Seasonal variations in bacterial biomass and diversity in the Arabian Sea oxygen minimum zone

A Thesis submitted to Goa University for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Marine Sciences

BY

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Taleigao Goa

2018

CERTIFICATE

This is to certify that the thesis entitled “Seasonal variations in bacterial biomass and diversity in the Arabian Sea oxygen minimum zone”, submitted by Mr. Mandar Damodar Bandekar, for the award of the degree of Doctor of Philosophy in Marine Sciences is based on original studies carried out by him under my supervision.

This thesis or any part therefore, has not been previously submitted for any degree or diploma in any universities or Institutions.

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DECLARATION

As required under the University Ordinance OA-19.8 (iv), I hereby declare that the present thesis entitled “Seasonal variations in bacterial biomass and diversity in the Arabian Sea oxygen minimum zone” is my original work carried out in the CSIR-National Institute of Oceanography, Dona Paula, Goa and the same has not been submitted in part or in full elsewhere for any other degree or diploma.

The literature related to the problems analyzed and investigated has been appropriately cited. Due acknowledgements has been made wherever facilities and suggestions has been availed of.

Mandar Damodar Bandekar

STATEMENT

I hereby state that all necessary corrections/modifications as advised by the examiners for my Ph.D thesis entitled “Seasonal variations in bacterial biomass and diversity in the Arabian Sea oxygen minimum zone” are incorporated.

Mandar Bandekar

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Mandar D. Bandekar

DEDICATED TO MY PARENTS

Table of Contents

Chapter 1

General introduction

1.1 Introduction

Vast oceans are a continuum of seawater. In it are 95 known elements in their dissolved forms. Thus seawater is the most complex fluid. Covering over 70% of planet Earth's surface the ocean has unique and strange ecosystems. Among the many uncommon or lesser known in popular science are the oxygen minimum zones (OMZs), the topic of interest of this research. OMZs are water mass at intermediate depths where dissolved oxygen (DO) is less than 20 µM (Stramma et al. 2008). In fact, the OMZs are reported and known to occur in many parts of the world oceans. As reported by Kamykowski and Zentara, (1990) they may be permanent, seasonal or rarely episodic.

The OMZs where, oxygen-depleted waters perennially persist are basins such as the Eastern Tropical North Pacific (ETNP, Wyrtki, 1966), the Eastern Tropical South Pacific (ETSP, Wyrtki, 1966), the Northern Indian Ocean (Wyrtki, 1973, Madhupartap et al. 1996, Naqvi and Jayakumar, 2000), and the Eastern South Atlantic (Karstensen et al. 2008). Be it known that OMZs are not totally devoid of oxygen, but with very low oxygen concentrations, often below the detection limit by both chemical and sensor methods and, is consumed immediately into the system (Stevens and Ulloa, 2008; Ulloa and Pantoja, 2009; Finster and Kjeldsen, 2010).

In spite of their restricted spatial extent, they are of great interest from an ecological and economic perspective. This is because low oxygen concentrations are lethal for most multi-cellular organisms, including fish as proposed by Danovaro et al. (2010) and Seibel (2011). Oxygen-producing photosynthetic organisms dominate marine surface waters and consequently, oxygen-dependent heterotrophic respiration is also by far the predominant

type of respiration in surface waters. As proposed by Orcutt et al. (2011) oxygen is usually present in deeper oceanic waters and close to the seafloor and is preferentially used. Lam and Kuypers, (2011) documented the community in OMZ waters to be diverse, complex, and distinctly different to oxygenated open-ocean and deep-sea communities. Generally, microorganisms living in OMZs are not energy-limited.

A great supply of biomass results from the photosynthetic phytoplankton community in surface waters during upwelling periods when there is entrainment of nutrient rich substrate to the euphotic zone. These reduced organic carbon compounds sinking to deeper waters represent a freely available source of energy for heterotrophic microorganisms. Since oxygen, the most widely used terminal electron acceptor during the respiration of organic matter is scarce, a pronounced OMZ should rather be considered as being limited in this particular terminal electron-acceptor. The scarcity or absence of oxygen leads to use of other oxidized compounds for respiration. Some of the most important compounds are listed in **Table 1.1**. Lam and Kuypers, (2011) described that high concentrations of nitrate (NO_3^-) in OMZ waters, play a greater role in respiration processes in comparison to other compounds which occur typically only in nano molar concentrations. Especially NO_3 and related oxidized nitrogen species like nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) are the second preferred terminal electron-acceptors after oxygen.

1.1 Denitrification and Anammox

Denitrification and anaerobic ammonium oxidation (anammox) (**Figure 1.1**) are the two important alternative respiratory processes used by the microorganisms inhabiting

the OMZs. Denitrification reactions are carried out by denitrifying bacteria by the sequential reduction of nitrate, nitrite, nitric oxide and nitrous oxide reductases specific to these nitrogen species (Crutzen, 1979). The Arabian Sea (AS) OMZ contributes to 20% of denitrification in the ocean affecting the concentration of nitrogen in the ocean (Codispoti et al. 2001).

Recent findings by Kuypers et al. (2005) and Thamdrup et al. (2006) suggest that anammox, coupling the reduction of NO_2 and the oxidation of NH_4 (van de Graaf et al. 1995), is an important contributor to the loss of nitrogen from the OMZ regions. Anammox is dependent on nutrient regeneration by other processes (Ward et al. 2009). Distribution of anammox bacteria in various oxygen-depleted waters and its potential to couple with denitrification, nitrification and dissimilatory nitrate reduction to ammonium emphasizes the critical role of anammox in the global N budget. Both processes account for approximately 30-40% of the fixed nitrogen loss in the global oceans (Codispoti et al. 2001; Castro-Gonzalez and Farias, 2004).

Ward et al. (1989) and Lam and Kuypers, (2011) reported that the close proximity of reduction/oxidation of nitrogen within OMZs and adjacent areas with increasing oxygen concentrations leads to an enhanced cycling of elements in these ocean regions. If a certain threshold of minimal oxygen concentration is reached, the second preferred electron acceptor after oxygen is used (Lam and Kuypers, 2011) and the required set of genes are then exclusively transcribed.

Table 1.1. Common electron acceptors used for respiration of organic matter, corresponding redox partners and concentrations in the world's oceans (Cypionka, 2010 and Lam and Kuypers, 2011).

Electron acceptor	Redox couple	Concentration
Oxygen	O_2 / H_2O	Variable (partly limiting)
Nitrate NO_3	NO_3^- / N_2	30 µM or less (partly limiting)
Manganese dioxide	MnO_2 / Mn_2^+	nM or less (limiting)
Nitrate	NO_3^- / NH_4	30 µM or less (partly limiting)
Iodate	IO_3^- / I^-	0.2-0.5 µM (not limiting)
Ferric oxide	Fe_2O_3 / Fe_2^+	nM or less (limiting)
Sulfate	$SO4_2^-$ / H_2S	28 mM (not limiting)
Carbon dioxide	CO_2 / CH_4	variable (not limiting)

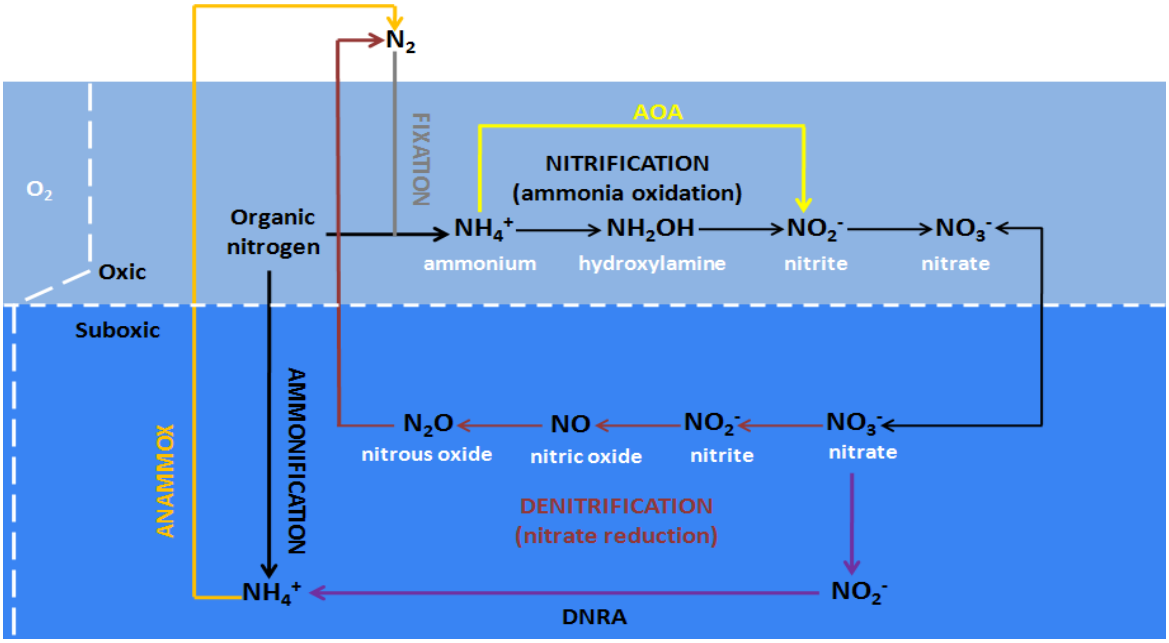


Figure 1.1. Schematic diagram of the nitrogen cycle in the Arabian Sea oxygen minimum zone modified from Colasanti, (2011). Dissimilatory nitrate reduction to ammonium (DNRA) and Ammonium oxidation by archaea (AOA).

1.2 Microbial communities in the OMZ

Handelsman et al. (1998) first formulated the metagenomic concept; it revolves around the sequencing of whole microbial communities of unknown composition, thus bypassing the need for isolation and cultivation of individual species. The acquired sequence information represents the most abundant genes and intergenic regions to be found in the microbial community of that particular environment. DeLong, (2009) states that metagenomic approach tries to correlate the abundance and variability of detected genes to biogeochemical and ecological patterns and processes, namely to the function of the whole environment. This is of major importance, since 99% of all microorganisms are not readily culturable, and in fact, most of the microbial species have never been described (Amann et al. 1995; Pace, 1997; Streit and Schmitz, 2004; Glockner and Joint, 2010). Metagenomic approaches along with sequencing technologies applied to microbial communities of low complexity indeed hold the potential to fully characterize an ecosystem.

1.3 The Northern Indian Ocean OMZ

In the Indian Ocean, the OMZs are found in both the AS and Bay of Bengal (BoB) (Paulmier and Ruiz-Pino, 2009). The BoB-OMZ is weaker than the AS-OMZ, with oxygen concentrations everywhere remaining above the denitrification threshold as reported by Naqvi et al. (2006) and Canfield et al. (2010). The AS-OMZ is the second-most intense OMZ in the world tropical ocean as reported by Kamykowski and Zentara, (1990), with near-total depletion of oxygen in the intermediate depths of 150-1000 m (Morrison et al. 1998). The AS-OMZ is driven by the upwelling of nutrient-rich waters as documented by Friederich and Codispoti, (1987); Helly and Levin, (2004); Karstensen et al. (2008); and

Ulloa and Pantoja, (2009). Naqvi, (1994) and Naqvi et al. (1998, 2008) details that, the AS-OMZ coincides with active denitrification zone and contributes to 40% of N_2 production. Microorganisms, inhabiting AS-OMZ (Riemann et al. 1999) are poorly addressed except for a few studies by Riemann et al. (1999); Fuchs et al. (2005); Divya et al. (2010, 2011) and Jain et al. (2014). A US Joint Global Ocean Flux Study (JGOFS) in the AS reported that organism distribution is strongly influenced by oxygen concentrations, especially in regions where OMZ is prominent. Due to the impact of these processes on the global N cycle and their sensitivity to environmental conditions, it is important to study the overall microbial ecology of the system and their role in the biogeochemical cycles.

In this context, the main aim of this research was to contribute to a better understanding of a more complete picture of bacterial diversity within the AS-OMZ in order to delineate the overall community structure and dynamics of natural assemblages. Through sequencing of *16S rRNA* and functional genes, diversity of unculturable bacterial community in the AS-OMZ, in regard to its seasonal and vertical variations was studied. In addition to measuring bacterial biomass, qPCR analysis was used to examine/evaluate the distribution of denitrifying and anammox bacteria necessary to facilitate bacteria-mediated denitrification and anammox processes.

1.4 Objectives

The objectives set forth for this study were based on the recognition that while AS-OMZ is acknowledged as among the larger OMZ region in the world oceans, the microbial community analyses are rather small and in many ways understudied. Hence, a focused investigation covering temporal variability as well as different depth strata at a regularly

monitored station, namely Arabian Sea Time Series (ASTS) station (17°0N and 67°59 E) was planned and executed for this study.

1. **To understand seasonal differences in bacterial biomass and phylogenetic diversity in the Arabian Sea Oxygen minimum zone region**

   The rationale behind this objective was to recognize the prevalence and abundance of uncultured population of bacteria and to realize the effect of seasonal variations on them. It is to be underlined here that any study on the diversity of bacteria in OMZ will prove useful for an understanding of different bacterial groups involved in different processes happening in the OMZ. Also such analyses bring forth whether there is any seasonal bearing on the diversity and abundance of community inhabiting the OMZ. Keeping in view of paucity of data on the overall bacterial diversity from the AS-OMZ region, this objective was planned to be pursued.

2. **To assess the functional diversity of major genes involved in denitrification and anammox processes**

   While it is essential to know the overall bacterial community structure (BCS) and its vertical and temporal distribution patterns, any attempt to get insights on what are the possible functional roles of the community is essential. Particularly so when the metagenomic based BCS description is the mode of diversity analyses. It is important to identify and understand the role of denitrifying and anammox bacteria, as presence of each of bacterial group are indicative of the on-going process in the OMZ. Diversity analyses based on functional genes will result in the elucidation of specific bacterial groups involved in different stages of denitrification and anammox process. Also, data

obtained during different seasons will help in elucidating the ideal conditions required for the better performance of the community.

3. **Quantitative analyses of denitrification and anammox genes through quantitative PCR**

It is hypothesized that detection and quantification of key genes involved in the processes of denitrification and anammox would yield valuable information on the role of these processes in the overall denitrification process in particular in nitrous oxide production. Such information based on quantitative analyses can be linked directly to the dominant bacterial groups involved in the process analyzed/looked for. It was therefore thought to get a sense on the probable dominant processes particularly from the AS-OMZ. Further, quantitative analyses carried out on the DNA extracted from various depths during different seasons would provide a comprehensive idea on the effect of seasonal variation on the bacterial community mediating the denitrifying and anammox processes.

