al. 2000; Dai et al. 2008). Higher
459 nitrification rate has been reported from the Schelde estuary in Belgium (Bie et al. 2002), where
460 the dissolved nutrient concentration and salinity were higher. In the samples taken during 2005
461 from the CE, Miranda et al (2008) have observed nitrification rate, lower than the present value.
462 During the past decade, the nutrient input as well as nitrification rate increased substantially in
463 the CE. Miranda et al (2008) observed nitrification rate of about 0 to 4.0 μmolday^{-1}in CE when
464 the dissolved ammonia concentration was up to 20 μM, but during our sampling in 2011 the
465 ammonia concentration reached up to 49 μM. It clearly indicates that anthropogenic input in the
466 estuary has been increasing over time. We observed a significant spatial and temporal variation
467 in nitrification rate ($P<0.01$), which was limited by ammonia, the substrate, and abundance of
468 AOB and NOB. This is in agreement with previous reports from the Elbe estuary in Germany
469 (Stehr et al. 1995) and the Seine estuary in France (Cébron et al. 2003). Similar to the abundance
470 of AOB and NOB, the activity was the highest during pre-monsoon and the lowest during
471 monsoon. A 10 to 40 fold increase in nitrification rate during the pre-monsoon season compared
472 to the monsoon season was observed. The nutrient level in the CE was less during the monsoon
473 season due to heavy inflow of rainwater. A previous study on flushing characteristics of the CE
474 Showed that the estuary flushes ~42 times a year, and thus it would have got freshened many
475 times during monsoon (Revichandran et al. 2012). The increased flushing during the monsoon
476 season along with heavy rain fall might have resulted in dilution of nutrients, in this case
477 ammonia, and hence the abundance of nitrifiers and their activity would have become low during
478 monsoon. On the other hand, the discharge becomes inactive during pre-monsoon, giving more

residence time for nutrients and microorganisms to interact, which resulted in higher abundance of AOB and NOB and the enhanced nitrification rate. High turbidity in the CE also might have supported intense nitrification by providing optimal substrate concentrations and habitat for estuarine nitrifiers (Balls et al. 1996) and reducing the inhibitory effect of light (Merbt et al. 2012).

Seawater influx, i.e. salinity, is considered as one of the major factors controlling nitrification process in many estuaries. In the CE, although no statistically significant correlation between salinity and nitrification rate was seen, it showed a preference to intermediate salinity. This is in agreement with reports from the Scheldt estuary in the Netherlands (Andersson et al. 2006) and the Fjord estuary in Denmark (Rysgaard et al. 1999), where higher nitrification was observed at intermediate salinity. However, differences in the optimum salinity for nitrification rate has been reported from many estuaries; for example low salinity in the Barataria Bay estuary in Mexico (Jones and Hood 1980) and high salinity in the Douro River estuary in Portugal (Magalhães et al. 2005). A recent study has classified the CE as a monsoonal estuary (Revichandran et al. 2012) where the river discharge shows large seasonal variation. Here, we observed that the nitrification in the CE is largely controlled by ammonia levels, which in turn is regulated through freshwater discharge (anthropogenic inputs) and flushing.

The present study reports for the first time the spatial and temporal variations in the abundance and activity of nitrifiers from the CE, a monsoon driven nutrient rich tropical estuary along the southwest coast of India. We observed that the levels of ammonia in the water column have significant influence on the abundance of AOB, NOB and nitrification rate. The DGGE analysis showed that the AOB in the CE are more adapted to varying environmental conditions

compared to AOA. From the study it could be concluded that the CE being a monsoon driven estuary, the nitrification rate and microorganisms involved are greatly influenced by seasonal variation brought in by river water discharge and flushing. Since nitrification rate was found to be increasing with increased nutrient concentration in the CE, the anthropogenic inputs have to be controlled to prevent eutrophication and associated environmental changes.