Chapter 2

Literature review

2.1 Preamble

This chapter covers the major and relevant literature pertaining to the characteristics of oxygen minimum zone (OMZs), microbial communities so far reported from OMZ in the global oceans and the possible/putative ecological roles played by the OMZ microbial assemblages

Permanent hypoxic conditions in marine waters, now known as the OMZ were first recognized in 1872-1876 during the Challenger Expedition (Dittmar, 1884). High sulphur content in the shelf of Walvis Bay reported during Meteor Expedition in 1925-27 was ascribed to decomposition of a dead whale. Murray Expedition in 1933-34 led to the discovery of OMZ in the Arabian Sea (AS). Wyrtki, (1962 and 1966), published particulars of the anoxia in World Ocean and in the Eastern Pacific.

Cline and Richards, (1972) coined the term "Oxygen Minimum Zones" to the regions in the world ocean, where oxygen saturation in the water column is at its lowest. This zone typically occurs in areas of upwelling and poor water circulation, where oxygen concentration is less than 2 mg L^{-1} (Paulmier and Ruiz-Pino, 2009). The OMZs are separated from each other by large geographic distance, continental barriers and by oxygenic waters. Water in the OMZ is exposed to the rain of sinking organic matter. Communities of bacteria and archaea adopted to inhabit the OMZ feed on this organic matter exhausting most of the *insitu* oxygen and thus utilizing the other electron acceptors.

OMZs occur in the world's oceans due to combination of factors. Due to mixing of surface waters by wind and wave action, oxygen levels at the sea atmosphere boundary is

relatively high, nevertheless increase in depth causes the oxygen level to diminish. Friederich and Codispoti, (1987) documented that high primary production in surface waters of the OMZ is driven by the upwelling of nutrient-rich waters. Upwelling transports cold, dense and nutrient-rich deep waters from the poles towards the ocean surface, replacing the warm and usually nutrient-depleted surface water resulting in a stratification of levels of dissolved oxygen (DO) within the oceans with zones of low oxygen (Helly and Levin, 2004; Wyrtki, 1962; Kamykowski and Zentara, 1991). This input of nutrients into photic surface waters enables high photosynthetic biomass production by the phytoplankton community. Significant proportion of biomass/organic material sinks out of the surface layer and is remineralized via microbial respiration at the intermediate depths (between 150-1000 m). This leads to severe oxygen depletion and permanent OMZs (Wyrtki, 1962; Dugdale, 1977). However, oxygen levels in the deep ocean are actually high. Thus, the OMZs are controlled by 3 main factors; (a) ocean temperature (dictates how soluble the oxygen is within the water), (b) concentration of organic matter (Higher concentration means more and rapid consumption of oxygen) and (c) abundance of bacteria which dictates how much oxygen gets consumed due to their metabolism of the organic matter. Sarmiento et al. (1988) suggested that sluggish water movement is among the other causes leading to persistence of the OMZs.

Stramma et al. (2008) reported that oxygen-depleted waters are predicted to increase in both frequency and size. One major reason is enhanced nutrient input due to anthropogenic activity (e.g. use of artificial fertilizers in farming), which results in the eutrophication of coastal waters, and thus enhances surface water productivity (Naqvi et al. 2000). Secondly, ocean warming in the course of global climate change will decrease

the solubility of oxygen. In addition to this effect, the predicted increase in surface water temperatures will also intensify the stratification of the ocean, which leads to a reduced gas exchange of surface with subsurface waters, eventually reducing the transfer of oxygen to deeper waters (Sarmiento et al. 1998; Grantham et al. 2004). Thus, the future oceans might potentially suffer a decrease in fish populations and other commercially important aquatic species, which could lead to under supply of seafood in coastal areas.

Kamykowski and Zentara, (1991) documented that the OMZs may be permanent (open ocean), seasonal or episodic (coastal). The OMZs are interesting because of their importance in controlling carbon and nitrogen cycling in the oceans. Helly and Levin, (2004) reported that 2% of the continental margin is intercepted by the OMZs. Of the total OMZ area, Indian Ocean, Eastern Pacific Ocean and South East Atlantic Ocean harbor 59%, 31% and 10% area respectively.

2.2 Permanent OMZs of the World Oceans

Paulmier and Ruiz-Pino, (2009) reported that the permanent OMZs are extending and currently cover 8% or 30.4 million km^2 of the World Ocean. The five main permanent OMZs (**Figure 2.1**) are found in the intermediate depths of Eastern Tropical North Pacific (ETNP, Wyrtki, 1966), Eastern Tropical South Pacific (ETSP, Wyrtki, 1966), Eastern Tropical South Atlantic (Karstensen et al. 2008) Arabian Sea (AS) and Bay of Bengal (BoB) (Wyrtki, 1973; Madhupartap et al. 1996; Naqvi and Jayakumar, 2000).

In the Pacific Ocean there are two large OMZ regions; one in the North Pacific off Central America, the ETNP-OMZ and one in the South Pacific off Peru and Chile, the

ETSP-OMZ (**Figure 2.2)**. Both the OMZs reach far into the central Pacific with the minimum oxygen values below 4.5 μmol kg^{-1} (0.1 ml l^{-1}). The ETNP-OMZ is one of the better studied OMZ in the ocean. This zone has been surveyed numerous times since the 1960s, with oxygen concentration measurements taken as far down as 1500 m (Richards 1965). An analysis of oxygen concentration measurements in the ETNP made by Cline and Richards (1972) identified a 600 m thick layer of water with almost no detectable oxygen. The ETSP-OMZ, one of the most extended OMZs (Kamykowski and Zentara, 1991; Helly and Levin, 2004), is today a permanent feature covering the areas off shore Peru and Chile. Morales et al. (1999) studied the OMZ off northern Chile focusing on the variability of the 40 μM oxygen isoclines. The entire Chilean OMZ thickness has not yet been documented. Anderson et al. (1982) suggests, that the oxygen concentrations can go lower than 40 μM, reaching oxygen <10 μM as in Peru, and can extend down to 400 m depth.

In the tropics, OMZs are located in poorly ventilated mid-depth layers. In the Indian Ocean, the OMZ is located mainly in the Northern hemisphere, where the ventilation age is 30 years or longer due to the closed northern boundaries (Fine et al. 2008). Kamykowski and Zentara, (1991) reported the AS-OMZ to be the second-most intense OMZ of the tropical regions. The BoB-OMZ is weaker than the AS-OMZ, with oxygen concentrations above the denitrification threshold (Naqvi et al. 2006). As per the reports of Tomczak and Godfrey, (1994), low oxygen values indicate a slow renewal rate of thermocline waters in the Northern Indian Ocean. In the Eastern Indian Ocean, a weak OMZ reaches across the equator off Indonesia. As in the Pacific, Howell et al. (1997) observed suboxic conditions with oxygen values below 4.5 μmol kg^{-1} occur in the AS (**Figure 2.2)**.

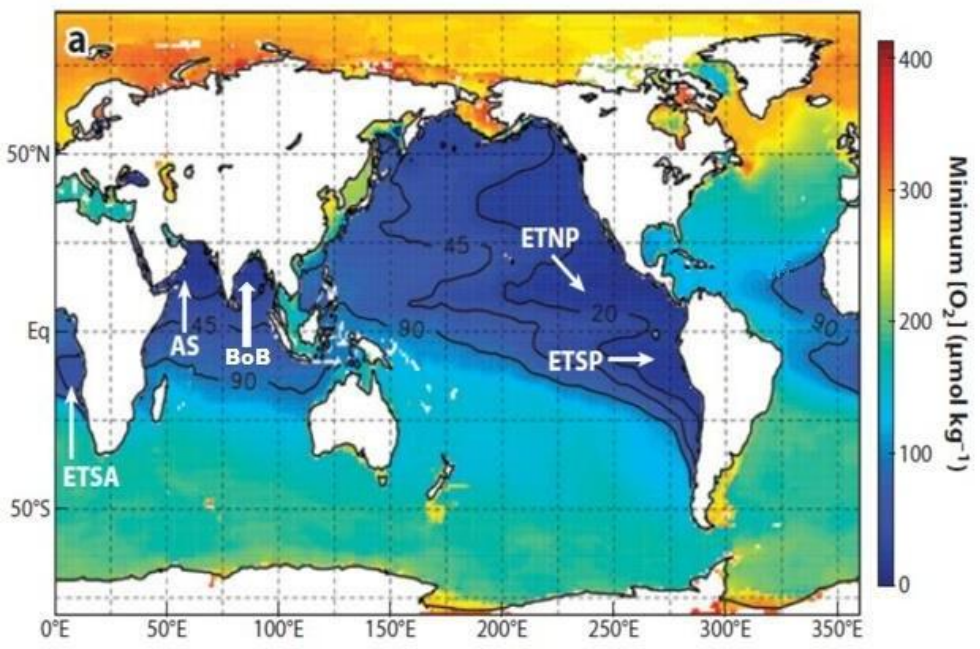


Figure 2.1. Oxygen minimum zones of the world oceans. Arabian Sea (AS), Bay of Bengal (BoB), Eastern Tropical South Pacific (ETSP), Eastern Tropical North Pacific (ETNP) and Eastern Tropical South Atlantic (ETSA). Contour lines depict minimum oxygen concentrations (µmol kg^{-1}) in vertical water column (modified from Lam and Kuypers, 2011).

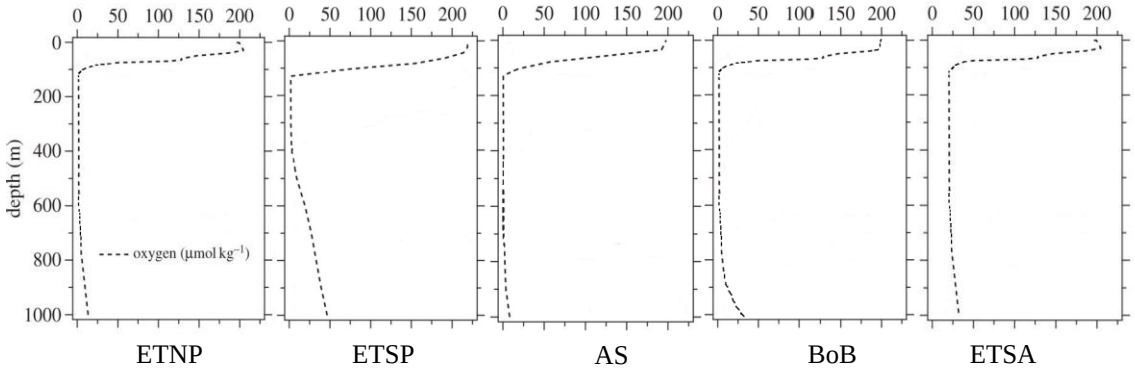


Figure 2.2. Vertical profiles of oxygen concentration in the world OMZs. Abbreviation explained in Figure 2.1.

Extended horizontal OMZs exist in the Eastern Tropical Atlantic in the depth range 200 to 800 m. The OMZ of the Eastern Atlantic is not suboxic and has relatively high oxygen minimum values of about 17 μmol kg^{-1} in the South Atlantic and more than 40 μmol kg^{-1} in the North Atlantic. The abundance of OMZs is considerably smaller (1% and 7%) for the South Atlantic and only 0% and 5% for the North Atlantic. Although more oxygenated with minimum values on the order of 20–40 μmol kg^{-1} the spatial distribution in the Atlantic is similar to that in the Pacific with the OMZ on the eastern side to the north and south of the equator. According to a theory proposed by Cannariato and Kennett, (1999), OMZs in the water column are expanding owing to increasing temperatures.

2.3 Biogeochemical characteristics of the OMZs

Nitrate (NO_3^-), phosphate and silicate concentrations are reported to be generally low in surface waters as they are consumed during the biological production. Below the surface layer, their concentrations increase since the organic material decomposition occurs and the released materials accumulate in subsurface waters with time. Nitrate reaches its maximal values in the mid-layers of the ocean where bacterial decomposition and its non-utility are high. NO_3^- and related oxidized nitrogen species like nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) are the second preferred terminal electron-acceptors after oxygen. Thus these electron acceptors facilitate anaerobic processes such as denitrification and anaerobic ammonium oxidation (anammox) in these layers. High concentrations of nitrate (NO_3^-) in seawater play a greater role in respiration process, than iodate (IO_3), manganese dioxide (MnO_2) and ferric oxide (Fe_2O_3), which occur typically only in nanomolar concentrations and are considered to be limiting (Lam and Kuypers, 2011). A

special characteristic of oxidized nitrogen species, in addition to their function as electron acceptors is that they also serve as a major and essential nutrient for growth and the building-up of biomass. Thus, a lack in nitrogen will also limit biomass production.

Reduction of oxidized nitrogen during respiration of organic matter is a stepwise process known as heterotrophic denitrification ($NO_3 \rightarrow NO_2 \rightarrow NO \rightarrow N_2O \rightarrow N_2$) which results in the formation of dinitrogen gas (N_2). This reaction is mediated by enzymes such as nitrite reductases (*nirS, nirK*) nitrate reductases (*narG, napA*) and nitrous oxide reductases (*nosZ*). N_2 is relatively inert and not available as a nutrient for most living organisms, except for those few organisms (diazotrophs), which are capable of reducing N_2 gas and form ammonia (NH_4^+). Eventually, through heterotrophic denitrification processes (Emery et al. 1955; Codispoti et al. 2001), the ocean losses fixed nitrogen to the atmosphere. Naqvi et al. (1990) reported denitrification and anammox in the intermediate depths of (150-1200 m) of the AS. Ward et al. (2009), Bulow et al. (2010) and Dalsgaard et al. (2012) reported denitrification as an important process for the loss of fixed nitrogen to in the AS-OMZ. Recent studies by Jayakumar et al. (2013) have explored *nir* gene diversity in the AS-OMZ and the ETSP.

Mulder et al. (1995) was the person to discover anammox. Thamdrup and Dalsgaard, (2002); Dalsgaard et al. (2005) reported the presence of anammox process in marine sediments and suboxic water columns. In contrast to the denitrification, van de Graaf et al. (1995) reported that anammox is a biochemical pathway in the microbial N cycle that allows coupling between ammonium oxidation with nitrite reduction through a hydrazine intermediate (Strous et al. 1998). Anammox bacteria are mostly chemotrophs

and consume NH_4 and NO_2 in respiration and form biomass by CO_2 fixation. Recent reports by Thamdrup et al. (2006) suggest anammox to be the lone process responsible for loss of nitrogen at ETSP. Lam et al. (2009), Kartal et al. (2007) and Jensen et al. (2011) suggest dissimilatory nitrate reduction to ammonium (DNRA) as important process for anammox in the OMZ of Peru and Namibia.

2.4 Macrofauna and microfauna in the OMZ

Extensive work is carried out on the biology of the OMZ. Abundance of zooplankton and feeding ecology of copepods in the tropical Pacific Ocean OMZ was studied by Wishner et al. (1995) and Gowing and Wishner, (1998). Inter-annual and seasonal variation of mesozooplankton was studied by Escribano et al. (2007) in the coastal upwelling zone off Chile. Escribano and Hidalgo, (2000) studied distribution of copepods and lipid profiles of the deep sea shrimp of the same location was reported by Allen et al. (2000). Distribution of mesopelagic fishes in the OMZ was investigated by Hunter et al. (1990). Studies on primary production in the Peruvian upwelling system by Fernandez et al. (2009) indicated that nitrogen and ammonia regenerated in euphotic layer which was made available for primary producers.

Benthic foraminifer generally found abundant in the OMZ regions has been an area of interest for most researchers and was first studied by Hermelin and Shimmield, (1990). The meiofaunal distribution and bioturbation in OMZ sediments is investigated in detail by Neira et al. (2001a). A number of novel benthic species have been identified by Oliver, (2001); Neira et al. *(*2001b) and Oliver and Levin, (2006). Diversity of benthic organisms found within the OMZ was lesser as reported by Quiroga et al. (2005). Effect of dissolved

oxygen concentration, sediment geochemistry and organic matter gradient on benthic macro-organisms is studied extensively by Levin, (2003). Within OMZs, macrobial life is well documented by. Wishner et al. (1995) report that the unique chemical characteristics of the OMZs to be the cause of low macrofauna diversity.

2.5 Prokaryotic studies in the OMZ

Although the diversity of macrobiota is low, bacterial abundance and diversity within the OMZs is apparently higher than in the adjacent upper and lower layers. Stevens and Ulloa (2008) delineated the bacterial community from the ETSP through 16S rRNA sequencing. In the OMZ waters, predominance of *Gammaproteobacteria*, SAR11, *Chloroflexi, Deltaproteobacteria, Acidobacteria* and *Planctomycetes* was evidenced. *Loktanella, Flavobacterium, Sulfitobacterium,* and *Alteromonas* were dominant in the non-OMZ columns. Longnecker et al. (2005) used denaturing gradient gel electrophoresis (DGGE) to study diversity of bacteria. Stewart et al. (2012) carried out a metatranscriptomic survey of the microbial community using high through put sequencing in the ETSP-OMZ. The upper layers were dominated by ammonia oxidizing archaea *Nitrosopumilus maritimus* and the OMZ transcripts were dominated by anammox bacterium, *Kuenenia stuttgartiensis*. Bryant et al. (2012) described that the microbial community, phylogenetic diversity and diversity of protein-coding gene analysed from the ETSP OMZ decreased with depth. Picoplankton composition from the AS-OMZ determined by Fuchs et al. (2005) showed that surface waters were dominated by uncultured group Svalbard.

Most prokaryotic studies in the OMZ are restricted to biogeochemical processes. In

the OMZ, nitrate is reduced to nitrogen forming nitrite in the process (Codispoti and Christensen 1985). Suzuki and Delong, (2002) proposed that prokaryotic microorganisms are present throughout the marine environment. Eubacteria of prokaryotes are the key players in various biogeochemical cycles and mediate the normal functioning of the ecosystem. Bailey (1991) reported high rates of primary production and reduction of sulphate by bacteria off central Namibia. Kuypers et al. (2005) also reported the massive loss of fixed nitrogen by anammox from this region. Molina et al. (2005) reported oxycline prokaryotes to be responsible for ammonium cycling in OMZ water column off Chile. Francis et al. (2005) reported the presence of ammonia oxidizing archaea in the OMZ water column. Diversity analysis of anammox bacteria in OMZs of AS showed the presence of *Candidatus Scalindua.* Woebken et al. (2007), observed *Scalindua* sp. followed by *Gammaproteobacteria, Alphaproteobacteria* and *Bacteriodetes* as the foremost group in the Namibian OMZ.

Castro-Gonzalez et al. (2005) studied the denitrifying bacterial communities from the ETSP-OMZ through the construction of *nirS* clone libraries. In this study the authors observed *nirS* sequences with high similarity to the sequences of *Paracoccus*, *Roseobacter, Pseudomonas, Marinobacter* and *Halomonas*. 16S rDNA clone library analysis of denitrifying genes by Liu et al. (2003b) in the oxygen-deficient zone off Mexico indicated that *nirS* sequences were affiliated to *Alcaligenes faecalis* and *Pseudomonas stutzeri*, whereas *nirK* clones were related to *Pseudomonas* sp. and *Alcaligenes xylosoxidans*. Jaeschke et al. (2010) study from the sediments of northwest Africa suggests that the abundance of anammox bacteria is higher in sediments at intermediate to deep water depths with lower rates of carbon mineralization. Sulphate reducing bacteria studied by Liu et al.

(2003a) in the sediments of eastern Pacific revealed that *dsrA* sequences clustered with *Desulfobulbus propionicus*, *Desulfosarcina variabilis* and *Desulfotomaculum putei*.

Quantitative study by Schippers and Neretin, (2006) on sulphate reducing, iron-reducing and manganese-reducing bacteria revealed that these bacteria dominate the surface and deeper sections of sediments. Bacterial sequences from sediments along the mid-Chilean margin were closely affiliated to *Desulfosarcina variabilis* (Hamdan et al. 2008). Walsh et al. (2009) reported the presence of SUP05, which was related to clams and mussels and multiple genes responsive to different redox states.

Contrary to bacteria, the archaeal community composition in the OMZs is less explored. Archaeal studies by Vetriani et al. (1999) discovered the presence of two main groups namely *Crenarchaeota* and *Euryarchaeota*. Euryarchaeal MGII and MGIII are the most common groups found in oxygen deficient zones. Majority of the sequences obtained from the coastal and open ocean OMZs are affiliated to cluster MGII. MGIII is less predominant in the OMZs and reported by Belmar et al. (2011) in the ETSP-OMZ.

Schafer et al. (2007), reported archaeal Marine Benthic Group D and C as the dominant groups. Presence of archaea from the ETSP was highlighted by Belmar et al. (2011). Small subunit ribosomal RNA gene (SSU rRNA) sequence data from the oxic/anoxic chemocline of the Black Sea reported the presence of archaea (Coolen et al. 2007). Suboxic zone of the Baltic Sea (Labrenz et al. 2010) reveal that putative nitrifying assemblages consisted of Crenarchaeota related closely to *Candidatus Nitrosopumilus maritimus*. Presence of ammonia-oxidizing archaea (AOA) in sediments and suspended particles of the Black Sea in the Namibian upwelling system was reported by Kuypers et

al. (2003); Francis et al. (2005); Coolen et al. (2007) and Woebken et al. (2007) respectively.

2.6 Arabian Sea oxygen minimum zone

The AS-OMZ was discovered in 1933-34 during Murray Expedition (Gage et al. 2000). The AS-OMZ is about 750 m thick extending up to an area of 2.5 million km^2. Oxygen depleted waters from the Persian Gulf forms the core of the OMZ. The AS, a biologically productive tropical basin, is among the significant suboxic regions in the world oceans. A permanent feature in its northeastern part is the oxygen-deficient waters in the intermediate depths (~150-1000 m column). Characteristically, the seasonally reversing southwest monsoons (SWM: June-September) and northeast monsoons (NEM: December-February), offshore upwelling and winter cooling fuel high biological productivity (Madhupratap et al. 1996) and organic carbon production (Hansell and Peltzer, 1998). Naqvi (1999) reported denitrification as the major cause for the loss of the fixed nitrogen in the AS-OMZ, particularly during SWM and NEM and is responsible for loss upto 60 Tg of nitrogen annually contributing to ~40% of the global pelagic N loss (Codispoti, 2007).

Salient features of AS-OMZ

- Largest suboxic regions with Dissolved oxygen (DO) levels below 0.5 ml L^{-1}
- Oxygen concentrations reaching as low as 0.1 μM in a vertical depth of 150 to 1000 m
- Major contribution to oceanic denitrification.
- Accounts for 40% of the global pelagic dinitrogen (N_2) production (Naqvi et al. 2008)

- Two major biological processes, heterotrophic denitrification and autotrophic anaerobic ammonia oxidation (anammox) are so far recognized.

2.7 Studies so far

A large volume of data on chemical characteristics of the AS-OMZ is collected during the IIOE (McGill 1973). Sengupta et al. (1975) investigated the quantitative relationships between nutrients and oxygen. Studies by Naqvi et al. (1987) revealed that most of the nitrate is lost during denitrification. Since then, a lot of studies have been carried out to quantitatively evaluate the rates of denitrification in the AS-OMZ.

The US JGOFS (Joint Global Ocean Flux Study) Arabian Sea Process Study over the entire monsoonal cycle of 1995 generated high quality dataset for understanding the conditions of the AS-OMZ. Results of other researchers were in agreement with the data sets from the US JGOFS. AS-OMZ is majorly responsible for production of nitrous oxide which to leads of anoxia and subsequently to denitrifying conditions (Naqvi and Noronha, 1991; Naqvi et al. 2000)

Within the OMZ, denitrifying and anammox bacteria convert nitrate to gaseous nitrogen disturbing the nitrogen budget (Naqvi et al. 1998). The role of microbial communities in the AS-OMZ is vital for elucidating the microbes-mediated global biogeochemical and climatic processes as detailed by Stewart et al. (2012) and Ulloa et al. (2012). Previous studies in the AS-OMZ focuses on anammox and denitrification bacteria (Jayakumar et al. 2009, Ward et al. 2009, Bulow et al. 2010, Pitcher et al. 2011) and general characterization of heterotrophic bacterial production (Ramaiah et al. 1996, 2000). Only a

few studies so far have focused on documenting the bacterial diversity from the sediments (Divya et al. 2011, 2017) and water column (Fuchs et al. 2005, Jain et al. 2014) of the AS-OMZ.

Molecular taxonomic analyses have reported the presence of marine archaeal assemblages in suboxic/anoxic pelagic waters which get enriched under such habitats (Karner et al. 2001, Coolen et al. 2007). *Crenarchaeota* (G-I.1a), *Euryarchaeota* (Marine Group II, MG-II) and *Thaumarchaeota* (G-I.1b) contribute significantly to marine carbon and nitrogen cycles (Francis et al. 2005, Ingalls et al. 2006, Brochier et al. 2008, Belmar et al. 2011). They are reported to be present in open ocean water column, marine sediments and estuarine and coastal sediments (Francis et al. 2005). However, the *16S rRNA* gene-based archaeal diversity analyses from the AS-OMZ are not many (Singh et al. 2010, Singh, 2013).

Jayakumar et al. (2009) observation of high diversity of *nirS* gene in comparison to regions with undetectable nitrite concentrations was in agreement with the findings of Ward et al. (2009) and Bulow et al. (2010). Jayakumar et al. (2009) found that denitrifying bacteria are widely distributed and show striking changes in diversity in association with the progression of denitrification. Woebken et al. (2008) reported that anammox bacteria are less diverse in comparison to denitrifying bacteria and are represented by one or two phylotypes.

Clones of *nirS* and *nirK* gene obtained by Jayakumar et al. (2009) from the AS-OMZ were closely related to the sequences of cultivated denitrifier *Pseudomonas aeruginosa*. The bacterial community data from the US JGOFS in the AS-OMZ reported

the dominance of SAR11 of *Alphaproteobacteria* and Cyanobacteria. Majority of the sequences from Riemann et al. (1999) study in the AS-OMZ were affiliated to *Gammaproteobacteria, Alphaproteobacteria* and *Bacteriodetes*. Magnetotactic bacteria were first reported in AS-OMZ also by Riemann et al. (1999).

Organic matter from the water column accumulates on the sea bed (Paropkari et a1.1992; 1993) causing the sea floor to be hypoxic which persists for many years (Wyrtki 1962; Kamykowski and Zentara, 1991; Helly and Levin, 2004). Hermelin (1992) suggests that the sediment pore is affected by the low levels of oxygen. Cirratulid mud balls in the northwest AS-OMZ was reported by Levin and Edesa (1997). DO is assumed to have an effect on the benthic foraminifera of the AS (Kurbjeweit et al. 2000). Effect of oxygen on benthic foraminifera from the AS-OMZ was studied by Panchang et al. (2006). Gooday and Bowser (2005) and Oliver (2001) reported the presence of gromiid protist, *Gromia pyriformis* and *Amygdalum anoxicolum*, respectively in the AS-OMZ. Ingole et al. (2010) found Polychetes as the dominant macro fauna in the sediments of the AS-OMZ. Discovery of *Thioploca* sp. in sediments of northern AS by Gallardo et al. (1998) suggests that bacteria play a significant role in the transformation of sulphur and this observation is supported by studies of Schmaljohann et al. (2001). Boetius and Lotche, (2000) enumerated the total microbial biomass in the sediments of the AS.

Previous studies have investigated the composition of denitrifiers and anammox assemblages in the OMZ using *16S rRNA* sequences and by analyzing markers genes involved in the denitrification process and anammox (Jayakumar et al. 2009). However, most of these studies were carried out on culturable bacteria isolated mostly from sediments

in the OMZ region. Given that most marine bacteria are unculturable; these studies only captured a partial picture of microbial community composition of the OMZ.

Metagenomics is the most widely used technique to study the total diversity, physiology, ecology and phylogeny of prokaryotes. This method involves isolation of DNA from environmental samples, amplification of targeted gene and construction of clone libraries eluding cultivation in the laboratory (Lorenz and Schleper, 2002; Rondon et al., 2000; Steele and Streit, 2005). The metagenomic approach is aimed to understand the link between communities in natural ecosystems and also to exploit the unknown microbial diversity. Metagenomic sequences help to understand that how complex microbial communities function and how microbes interact within these niches. This technique has very promising approach to understand the physiology of uncultured in natural environments.

From the foregoing, it can be summarized that considerable gaps exist in our knowledge of AS-OMZ microbial community. Thus OMZ, in the Indian Ocean are important frontiers for discovery of new clades of bacteria and archaea and the processes they are involved in. Pelagic studies in the AS-OMZ are limited due to the limited access of samples. Metagenomic and molecular approach help in understanding the bacterial/archaeal diversity of the AS-OMZ. Information on their type and various functions and their overall role in the biogeochemical processes including their response to seasonality is essential.

Chapter 3

Bacterial biomass and diversity

3.1 Introduction

Microbial assemblages are key components in marine ecosystems and play an important role in nutrient cycling (Azam et al. 1994; Jiao et al. 2010). Fuhrman (2002) stated that they process more than half of the total primary production. Their biomass is often comparable to that of phytoplankton in the euphotic zone (Simon et al. 1992). Bacterial community composition is an important variable in most marine ecosystems, controlling the rates and patterns of dissolved and particulate organic matter hydrolysis. Knowledge of spatial and temporal variation in bacterial community diversity, biomass and specific ecological functions is essential for understanding the role of bacteria in marine biogeochemistry.