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716 **Table I.** List of FISH Probes, formamide concentrations and references used in the study

Organisms	*Probe Name*	*Sequence*	*Formamide (%)*	*Reference*
Bacteria	*EUB 338*	*GCTGCCTCCCGTAGGAGT*	*55*	*Amann et al. 1990*
Archaea	*ARCH 915*	*GTGCTCCCCCGCCAATTCCT*	*40*	*Stahl and Amann 1991*
AOB				
β-AOB	*Nso 190*	*CGATCCCCTGCTTTTCTCC*	*55*	*Obarry et al. 1996*
Nitrosococcus mobilis	*NmV*	*TCCTCAGAGACTACGCGG*	*40*	*Pommerening-RāSer et al. 1996*
NOB				
Nitrobacter	*NIT*	*CCTGTGCTCCATGCTCCG*	*45*	*Wagner et al. 1996*
Nitrospira	*Ntspa 712*	*CGCCTTCGCCACCGGCCTTCC*	*40*	*Daims et al. 2000*

717

720 **Table II**. Three way ANOVA and their first order interaction effects between station (A),

721 surface and bottom (B) and months (C)

	Between			**Interaction effect of**		
	Stations(A) (3,15)	Surface and bottom(B)(1,15)	Months (C)5,15	A and B(3,15)	B and C (5,15)	A and C (15,15)
Eubacteria	24.37**	1.05	25.37**	2.44	0.62	6.17**
Archaea	11.71**	1.31	2.12	1.79	0.39	0.91
β- AOB	51.24**	8.58	125.6**	1.06	0.54	10.12**
N. mobilis	4.43*	0.12	11.01**	0.51	0.89	1.61
Nitrobacter	13.44**	40.49**	34.57**	1.99	0.88	2.98
Nitrospira	25.39**	49.91**	49.18**	1.99	3.72	3.77**
Ammonia	5.14**	0.87	17.12**	2.14	0.82	1.94
Nitrite	4.54*	0.75	8.51**	0.51	1.53	2.79
Nitrate	53.42**	9.08**	20.74**	2.13	0.31	31.15**
Salinity	38.53**	16.63**	44.34**	5.34	4.23	6.98
DO	5.99**	14.48**	15.89**	1.46	1.55	3.55
SPM	4.73*	14.55**	4.98**	3.27**	1.16	2.44

722 *- Calculated F statistic is significant at 5% level of significance P<0.05

723 ** - Calculated F statistic is significant at 1% level of significance P<0.01

729

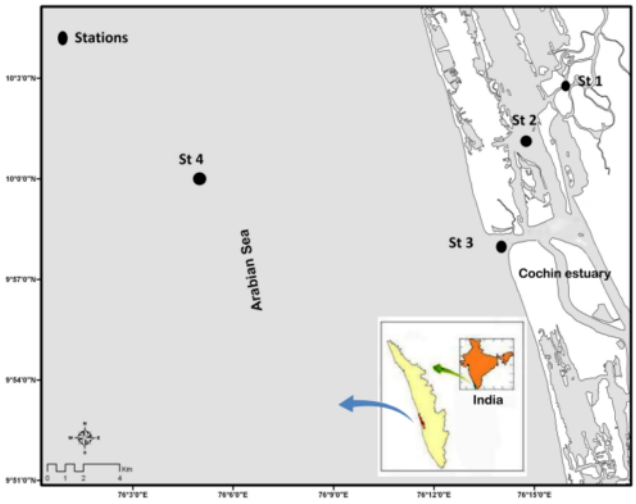
730

731 **Table III.** Correlation between environmental parameter and microorganisms

	Archaea	Eubacteria	β-AOB	*N. mobilis*	*Nitrobacter*	*Nitrospira*
Ammonia	0.31**	0.12	0.63**	0.28**	0.42**	0.33**
Nitrite	0.09	0.20	0.1	0.13	-0.03	-0.02
Nitrate	0.43**	0.40**	0.39**	0.23	0.21	0.18
Salinity	-0.3	-0.49	-0.19	-0.23	-0.13	-0.3**
DO	-0.1	-0.06	-0.03	-0.28**	-0.3**	-0.3**
SPM	-0.2	-0.23	0.15	-0.02	0.04	0.01
Silicate	0.11	0.26	0.13	0.16	-0.03	0.05
Phosphate	-0.1	0.12	-0.18	-0.01	-0.2	-0.2