The dynamics and seasonality in biology, physics and chemistry of the Arabian Sea (AS) are modulated seasonally due to upwelling, winter cooling (Prasanna Kumar et al. 2001) and semi-annual reversal of monsoonal winds (Madhupratap et al. 1996). The physical forces that affect mixed-layer dynamics and nutrient entrapment lead to extremes in patterns of primary productivity which affects the organic carbon production and downward flux (Hansell and Peltzer, 1998). Intense mineralization of surface derived organic matter and limited supply of oxygen generate prominent oxygen minimum zones (OMZs) at intermediate depths (150 to 1500 m; Naqvi, 1994). These seasonal changes in turn affect the abundance and distribution of microbial communities as detailed by Ducklow et al. (2001), Ramaiah et al. (2009), Singh and Ramaiah, (2011) and Divya et al. (2017). AS-OMZ is vital for elucidating the bacteria-mediated global biogeochemical and climatic processes (Stewart et al. 2012, Ulloa et al. 2012) but not much is understood about their community structure and metabolism from the AS-OMZ.

Previous studies by Ramaiah et al. (1996, 2000) are important in recognizing the heterotrophic bacterial production and abundance. Analyses of the diversity of bacterial community of the AS-OMZ are rather scarce except for the studies by Fuchs et al. (2005), Jayakumar et al. (2009) and Jain et al. (2014). The available studies from the AS−OMZ have focused mainly on the vertical distribution and activity of anammox bacteria (Pitcher et al. 2011), with no emphasis on the overall bacterial biodiversity. In order to understand the seasonal differences in the total bacterial biomass in terms of their cellular abundance and to decipher the phylogenetic diversity of bacterial communities in the AS-OMZ this study was carried out. Besides enumerating the abundance by Epifluorescence microscopy, clone libraries of *16S rRNA* or SSU rRNA were carried out from water samples collected from five depths from the Arabian Sea Time Series station (ASTS; 17°0.126'N, 67°59.772'E), during three different seasons.

3.2 Materials and methods

3.2.1 Sample collection

Sampling was carried out under the Sustained Indian Ocean Biogeochemistry and Ecosystem Research (SIBER) Program during May 2012 (spring intermonsoon, SIM), September 2012 (fall intermonsoon, FIM) and February 2013 (northeast monsoon, NEM). Samples were collected using pre-cleaned Niskin bottles (washed with tap water and rinsed with distilled water after each sampling) for analysis of bacterial abundance and extraction of genomic DNA during the cruises of ORV '*Sagar Kanya*' (SK-294) and RV '*Sindhu Sankalp*' (SSK-038 and SSK-046). Samples from surface (5 m), deep chlorophyll maxima (DCM varied between ~43 and 50 during the 4 seasons), upper OMZ/core OMZ (250 m and 500 m) and lower OMZ (1000 m) from the Arabian Sea Time Series (ASTS)

(17°0.126' N, 67°59.772'E, **Figure 3.1**) were strained through 200 µm pore sized bolting silk. Immediately after collection, 2.5/3 L from each depth was filtered peristaltically through a Sterivex cartridge fitted with 0.22 µm pore size membrane filter (Millipore, USA). The Sterivex cartridge was then filled with 1.8 ml of DNA storage buffer (50 mM Tris pH 8.3, 40 mM EDTA and 0.75 M sucrose), sealed, and stored frozen at -80°C until nucleic acid extraction in the laboratory.

3.2.2 Measurement of nutrients and dissolved oxygen

Measurements of the physico-chemical parameters of seawater for each sample were recorded from different sensors fitted on to the CTD rosette. The Winkler titration method modified by Carpenter, (1965) was followed to measure Dissolved oxygen (DO). Standard methods of Grasshoff et al. (1983) were used to measure the nutrients (nitrate, nitrite, ammonia, silicate and phosphate) from frozen samples transported to the on-shore lab in the CSIR-National Institute of Oceanography (NIO) using a Skalar auto analyser (Skalar Anlytical). Values of total organic carbon (TOC) concentrations from cruises TTN-043 (NEM; January 1995), TTN-045 (SIM; March 1995), and TTN-50J (FIM; August 1995) of the Joint Global Ocean Flux Study (JGOFS) Arabian Sea Process Study (ASPS) were used for all depths we sampled during this study.

3.2.3 Enumeration of total bacterial cells

Modified method of Porter and Feig, (1980) was used for total bacterial counts (TBC). Formaldehyde (2% final concentration) was added to subsamples of 50 ml from each depth and preserved at 4°C in the dark until analysis. 1-5 ml aliquots of these samples were incubated with 4, 6-diamidino-2-phenylindole (DAPI; 20 µl of 1 mg ml^{-1} working

solution per ml) for 20 mins and filtered onto black 0.22 μm pore-size polycarbonate membrane filters (Millipore). Membrane filters were then washed with 1 ml phosphate buffer saline (PBS); air dried and covered using cover slip. The filter was observed under 100X lens using UV light with a drop of oil. Microscopic counts were made using epifluorescence microscope (Olympus BX-51). Minimum of 20 randomly chosen microscopic fields from each sample was used to obtain a reliable mean of TBC.

3.2.4 DNA extraction

DNA extraction was performed using a modified method of Ferrari and Hollibaugh, (1999). Briefly, lysozyme (40 μl of 50 mg ml^{-1}) was added to filtered sterivex cartridge and incubated at 37°C for 45 min. Sodium dodecyl sulfate (100 μl of 20% solution), proteinase K (100 μl of 10 mg ml^{-1}) and RNase (60 μl of 1 mg ml^{-1}) were sequentially added, and the filters were incubated at 55°C for 60 min. The lysates were purified twice by extraction with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and the residual phenol was removed by adding an equal volume of chloroform-isoamyl alcohol (24:1). Finally, nucleic acid was precipitated overnight in 500 μl of absolute ethanol at −20°C, and centrifuged at 4°C. The pellet was collected and 40 μl of TE buffer was added. The DNA was checked by agarose (0.8%) gel electrophoresis. Nucleic acid extracts were stored at −80°C until further analyses.

3.2.5 PCR amplification

16S rRNA gene from all DNA extracts was amplified using primer pairs, 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT). PCR reactions were performed in a final volume of 50 μl using a Veriti (Applied Biosystem,

USA) thermal cycler. The PCR mixture (50 µl) contained 1 µl of extracted DNA (5 to 50 ng µl−1), 1 µl of each primer at a concentration of 0.5 µM, 25 µl of ReadyMix *Taq* PCR mix (Sigma Aldrich) (1.5 U *Taq* DNA polymerase; 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM deoxynucleoside triphosphate [dNTP], stabilizers) and 22 µl of milliQ water. Temperature parameters for amplification were: 94°C for 4 min for initial denaturation followed by 35 cycles each at 94°C for 1 min denaturation, 1 min of annealing at 55°C and 1 min of extension at 72°C. Negative control having the reaction mixture without the template DNA was also run along with the samples for ascertaining reagent and sample purity. The resulting PCR amplicons were run through 1% agarose gel using 1kb molecular ladder ranging from 300 bp to 10000 bp (**Figure 3.3**).

3.2.6 Purification of PCR products

In order to ensure good quality DNA for cloning, Axyprep-96 PCR Clean up kit (Axygen Scientific Inc, Union City, USA) purification kit was used for PCR clean-up. Three volumes of PCR-A buffer were added to one volume of PCR product and vortexed. The above solution was pipetted in a PCR column placed in a 2 ml eppendorf tube and centrifuged at 14000 rpm for 1 min. After discarding the filtrate, 700 µl of buffer W2 was pipetted onto the column and centrifuged for a min (14000 rpm). Filtrate was discarded and 400 µl of buffer W2 was pipetted onto the column and centrifuged at 1400 rpm (1 min). The PCR column was transferred into a fresh eppendorf tube and 25-30 µl of the eluent (pre-warmed at 65ºC) was added. The tubes were allowed to stand at room temperature (RT) for 5 min for efficient elution of DNA. The tubes were centrifuged for one min (14000 rpm) and the purified PCR product was stored at -20 until cloning.

3.2.7 Cloning and colony-PCR of *16S rRNA* gene

Purified *16S rRNA* gene products of bacteria were cloned into pCR4-TOPO vector using a TOPO-TA cloning kit (Invitrogen, Carlsbad, California, USA) and transformed by chemical transformation into TOP-10 cells as per manufacturer's instructions. The clones were grown overnight at 37°C on Luria Bertani (LB) plates. A minimum of 65 clones of bacteria per depth were collected for clone library construction. All positive clones/transformants from each sample were randomly picked, and subjected to colony-PCR using primer set pucM13F (GTTTTCCCAGTCACGAC) and pucM13R (CAGGAAACAGCTATGAC). Temperature conditions for colony-PCR are: initial denaturation step of 10 min at 94°C, followed by 30 cycles of 94°C for 1 min, annealing at 55°C for 1 min with elongation step at 72°C for 1 min and final extension at 72°C for10 mins.

3.2.8 Sequencing *16S rRNA* gene

The PCR products were purified using Axyprep-96 PCR Clean up kit. Sequencing was performed with 15-50 ng of the PCR amplicons adding one pmol each of forward and reverse primer of pucM13F / pucM13R in an ABI3130 Genetic Analyzer following the dideoxy chain termination technique. Temperature profile for sequencing is as follows: denaturation (96°C for 1 min), denaturation at 96°C for 10 secs (30 cycles), annealing (55°C for 10 sec), elongation (60°C for 4 mins) and final extension (60°C for 1 min). Obtained sequences were assembled into contigs using DNA Baser sequence assembly software. Vector sequences were trimmed and bidirectional sequence pair was assembled to get a complete sequence of approximately 1400 bp of cloned product. The contigs were checked for 5' to 3' direction. The contigs showing the opposite direction

were changed to their reverse complement using the Sequence Massager tool. All the sequences of poor read or small in size were omitted from further analysis.

3.2.9 Sequence analyses

Vector contamination was removed from the sequences using the VecScreen tool (http://www.ncbi.nlm.nih.gov/tools/vecscreen/). Non-chimeric consensus sequences (Decipher, http://decipher.cee.wisc.edu/FindChimeras.html) without vector and primer residues and with a quality score of 20 (which translates into more than 99.5% correct bases, Allex, 1999), were used for further analyses. The taxonomic classification of sequences was done using 1,000 pseudo-bootstrap replications with a bootstrap value of 80%, which results in a standard error of only 1.3%. The sequences were compared with other databases (SILVA, NCBI, Greengenes) using mothur. Alignments were trimmed using Gblocks software (Castresana, 2000) to remove the poorly aligned and divergent regions. The sequences were clustered into phylotypes (operational taxonomic units, OTUs) using mothur by applying the average neighbor rule (Schloss and Westcott, 2011) at 97% sequence similarity cut-off.

3.2.10 Diversity and statistical analyses

Alpha diversity within each sample was calculated on the observed species matrix using Shannon and Pielous indices using mothur (Schloss et al. 2009). Beta diversity was calculated using ∫ LIBSHUFF (Schloss 2008, Schloss et al. 2009) and Bray-Curtis similarity index to compare clone libraries between samples at OTU levels. The similarity matrix was used to perform cluster analysis in Primer 6 (PRIMER-E, Plymouth, UK) using a group-average linking method and non-metric multidimensional scaling (NMDS).

Similarity profile (SIMPROF) test was carried out to check statistically significant differences between the clusters using Primer 6 (Clarke and Gorley, 2006, Clarke et al. 2008). The SIMPROF output was superimposed on an NMDS plot to best reflect the group formation (Clarke and Gorley, 2006, Clarke et al. 2008). The advantage of the SIMPROF test is that it looks for statistically significant evidence of clusters of communities rather than using an arbitrary cutoff of similarity to define sample groups (Clarke et al. 2008). Canonical correspondence analysis (CCA) was performed using Past-3 software (https://folk.uio.no/ohammer/past/) to check out the influence of environmental parameters on bacterial community structure.

3.2.11 Nucleotide accession numbers

The bacterial sequences obtained and described in this study were submitted to the NCBI GenBank database and are available under the accession numbers. KJ589647 to KJ590044 (SIM, FIM, and NEM), KR269603 to KR269693 (SIM), KR919859 to KR920002 (FIM) and KR673365 to KR819266 (NEM).

3.3 Results

Temperature, salinity, pH, DO and TOC were consistently higher in the surface layers (5 m and DCM) than in the OMZ (250 m, 500 m and 1000 m) during all 3 seasons. Conversely, nitrate, phosphate and silicate were significantly lower in the surface layers than in the OMZ (**Table 3.1**). In general, DO profiles during all three seasons are typical of an OMZ, i.e., surface (5 m) and DCM depths (~35-50 m) are well oxygenated (average DO, 185.24 ± 31.1 μmol L^{-1}) followed by a steep oxycline between DCM and 250 m (average DO, 5.56 ± 5.5 μmol L^{-1}). Surface layers (5 m and DCM) are almost devoid of

nitrate, while it was replete in the OMZ depths during all three seasons. However, an evident nitrite accumulation or secondary nitrite at 250 m was seen only during NEM. Most environmental parameters analyzed during this study were significantly different between surface layers and the OMZ, except nitrite and ammonia.

SIM recorded the lowest numbers of bacteria ranging from 0.53 (± 0.06, SD) at the surface to 0.36 (± 0.02) x 10^9 l^{-1} below 250 m whereas FIM recorded the highest number ranging from 1.4 (± 0.045) (in surface waters) to 0.6 (± 0.03) x10^9 l^{-1} (below 250 m). NEM also observed higher bacterial abundance ranging from 0.8 (± 0.08) x 10^9 to 0.57 (± 0.07) x 10^9 l^{-1} (**Figure 3.2**). Significant ($p<0.05$) seasonal variation in bacterial abundance was revealed by RM-ANOVA. Single group Student's *t*-tests indicated significant changes in bacterial abundance with depth.

The number of bacterial OTUs (S) from the ASTS location varied from 7-14, 7-11 and 5-12 during SIM, FIM, and NEM, respectively (**Table 3.2**). Maximum number of OTUs (14) was recorded from 500 m sample during SIM and minimum (5) were from 5 m during NEM. The numbers of OTUs in the OMZ depths (250 m-1000 m) were higher than those from surface depths (5 m and DCM). Bacterial diversity (H') differed during SIM, FIM, and NEM ranging from 2.58 to 3.63, 2.55 to 3.31 and from 2.04 to 3.43 respectively (**Table 3.2**). The highest and the lowest diversity were recorded during SIM (500 m) and NEM (5 m) respectively. In general, bacterial diversity in OMZ depths was higher than that in surface layers (student's $t = 6.0916$, $df = 3$, $P<0.001$). Evenness values ranged from 0.85-0.95 at the ASTS. In fact, J', close to 1, was observed at all depths during all seasons. The lowest equitability ($J = 0.85$) of bacterial population was inferable from DCM during FIM.

Hierarchical clustering revealed clear patterns of vertical as well as temporal partitioning (**Figure 3.4**). SIMPROF routine applied with hierarchical cluster analysis segregated the libraries into two statistically significant clusters at 5% significance level. The first cluster consisted of communities residing in the surface layers (5 m-DCM) and, the second cluster comprising those from the OMZ depths (250 m, 500 m and 1000 m). Two- dimensional non-metric multidimensional scaling (2D-NMDS) ordination profiles indicated (**Figure. 3.5**) patterns similar to those seen from hierarchical clustering analysis with a stress value of <0.15. These analyses suggest a good fit of data points in 2D ordination. The LIBSHUFF ($p<0.0016$) analyses showed a significant difference between surface and OMZ bacterial community during all three seasons. Bacterial community in surface layers differed significantly between seasons (LIBSHUFF, $p<0.0001$), whereas no such significant seasonal differences seen in those from OMZ (**Table 3.3**).

Clone libraries from 5 m and DCM depth were grouped as the surface samples. Similarly, clone libraries from 250 m, 500 m and 1000 m were grouped as OMZ samples for each season. In the surface samples 335 bacterial sequences clustered into 177 OTUs at 97% similarity level. Of these, three OTUs were shared or common during all three seasons (**Table 3.4**) and covered ~11-17% of the sequences from individual seasons (**Figure 3.6a**). Also, 20 OTUs shared between any two seasons (**Table 3.4**) comprised 19-38% of the sequences in single seasons. The percentage of season-specific OTUs in the surface samples varied between 45 and 68% (**Figure 3.6a**). Within the OMZ samples (250 m, 500 m and 1000 m), eight bacterial OTUs shared among all three seasons (**Table 3.5**). These OTUs covered 14, 27, and 34% of sequences during NEM, FIM, and SIM respectively (**Figure 3.6b**). Further, 20 OTUs were common between any two seasons

(**Table 3.5**) covering 15-20% of the sequences. The percentage of season-specific OTUs in the OMZ samples varied between 51-67% (**Figure 3.6b**).

The three OTUs shared among surface groups during all seasons were affiliated to genus *Synechococcus* of class *Cyanobacteria*. The bacterial OTUs shared between any two seasons were affiliated to *Alteromonas* (*Class Gammaproteobacteria*), *Burkholderia* (*Class Betaproteobacteria*), *Synechococcus*, *Acidobacterium* (*Class Acidobacteria*), and uncultured_*Alphaproteobacteria* (**Table 3.3**). In the OMZ samples, OTUs shared between all three sampling seasons were related to *Alteromonas*, *Rhodobacter* (*Class Alphaproteobacteria*), *Sphingomonas* (*Class Alphaproteobacteria*), and *Burkholderia*. The OMZ OTUs common to any two seasons were affiliated to *Sphingomonas*, *Nitratireductor* (*Class Alphaproteobacteria*), *Acidimicrobium* (*Class Actinobacteria*), uncultured_*Gammaproteobacteria*, and Marine Group A (**Table 3.4**).

Bacterial community dynamics with the environmental variables were analyzed using canonical correspondence analysis (CCA, **Figure 3.7**). The sum of all Eigen values indicated an overall variance of 0.83 in the dataset. The first two ordination axes of CCA accounted for 67.25% of the explained total variance. The first canonical axis accounted for 48.93% of the total explained variance and reflected a strong gradient caused by DO (R = 0.954) and TOC (R = 0.939). CCA indicated that among the seven different parameters, DO and TOC seemed to separate the bacterial communities into surface and OMZ communities. Further, TOC explained the temporal variations of the bacterial community in the surface waters. Depths 5 m and the DCM were positively correlated with DO during all three seasons whereas 250 m, 500 m and 1000 m interconnected with nutrients (silicate, phosphate, ammonia, nitrate, and nitrite).

Overall, a total of 11 phyla and 24 orders were recorded. Among these 11 Phyla, five were found in water samples from 5 m, 7 in DCM, 9 in samples from 250 m, 10 in samples from 500 m and 7 in samples from 1000 m. Of the 24 orders, 9 were from 5 m, 11 were from DCM, 16 in 250 m and 19 each from 250 m and 500 m. Phyla and genera of bacteria exclusive to each depth are explained in **figures 3.8** and **3.9**. The seasonal distribution patterns of bacterial sequences from 5 m to 1000 m depth (**Figure 3.8**) indicate that maximum numbers of sequences were affiliated to Phyla *Proteobacteria* (*Gammaproteobacteria*, *Alphaproteobacteria*) and *Cyanobacteria* (*Synechococcus*). The relative proportions of sequences in these groups varied temporally, as well as vertically. The percentages of *Gammaproteobacteria* were much higher in the OMZ than in the surface layers. During NEM, the percentages of *Synechococcus* and *Gammaproteobacteria* were the highest in the surface layers. Notably, the percentage of *Gammaproteobacteria* within OMZ (250 m, 500 m and 1000 m) hardly varied between seasons. Bacterial community at the order level in surface layers (5 m-DCM), is dominated by members of *Synechococcales* (25-58%) and *Alteromonadales* (9-42%). *Rhodobacterales*, SAR 11 and *Sphingomonadales* were found in small proportions in the surface layers and represented <8% of the total sequences (**Figure 3.9)**. Furthermore, members of *Acidimicrobiales*, Marinimicrobia_incertae_sedis, *Verrucomicrobiales*, *Rhodospirillales*, *Caulobacteriales*, *Burkholderiales*, *Myxococcales*, *Desulfobacteriales*, *Oceanospirillales*, *Vibrionales*, *Legionellales*, and *Nitrospinales* formed the minor component (<4%) of the surface bacterial community.

Majority of sequences in OMZ depths were affiliated to *Alteromonadales* (33-55%), followed by SAR-11 (3-26%), *Sphingomonadales (* 2-25%), *Oceanospirillales* (2-

12%), *Acidimicrobiales* (3-11%), *Rhodobacterales* (3-7%), *Acidobacteriales* (2-7%) and Marinimicrobia_incertae_sedis (2-10%). Interestingly, members of order *Thiotrichales* (2-5%) were found exclusively in 500 m (all seasons) and 1000 m (FIM and NEM) depths. *Verrucomicrobiales*, *Planctomycetales*, *Synechococcales*, *Nitrospinales*, *Flavobacteriales*, *Rhizobiales*, *Rhodospirillales*, *Caulobacteriales*, *Burkholderiales*, *Myxococcales*, *Desulfobacteriales*, *Legionellales*, and *Xanthomonadales* formed the minor component (< 4%) of the OMZ bacterial community.

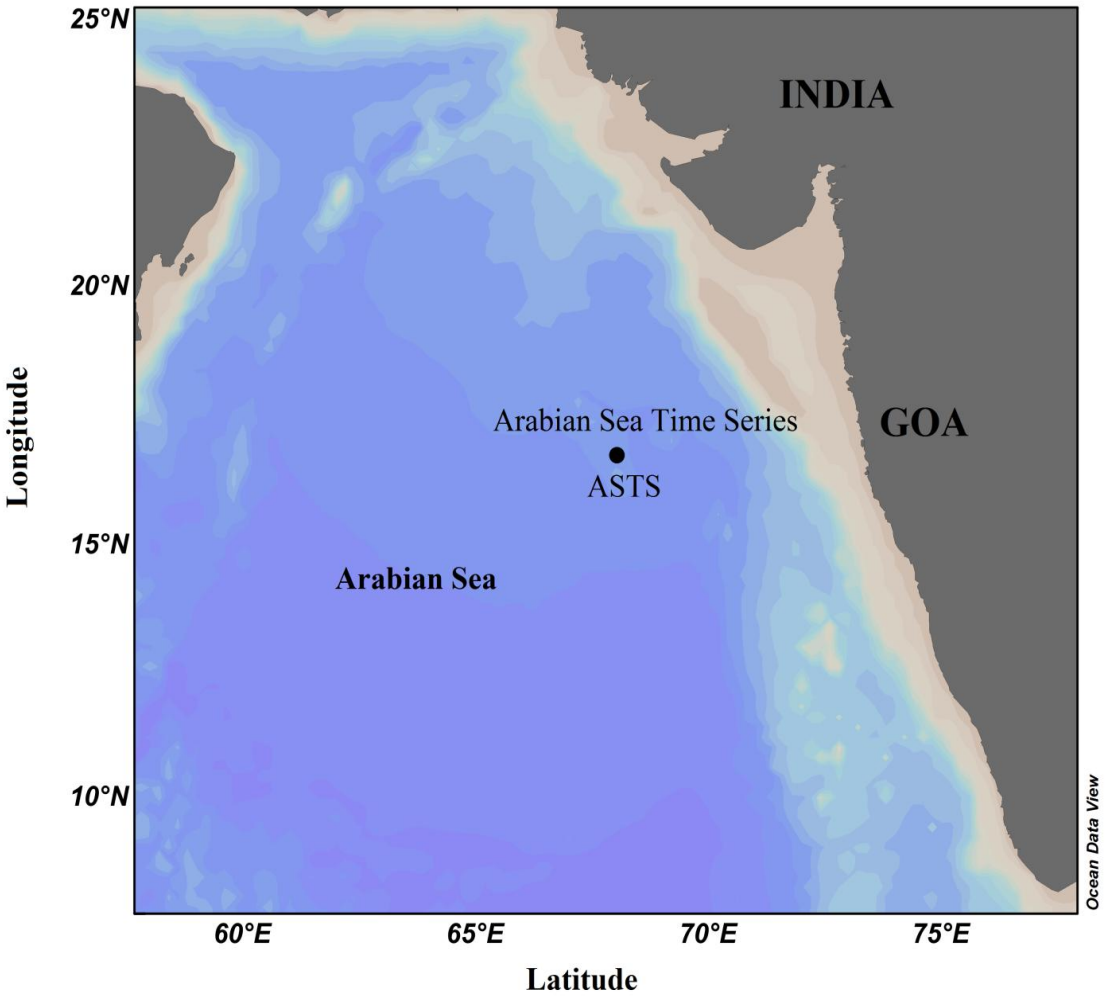


Figure 3.1. Map showing Arabian Sea Time Series (ASTS) location. Water samples were collected at five different depths from surface to 1000 m to represent surface (5 m), Deep Chlorophyll maxima (DCM), core OMZ (250 m, 500 m) and at 1000 m. Sampling was carried out during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM).

Table 3.1. Variations in physico-chemical parameters at the Arabian Sea Time Series (ASTS) location during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM). TOC data are from the Joint Global Ocean Flux Study (JGOFS) Arabian Sea Process Study.

Parameters	Seasons	5 m	DCM	250 m	500 m	1000 m
Physical						
Temperature	SIM	30.19	27.04	18.24	12.96	8.64
	FIM	27.74	25.89	16.00	12.02	10.29
	NEM	25.87	25.66	15.28	12.11	8.59
Salinity	SIM	36.31	36.35	35.72	35.71	35.34
	FIM	36.51	36.43	36.08	35.61	35.51
	NEM	36.65	36.60	35.75	35.6	35.31
DO ($\mu M\ l^{-1}$)	SIM	199.99	203.76	11.79	4.58	9.49
	FIM	205.97	149.30	3.47	0.76	3.06
	NEM	206.62	202.64	1.4	1.51	9.10
pH	SIM	7.93	8.05	7.76	7.59	7.52
	FIM	8.02	8.04	7.22	7.19	7.15
	NEM	8.1	8.22	7.51	7.43	7.31
Nutrients						
Nitrate ($\mu M\ l^{-1}$)	SIM	ND	0.11	22.41	15.33	38.91
	FIM	ND	8.14	25.002	32.79	38.28
	NEM	0.006	13.37	19.05	25.98	32.89
Nitrite ($\mu M\ l^{-1}$)	SIM	0.094	0.07	0.01	0.002	0.08
	FIM	ND	0.29	ND	ND	ND
	NEM	0.04	0.11	2.57	0.05	0.07
Phosphate ($\mu M\ l^{-1}$)	SIM	0.23	0.24	2.00	1.6	2.69
	FIM	0.41	0.79	2.35	2.55	2.92
	NEM	ND	0.62	2.22	2.58	3.16
Silicate ($\mu M\ l^{-1}$)	SIM	3.07	4.08	18.47	19.44	66.55
	FIM	0.62	2.34	21.79	34.99	50
	NEM	2.90	7.71	29.36	50.29	100.37
Ammonia ($\mu M\ l^{-1}$)	SIM	0.009	0.13	0.09	1.008	0.13
	FIM	0.24	0.27	0.19	0.27	0.17
	NEM	ND	0.53	ND	ND	ND
TOC ($\mu M\ l^{-1}$)	SIM	83.8	79	54.2	47.8	44.7
	FIM	77.2	71.98	52.43	43.36	43.01
	NEM	69.5	67.9	45.3	38.2	38

*DO = dissolved oxygen; ND = not detected; TOC=Total organic carbon

Table 3.2. Operational taxonomic units (OTU), species richness *(S)*, Shannon diversity index (*H*') and Pielou's evenness index (*J*') derived from bacterial clone libraries at the Arabian Sea Time Series (ASTS) station during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM).

Parameters	**Seasons**	**Depths**				
		5 m	DCM	250 m	500 m	1000 m
Bacteria						
Total OTUs (*S*)	SIM	7	7	12	14	11
	FIM	7	9	8	9	11
	NEM	5	7	11	12	12
Shannon diversity (*H'*)	SIM	2.58	2.59	3.31	3.63	3.19
	FIM	2.55	2.71	2.85	2.97	3.31
	NEM	2.04	2.56	3.27	3.43	3.39
Pielous eveness (*J'*)	SIM	0.92	0.92	0.92	0.95	0.92
	FIM	0.91	0.85	0.95	0.93	0.95
	NEM	0.88	0.91	0.94	0.95	0.94

Table 3.3. ∫-LIBSHUFF comparisons of bacterial clone libraries constructed from the surface (5 m and DCM) and OMZ (250 m-1000 m) depths during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM).

		Surface			**OMZ**		
		SIM-S	FIM-S	NEM-S	SIM-O	FIM-O	NEM-O
Bacteria							
	SIM-s	0	0.2783	0.0525	0.0902	**<0.0001**	0.0001
Surface	FIM-s	**<0.0001**	0	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	NEM-s	**<0.0001**	**<0.0001**	0	0.0006	**<0.0001**	**<0.0001**
	SIM-o	**<0.0001**	**<0.0001**	**<0.0001**	0	0.0036	0.0022
OMZ	FIM-o	**<0.0001**	**<0.0001**	**<0.0001**	0.0262	0	0.0352
	NEM-o	**<0.0001**	**<0.0001**	**<0.0001**	0.0615	0.0071	0

With an experiment wise error rate of 0.05 and taking into account a Bonferroni's correction for multiple comparisons, the libraries were considered significantly different when either of the two *P* values generated for an individual pairwise comparison was lower than 0.0016 (significant values are marked in bold).