732 ** Calculated correlation coefficient is significant at 1% level, P< 0.01

Figure 1



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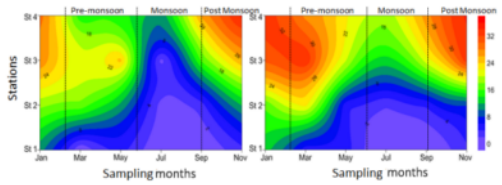

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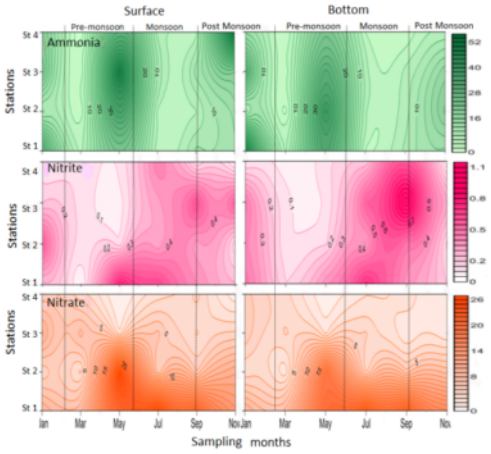

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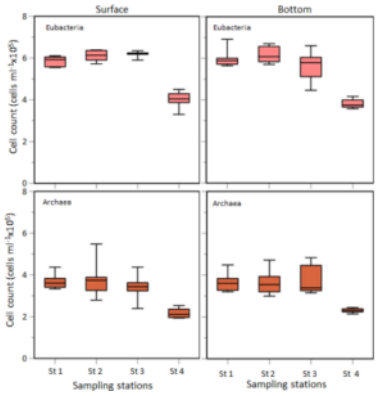

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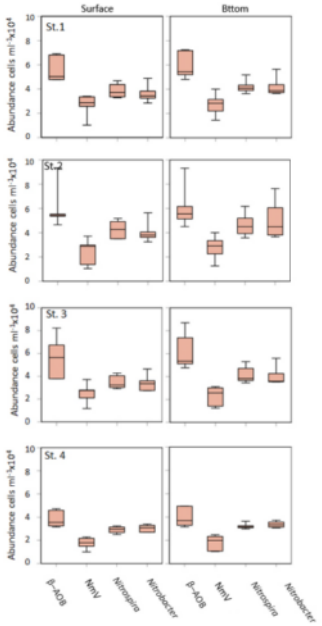

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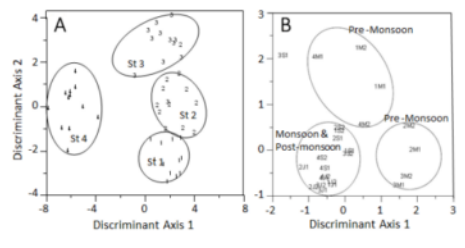

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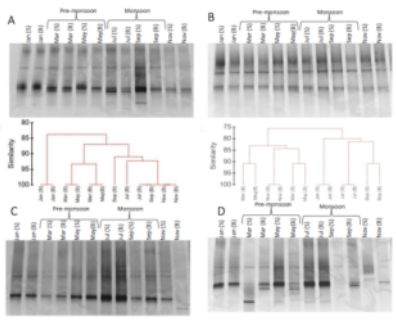

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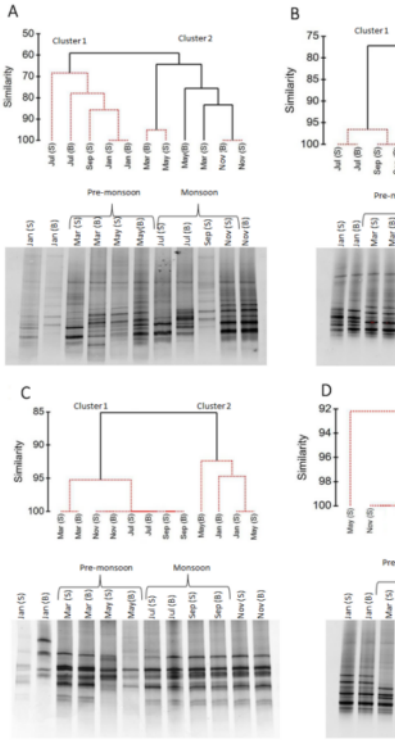

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