Table 3.4. Phylogenetic affiliation of bacterial OTUs (surface) common in all season and two seasons from the Arabian Sea Time Series (ASTS) location.

OTUs	Phylogenetic affiliation	Accession number	Environment
OTUs common in all season			
OTU-01	Uncultured Cyanobacterium	EF_123580.1	Caribbean Sea
OTU-02	Uncultured *Prochlorococcus* sp.	JN_547420.1	Red Sea
OTU-03	Uncultured *Prochlorococcus* sp.	KF_001530.1	Tyrrhenian Sea
OTUs common in 2 seasons			
OTU-04	Uncultured Alphaproteobacterium	JF_824769.1	northern gulf of Mexico
OTU-05	Uncultured *Alteromonas* sp.	HQ_161387.1	Eastern Mediterranean Sea
OTU-06	Uncultured *Synechococcus* sp.	AY_125366.1	Oligotrophic waters
OTU-07	*Alteromonas* sp.	FJ_170025.1	South China Sea
OTU-08	Uncultured *Prochlorococcus* sp.	JN_547417.1	Red Sea
OTU-09	Uncultured *Alteromonas* sp.	KF_545037.1	Deep sea sediment
OTU-10	Uncultured bacterium	EF_574727.1	Coco's Island
OTU-11	Uncultured Gammaproteobacterium	GQ_250617.1	Gulf of Mexico
OTU-12	*Burkholderia* sp.	KT_159931.1	Swamp forest
OTU-13	Halophilic bacterium	AB_042502.2	Antarctic habitats
OTU-14	Uncultured Cyanobacterium	HQ_242063.1	North East Pacific
OTU-15	*Idiomarina baltica*	AJ_440215.1	Central Baltic Sea
OTU-16	Uncultured Alphaproteobacterium	HQ_242175.1	North East Pacific
OTU-17	Alphaproteobacteria	JQ_515459.1	Caribbean reef
OTU-18	Uncultured Cyanobacterium	EU_980186.1	Lake Taihu China
OTU-19	Uncultured bacterium	JX_668703.1	Polar marine water
OTU-20	Uncultured Cyanobacterium	GQ_348575.1	Oceanic dead zones
OTU-21	Betaproteobacterium	HM_163254.1	Sediment sample
OTU-22	Uncultured Acidobacterium	KF_183225.1	Soil Bacterial Community
OTU-23	*Alteromonas macleodii*	KC_871610.1	Atlantic Ocean

Table 3.5. Phylogenetic affiliation of bacterial OTUs (OMZ) common in all season and two seasons from the Arabian Sea Time Series (ASTS) location.

OTUs	Phylogenetic affiliation	Accession numbers	Environment
OTUs common in all season			
OTU-01	Uncultured *Alteromonas* sp.	AB_262378.1	Ishigakijima Island
OTU-02	*Alteromonas* sp.	JX_533666.1	South china Sea
OTU-03	*Erythrobacter* sp.	AM_990645.1	South Pacific Gyre
OTU-04	Uncultured bacterium clone	KM_110216.1	South Atlantic Ocean
OTU-05	Uncultured *Burkholderia* sp.	KF_305507.1	Thermal Spring
OTU-06	Uncultured bacterium clone	JX_441387.1	Indian Ocean
OTU-07	Uncultured Proteobacterium clone	KM_018890.1	Red Sea
OTU-08	*Methylarcula* sp.	DQ_412076.1	Loihi Seamount
OTUs common in 2 season			
OTU-09	Sphingobium sp.	KF_672731.1	Haihe estuary
OTU-10	Uncultured Gammaproteobacterium clone	JN_232986.1	Cape Lookout
OTU-11	Uncultured marine microorganism clone	JN_166297.1	Subtropical North Pacific
OTU-12	Uncultured Marine Group A	HQ_674579.1	Subarctic pacific Ocean
OTU-13	*Euzebya* sp.	KP_735966.1	Pacific Ocean
OTU-14	*Nitratireductor* sp.	EU_440986.1	Southwest Indian Ridge
OTU-15	Uncultured marine microorganism clone	JN_166353.1	Subtropical North Pacific
OTU-16	Uncultured Alphaproteobacterium	GQ_347307.1	Oceanic dead zones
OTU-17	Uncultured bacterium clone	JX_391520.1	Marine sediments
OTU-18	*Oleibacter marinus*	NR_112787.1	Tropical ocean
OTU-19	Uncultured Alphaproteobacterium clone	GQ_337156.1	Deep Arctic Ocean
OTU-20	Uncultured bacterium clone	HQ_674365.1	Pacific Ocean
OTU-21	Uncultured bacterium clone	JX_441447.1	Indian Ocean
OTU-22	*Alterierythrobacter* sp.	EU_440971.1	Southwest Indian Ridge

OTU-23	Uncultured Marine Group A	HQ_673196.1	Subarctic pacific Ocean
OTU-24	*Donghicol Aeburneus*	NR_043928.1	East Sea in Korea
OTU-25	Uncultured Gammaproteobacterium clone	KP_076569.1	Coastal Arabian Sea
OTU-26	Erythrobacter sp.	DQ_285076.1	Marine environment
OTU-27	Uncultured bacterium clone	AY_375071.1	Deep sea sediments
OTU-28	Uncultured Actinobacterium	FJ_615153.1	Saanich Inlet OMZ

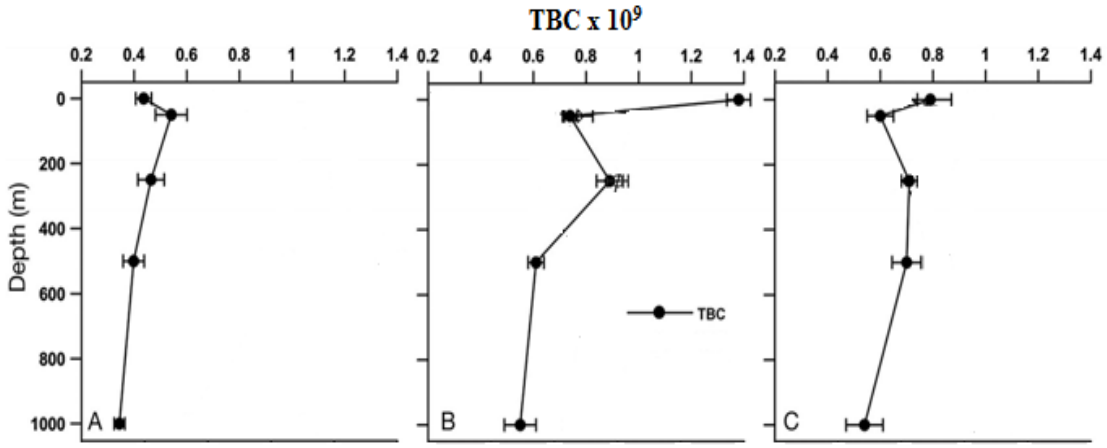


Figure 3.2. Total bacterial counts (TBC) at different depths at the Arabian Sea Time Series (ASTS) station during (A) Spring intermonsoon (SIM) (B) Fall intermonsoon (FIM) and (C) Northeast monsoon (NEM).

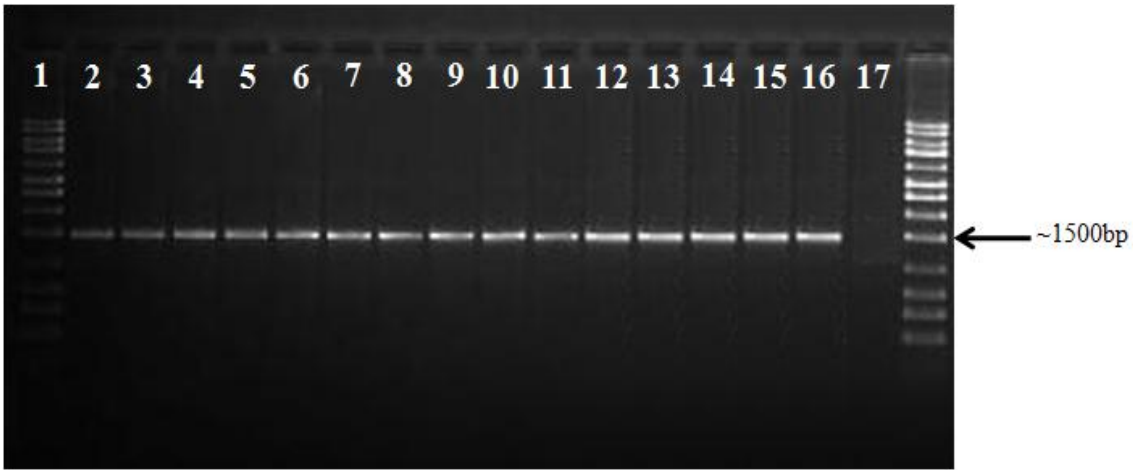


Figure 3.3. PCR amplified product showing *16S rRNA* gene of 15 metagenomic samples. Lane 1 with 1 kb DNA ladder (300 to 10000 bp); lane 2 to 6 (SIM-5 m, SIM-DCM, SIM-250 m, SIM 500 m, SIM-1000 m); lane 7 to 11 (FIM-5 m, FIM-DCM, FIM-250 m, FIM-500 m, FIM-1000 m); lane 12 to 16 (NEM-5 m, NEM-DCM, NEM-250 m, NEM-500 m, NEM-1000 m) depict the PCR amplified at ~1500 bp; lane 17 with PCR control.

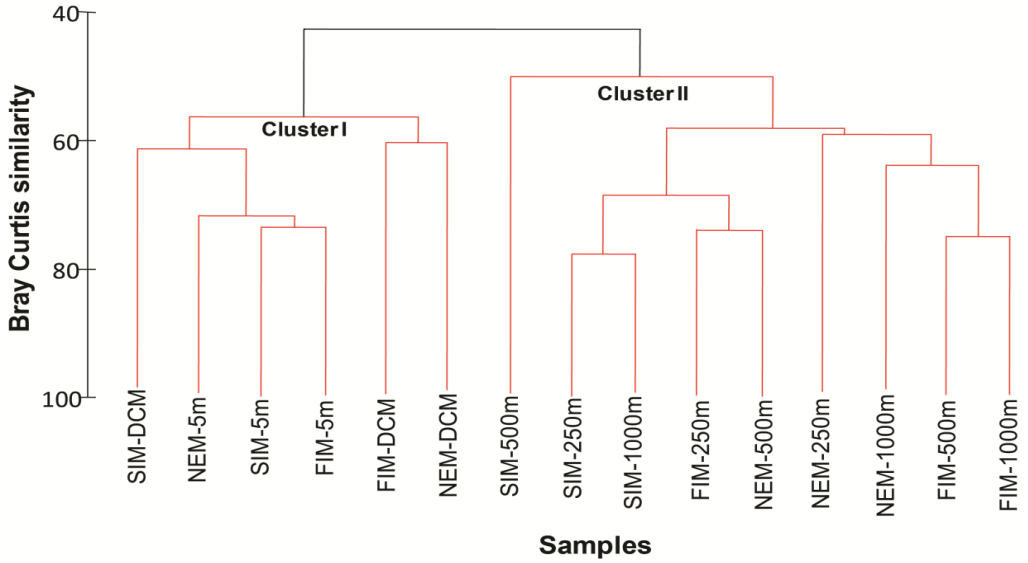


Figure 3.4. Hierarchical cluster analyses of bacterial community profiles using group-average linking method. Similarity profile (SIMPROF) forms 2 statistically significant groups (represented by black line): Cluster-I (Surface group) and Cluster-II (OMZ group).

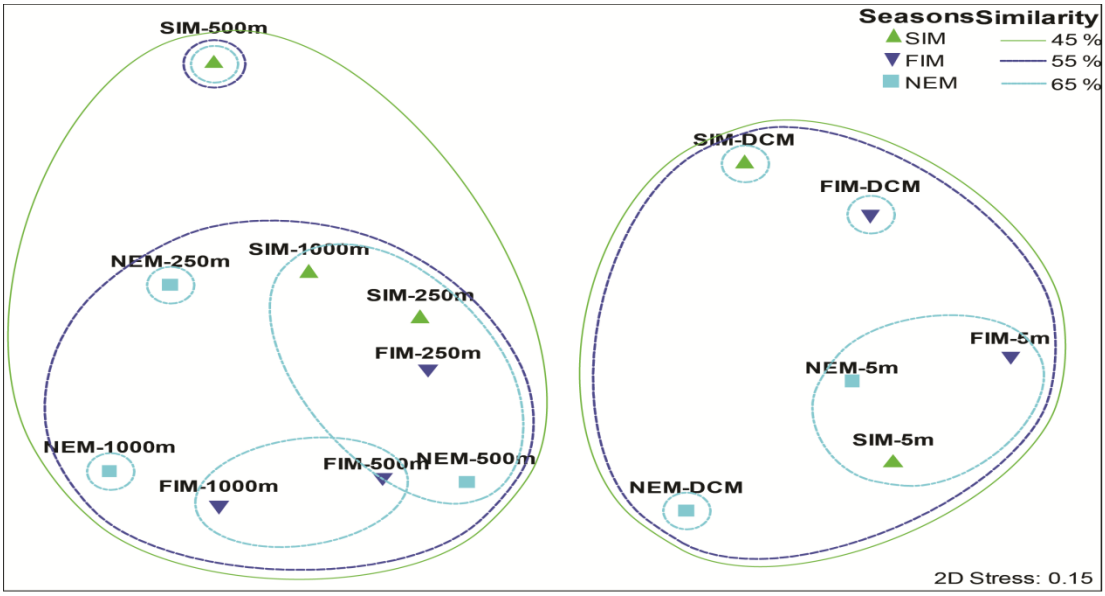


Figure 3.5. Non-metric multidimensional scaling (NMDS) plot of bacterial community profiles was constructed based on the relative abundance of OTUs using Bray–Curtis similarity index. The samples within complete green, dashed navy blue and dashed sky blue circles were 45, 55, and 65% similar in bacterial community structure.

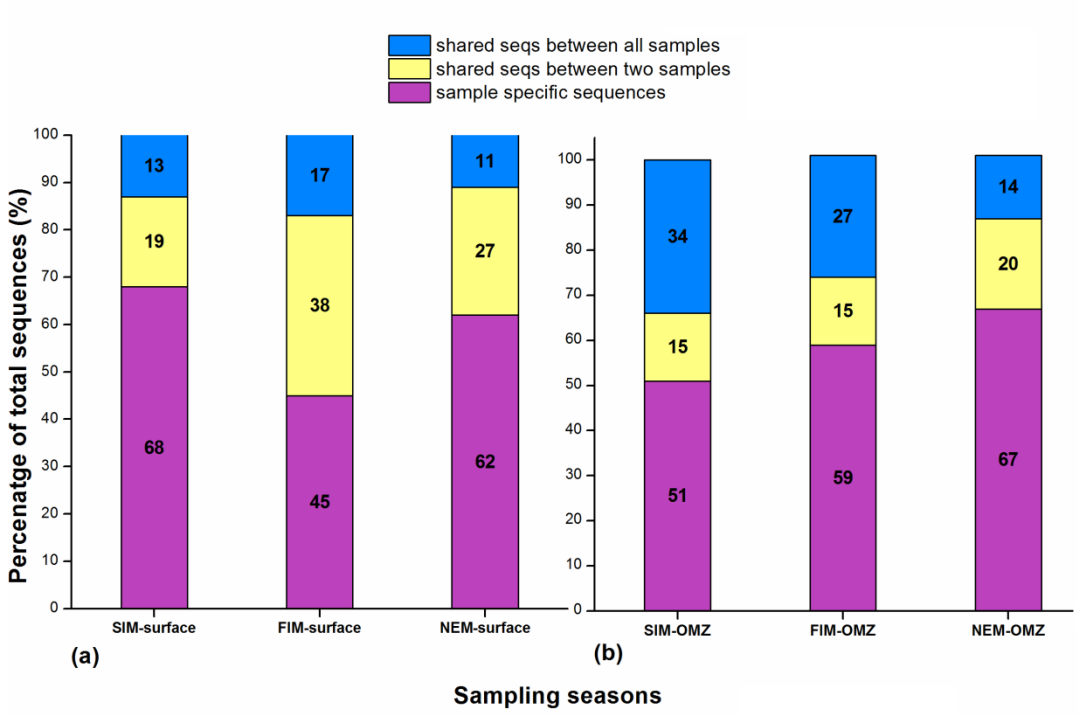


Figure 3.6. Percentage of bacterial sequences in the clone libraries from (a) surface and (b) OMZ samples during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM) at the Arabian Sea Time Series (ASTS) location.

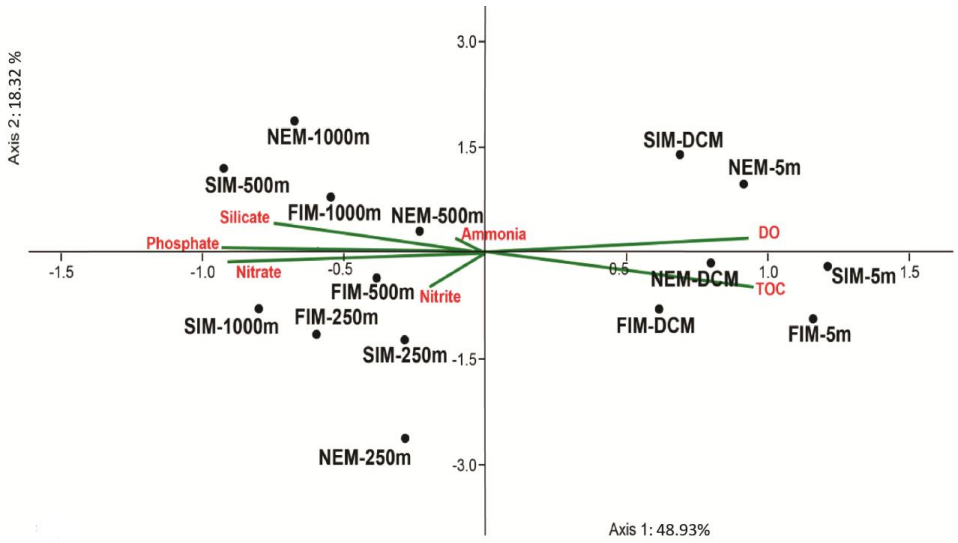


Figure 3.7. Canonical correspondence analysis (CCA) ordination diagram of bacterial communities, associated with environmental variables. Red is environmental parameters, black is season and depth.

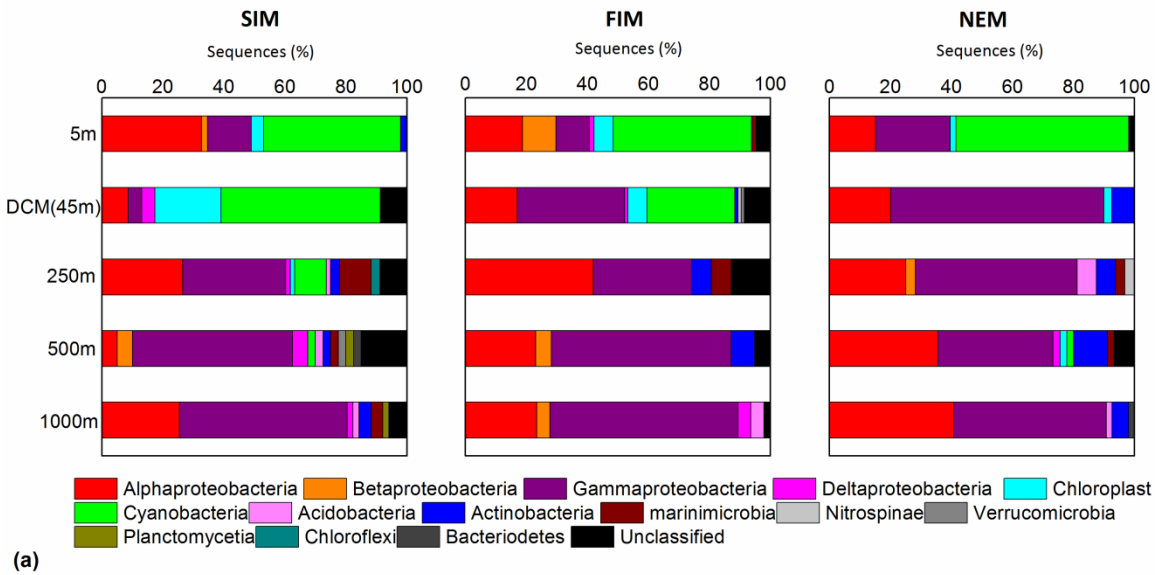


Figure 3.8. Percentages (x-axis) and depth-wise distribution (y-axis) of major bacterial classes at the Arabian Sea Time Series (ASTS) station during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM).

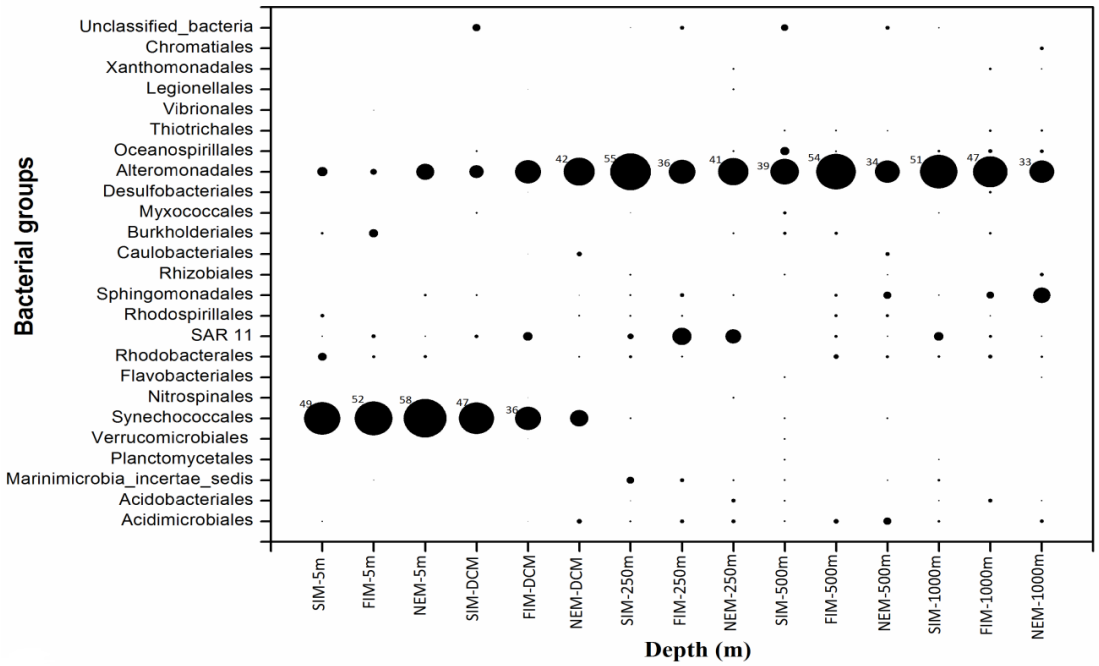


Figure 3.9. Seasonal and depth wise distributions of percentages of bacterial orders at the Arabian Sea Time Series (ASTS) during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM).

3.4 Discussion

Seasonal variability of DO in the intermediate depths of the ASTS corresponded with previous reports from the central and eastern AS (de Sousa et al. 1996). Concentrations of DO were the lowest during NEM than during SIM and FIM. High surface productivity (Bhattathiri et al. 1996) and slow renewal of intermediate waters (Naqvi et al. 1990) lead to severe oxygen depletion during the winter season in the AS. Nitrate was almost undetectable in the surface waters during all three seasons. During the intermonsoon period, intense solar heating and weak winds stratify the surface layer of the AS leading to depletion of nutrients/nitrate in the upper euphotic zone (Muraleedharan and Prasanna Kumar, 1996). However, during winter, nutrient-rich subsurface layers are pumped to the surface due to convective mixing which leads to high primary production (Prasanna Kumar and Prasad, 1996). Previous reports suggest that high nitrite and low DO during the NEM (250 m) imply intense denitrification process, while nitrite in the thermocline region indicates nitrification process (Sen Gupta et al. 1975).

Bacterial abundance at the ASTS varied seasonally, with the lowest and highest levels recorded during SIM and FIM, respectively. In the overall, the seasonal trend of bacterial abundance at ASTS matches well with earlier data from the Central Arabian Sea (Ducklow et al. 2001, Ramaiah et al. 1996, 2005). During SIM the bacterial abundances are reported to be sustained by the slow-to-degrade dissolved organic carbon (DOC, Ramaiah et al. 2000). The SIM being a transitional period, the primary production is low due to stratification and prevalence of oligotrophic conditions (Madhupratap et al. 1996). Typically, bacterial abundance is higher at the end of the southwest monsoon (SWM, i.e. FIM). The decay of SWM phytoplankton blooms and increased exudation at the end of the

season (Ducklow, 1993) also lead to prolonged bacterial proliferation well into the FIM. The presence of a secondary peak in bacterial abundance at 250 m during the FIM is similar to earlier reports (Ramaiah et al. 2005), but was not observed during other seasons.

Environmental conditions and competition across the oxygen gradient could be responsible for differences between the surface and OMZ bacterial communities. The availability of low energy in the OMZ depths due to the lesser amount of available organic matter might play a role in the predominance of a few phylotypes of bacteria. Earlier observations report that microbial communities from different depths within the OMZ clustered together, while those from surface layers were less similar (Bryant et al. 2012, Stewart et al. 2012). In this regard, our results indicate that clustering among the AS-OMZ bacterial community at different depths (within OMZ) is a perennial phenomenon in the ASTS. DO and organic matter are considered as the crucial factors responsible for the changes in microbial community composition in aquatic ecosystems (Stevens and Ulloa 2008). CCA analyses used in this study suggest that DO and TOC play a vital role in influencing the bacterial community at the ASTS.

Higher bacterial diversity at the ASTS was recorded during SIM. SIM is a transition period from winter to summer and is a period of low primary productivity caused by water column stratification and oligotrophic conditions (Madhupratap et al. 1996). During SIM, the bacterial community is mainly sustained by the slow-to degrade dissolved organic carbon (DOC) from earlier phytoplankton blooms of the NEM (Ramaiah et al. 2000). Inter-species competition for limited resources might have resulted in the high bacteria diversity during SIM. Over 60 chimera free clones were obtained for sequencing from each depth

so as to have larger sample size to be statistically relevant. Higher H' values recorded in the ASTS-OMZ layers than in the surface layers are similar to those reported earlier by Stevens and Ulloa, (2008) from the Eastern Tropical South Pacific (ETSP)-OMZ. The overall bacterial diversity in the ASTS is higher in the OMZ depths than in the surface layers. Similar results were reported by Stevens and Ulloa, (2008), Brown et al. (2009) and Kemble et al. (2011) in the OMZ of ETSP and Hawaii Ocean Time-Series (HOTS) respectively. Higher bacterial diversity in the OMZ is linked to the use of a broader range of terminal electron acceptor compared to the surface depths where oxygen is the dominant electron acceptor (Stevens and Ulloa, 2008).

Bacterial communities found in this study were similar to those found in a variety of pelagic marine environments (Giovannoni and Rappe, 2000). Bacterial community structure at the ASTS is dominated by phylotypes affiliated with *Gammaproteobacteria*, *Alphaproteobacteria*, and *Cyanobacteria*. The dominance of *Alphaproteobacteria* and *Gammaproteobacteria*, in a variety of pelagic marine environments, is well known (Giovannoni and Rappe, 2000), including the ETSP-OMZ (Stevens and Ulloa, 2008, Ganesh et al. 2014) and Southern AS (Fuchs et al. 2005).

Most surface layer (5 m-DCM) OTUs were affiliated with *Cyanobacteria*, specifically to *Prochlorococcus*. *Prochlorococcus* and *Synechococcus* as also reported by Johnson et al. (1999), Goericke et al. (2000), Ulloa et al. (2008), Galán et al. (2009), Lavin et al. (2010) and Ulloa et al. (2012) are known to be inhabiting the euphotic zone of OMZs. Phylotypes of *Synechococcus* often were restricted to the upper part of the euphotic zone without an explicit vertical depth-wise partitioning. The dominance of *Prochlorococcus*

over *Synechococcus* in the AS-OMZ is probably due to its specific physiological adaptation to the low light and nutrient conditions (Moore and Chisholm, 1999; Moore et al. 2002). *Alteromonas* affiliated to *Gammaproteobacteria* is the major phylotype in the surface sample. The surface ecotype of *Alteromonas macleodii*, an r-strategist, is known to regulate and degrade sugars and amino acids, colonizing smaller particulate organic matter with much slower sinking rates (Perez et al. 2012). The variation in abundance of members of *Altermonodales*, in the surface layers could be attributed to the seasonal changes in primary productivity patterns and organic carbon concentration in the surface layers of the North Eastern Arabian Sea (Hansell and Peltzer, 1998). Also, sequences affiliating to bacteria that appear to be involved in the sulfur cycle were observed. Sequences affiliating to *Rhodobacterales* (Moran et al. 2003), and SAR11 (Howard et al. 2006) of the class *Alphaproteobacteria* were found. Another group of bacteria contributing to the variation in the surface ASTS bacterial community was *Sphingomonadales*.

In this study, most phylotypes affiliated with *Alphaproteobacteria* in the OMZ include *Rhodobacterales*, *Sphingomonadales* and members of the SAR11. *Rhodobacterales* and members of the SAR11 group are known to limit the flux of sulfur from the ocean by demethylating dimethylsulfoniopropionate (DMSP), which is degraded to volatile dimethyl sulfide (Moran et al. 2003, Howard et al. 2006). Demethylated DMSP can serve as a substrate in sulfur oxidation in the deeper layer. Interestingly, sequences affiliated to, sulfur-oxidizing bacterial clades, such as *Thiotrichales* of the class *Gammaproteobacteria* and sulfur-reducing bacterial clade such as *Desulfobacteriales* of the class *Deltaproteobacteria* were found in the AS-OMZ. Sulfate-reducing bacteria are reported to survive in suboxic conditions (Santegoeds et al. 1998, Minz et al. 1999) and

play a vital role in the cryptic sulfur cycle in the OMZ (Canfield et al. 2010). Significant numbers of OMZ-OTUs at the ASTS were affiliated with *Sphingomonadales* (5%). Although the presence of *Sphingomonadales* is reported in the upper OMZ (Riemann et al. 1999) their exclusive inhabitation and ecological significance in OMZ depths are not apparent.

A substantial number of OMZ-OTUs affiliated with *Alteromonadales* (16%) account for stable bacterial community structure in the OMZ depths. The deep ecotype of *Alteromonas macleodii* is better suited to microaerophilic conditions (Martinez et al. 2008) and could degrade both labile and recalcitrant compounds (McCarren et al. 2010) colonizing large particles that sink rapidly to meso and bathypelagic depths. The ability of *Alteromonadales* to degrade recalcitrant compounds (Perez et al. 2012) and large sinking particulate matter which is abundant in the northern AS (e.g., Transparent exopolymer particles, Kumar et al. 1998, Ramaiah et al. 2005) might help sustain their abundance perennially in the AS-OMZ.

Few sequences from the anammox-group of the *Planctomycetes* were found exclusively in the OMZ during SIM, but anammox appear to be present in the AS-OMZ as revealed by the study of their functional genes (Jayakumar et al. 2013). On the other hand, we found sequences from the denitrifying-group only in the OMZ depths. OMZ-OTUs affiliated with *Nitratireductor. Sp*, a known denitrifying bacterium of the order *Rhizobiales* was detected. This is consistent with geochemical results that show denitrification pathway to be the primary sink for fixed nitrogen in the AS-OMZ (Bulow et al. 2010). The other OMZ-OTUs present in minor fractions were *Burkholderiales*, *Acidimicrobiales*, uncultured *Gammaproteobacteria*, and *Rhodobacterales*.

Results of this study demonstrate that the AS-OMZ bacterial communities are diverse and different communities prevail in the surface and OMZ depths. *Proteobacteria* (Class: *Gammaproteobacteria* and *Alphaproteobacteria*) and *Cyanobacteria* were the dominant bacterial phyla in the surface depths. Many clades of of class *Gammaproteobacteria* and *Alphaproteobacteria* were predominant in the OMZ depths. Bacterial community in surface layers varied a lot between seasons and no such differences were discernible in OMZ depths. Even as seasonal variation in the OMZ bacterial community is minimal, bacterial diversity in the OMZ depths is higher than the surface layers. Presence of denitrifiers (*Nitratireductor*) and anammox bacteria (*Planctomycetes*) although in small numbers in the OMZ depths brings forth their crucial role in the AS-OMZ ecosystem functioning.

Chapter 4

Archaeal diversity

4.1 Introduction

Microbial diversity analysis post 1980s has undergone a substantial change owing to the application of different molecular tools. These tools have helped in understanding and appreciating the role and presence of microbial communities in various ecosystems. While a great deal of information has been obtained on Bacteria, information on the role, type and ecosystem specialization of Archaea has only recently been intensified. Archaea are a phenotypically diverse group of microorganisms aligned into two kingdoms, *Euryarchaeota* and *Crenarchaeota* (DeLong, 1992; Fuhrman et al.1992). There are four general phenotypic groups of archaea: the methanogens, the extreme halophiles, the sulfate-reducing archaea, and the extreme thermophiles. So far, the archaeal habitats were believed to be limited to shallow or deep-sea anaerobic sediments (methanogens), deep-sea hydrothermal vents (methanogens, sulfate reducers, and extreme thermophiles) and highly saline land-locked seas (halophiles) until, Beman et al. (2008); Molina et al. (2010) reported the presence of archaea in low oxygen conditions.

The *16S rRNA* gene-based archaeal diversity analyses reveal the ubiquitous (Stein and Simon, 1996) presence of archaea in the open ocean, marine sediments and freshwater lake sediments (Schleper et al. 2005). First reports on presence of archaea in the anoxic zone was reported by Vetriani et al. (2003) suggesting its involvement in the anaerobic oxidation of methane. Further, Karner et al. (2001); and Molina et al. (2010) reported the presence of archaea in low oxygen conditions, which according to Park et al. (2010) are enriched under low oxygen concentrations. Francis et al. (2007) and Ingalls et al. (2006) reported *Crenarchaeota* and *Euryarchaeota*, to contribute significantly in carbon and nitrogen cycle, the two most biologically essential elements.

Previous studies have led to believe that diversity and distribution of Archaea are limited in comparison to Bacteria. Studies of Karner et al. (2001) suggest that there are 1.3×10^{28} archaeal and 3.1×10^{28} bacterial cells in the ocean. Therefore, the expected metabolic capabilities of Archaea must be almost analogous to Bacteria. The *16S rRNA* gene sequence analyses are useful to demonstrate that most marine prokaryotes are undescribed species that have not been cultivated 'yet' (Clementino et al. 2008; DeLong, 1992; Fuhrman et al. 1993).

So far, there has not been a single investigation from the open ocean Arabian Sea (AS). Singh et al. (2010) studied the seasonal and spatial variability of archaeal community in the central west-coast of India using DGGE. However, detailed analyses to understand archaeal community structure and/or archaeal diversity of the AS oxygen minimum zone (OMZ) and its overlying surface waters are not many.

Phylogenetic diversity of archaea has been reported from different marine habitats (Fuhrman et al. 2008; Martin-Cuadrado et al. 2008; Alonso-Saez et al. 2008) following the 16S rRNA gene analysis. In order to elucidate the overall archaeal community, their seasonal variability in the AS-OMZ, the *16S rRNA* gene analysis was done. The rationale for this objective was to profile the archaeal community and recognize if they vary with depth at the Arabian Sea Time Series (ASTS) location. Through such elucidations, it was aimed to explain the temporal and vertical patterns of archaeal members and their possible functional role in the AS-OMZ.

4.2 Materials and methods

4.2.1 Sampling and Extraction of DNA

For archaeal diversity analyses, the DNA extracts from water samples collected from the ASTS during all three seasons were as described in detail in subsection 3.2.1 and 3.2.4.

4.2.2 Amplification, cloning and sequencing of *16S rRNA* gene

PCR amplification was performed on all of the DNA extracts using the universal archaea specific primer set 21F (5'-TTCCGGTTGATCCYGCCGGA-3') and 958R (5'YCCGGCGTTGAMTCCAATT-3'). The PCR mixture (50 µl) contained 1 µl of extracted DNA (5 to 50 ng µl), 1 µl of each primer at a concentration of 0.5 µM, 25 µl of ReadyMix *Taq* PCR mix (Sigma Aldrich) (1.5 U *Taq* DNA polymerase; 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM deoxynucleoside triphosphate [dNTP], stabilizers) and 22 µl of milliQ water. Thermal cycling for PCR amplification was as per Delon, (1992) Amplification was done only for 30 cycles to minimize the bias in PCR amplification. Amplified product was electrophoresed through 1% agarose gel for confirming the amplification. All amplified PCR products of each sample were purified with Axyprep PCR purification kit as per manufacturer's instructions explained in subsection 3.2.6.

Purified PCR products were cloned into pCR4-TOPO vector using a TOPO-TA cloning kit (Invitrogen, Carlsbad, California, USA) according to manufacturer's instructions described in subsection 3.2.7. Transformation was done into TOP-10 cells. A minimum of 30 positive clones per depth were picked up randomly for library construction and subjected to the colony-PCR with primers sets pucM13F/pucM13R. Details of colony PCR explained in subsection 3.2.7. The colony-PCR product was purified and sequenced in both directions using universal forward sequencing primers using as ABI 3130 Genetic

Analyzer following dideoxy chain termination technique. Vector sequences were trimmed off and bidirectional sequence pair was assembled to get complete sequence of approximately 950 bp of cloned product.

4.2.3 Phylogenetic Analysis of Libraries:

VecScreen tool (http:// www. ncbi. nlm. nih. gov /tools/vecscreen/) was used to remove vector contamination. Resulting *16S rDNA* sequencing results were compared to those in National Centre for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), for their matches using BLAST programs and the identification confirmed. The sequences were compared with other databases including SILVA, and NCBI, using MOTHUR and assigned to a Phylum using 97% sequence similarity cut-off. Alignments were automatically trimmed using Gblocks (Castresana, 2000) to remove poorly aligned positions and divergent regions. The sequences were assigned into phylotypes (operational taxonomic units, OTUs) with MOTHUR (http://www.mothur.org) by applying the average neighbor rule (Schloss and Westcott, 2011) in which all sequences within an OTU have to fulfill the preset similarity criterion of 97% sequence similarity.

4.2.4 Statistical analyses

Alpha diversity was calculated on the observed species matrix using Shannon and Pielous indices using mothur (Schloss et al. 2009). Bray-Curtis similarity index was used to compare clone libraries between samples at OTU levels. The similarity matrix was used to perform cluster analysis in Primer 6 (PRIMER-E, Plymouth, UK) using a group-average linking method and non-metric multidimensional scaling (NMDS). Similarity profile (SIMPROF) test was carried and the output was superimposed on an NMDS plot to best

reflect the group formation (Clarke and Gorley, 2006, Clarke et al. 2008).

4.2.5 Multivariate analyses

Canonical correspondence analysis (CCA) was performed using Past-3 software (https://folk.uio.no/ohammer/past/) to understand the influence of environmental parameters on archaeal community structure. All data were normalized to reduce the importance of minor differences in relative concentration/s of individual parameters.

4.2.6 Nucleotide Sequence Accession Numbers

Accession numbers for our archaeal sequences are KY491128 to KY491162 and KY510292 to KY510329 (SIM), KY510330 to KY510359, KY510360 to KY510397 and KY510398 to KY510432 (FIM), KY491050 to KY491087 and KY491088 to KY491127 (NEM).

4.3 Results

From all DNA samples extracted from water samples using archaea biased primers, the archaea *16S rRNA* gene fragments were successfully amplified for seven DNA extracts **(Figure 4.1)**. Further, the employing of archaea specific primers designed by DeLong (1992) proved very effective in this endeavor. From all the seven DNA samples, PCR amplicons of 950 bp were obtained.

Cloning efficiency was evaluated from checking out the insertion of PCR amplified *16S rRNA* gene insertion by random pick outs of a minimum of 30 colonies from each library. Picking of white colonies for examining the transformation efficiency, that over 98% of the colonies picked out were positive for PCR amplified *16S rRNA* gene insertion. After this confirmation, in total 260 clones were randomly picked out for sequencing, after

colony-PCR of which 254 non chimeric sequences were used for further analyses.

In spite of repeated attempts, amplification of archaeal *16S rRNA* genes was not successful from DNA extracted from the surface, DCM, and 500 m. As an exception, the 500 m FIM sample was positive for archaeal PCR amplification. Further analyses, was carried out using 256 archaeal clone sequences obtained from all 250 m, 1000 m and FIM 500 m depths. Sequence similarity between these 256 was quite weak (~82%), and the average substitution differences were 16% (±7% at a 97-95% similarity as the cut-off). With this level of cut-off as many as 182 OTUs were formed of which 57, 87 and 54 OTUs were obtained from the clone libraries of SIM-OMZ (74 seqs), FIM-OMZ (104 seqs) and NEM-OMZ (78 seqs).

The highest number of archaeal OTUs was observed at 250 m during SIM and, the lowest numbers at 1000 m during FIM (**Table 4.1**). Higher diversity was at 250 m than 500 m or 1000 m depths (student's $t = 2.6058$, $df = 5$, $P<0.05$). In the overall, archaeal diversity in the OMZ depths was higher than that of the bacterial diversity (Student's $t = 4.6996$, $df = 14$, $P< 0.001$). Further, the evenness values close to 1 was evidenced among all archaeal clone libraries.

Two-dimensional non-metric multidimensional scaling (2D-NMDS) ordination employed to decipher temporal similarities/dissimilarities among archaeal (**Figure. 4.2**) community profiles indicated patterns with a stress value of <0.10. These analyses suggest a good fit of data points in 2D ordination. With the first two ordination axes of CCA accounting for 58% of the explained total variance, it is apparent that the archaeal communities are influenced by the environmental variables (**Figure 4.3**). The first axis

accounted for 39.53% of the total explained variance reflecting a strong gradient caused by nitrate (R = 0.63) phosphate (R = 0.56) and silicate (R = 0.74). An overall variance of 0.82 in the dataset is evident from the sum of all Eigen values. LINKTREE analysis revealed that nitrate (<22.4 or>25 μM) or phosphate (<2.23 or >2.35 μM) was responsible for the first split (A). The second split (B) segregated the OMZ archaeal profiles into two subgroups based on DO (<3.47 or >9.11 μM) or silicate (<50 or >66.6 μM) or ammonia (<0.133 or >0.17 μM) or nitrite (<0 or >$7.4E^{-2}$ μM) (**Figure 4.4**).

Out of 182 archaeal OTUs, only four were common during all seasons (**Table 4.2**) covering ~13-19% of the sequences from individual seasons (**Figure. 4.5**). Besides, 16 OTUs were common between any two seasons (**Table 4.2**) contributing 14-20% of the sequences. The percentage of season-specific OTUs varied between 64-73% (**Figure 4.5**). The four archaeal OTUs common to all seasons were affiliated to genera *Methanocaldococcus*, *Methanolobus*, *Methanohalophilus* and *Methanothermococcus*. Majority of the archaeal OTUs shared between any two seasons were affiliated with *Methanocaldococcus*s, *Methanolobus*, *Methanosaeta*, *Methanothermococcus* and *Methanotorris* (**Table 4.2**).

Euryarchaea (MG II) dominated (98%) the archaeal clone libraries and maximum numbers of sequences were affiliated to *Methanomicrobia*, *Methanococci*, and *Archaeoglobi* (**Figure 4.6**). *Thaumarchaea* contributed to only 2% of the total archaeal sequences. A total of nine archaeal orders were identified from the OMZ (250 m, 500 m and 1000 m) samples (**Figure4.7**). *Methanosarcinales*, *Thermococcales*, *Methanococcales*, and *Archaeoglobales* were the most dominant and found in all OMZ depths during all seasons. *Thermoplasmatales* (2-23%) and *Methanomicrobiales* (3-18%)

were the second dominant group and were absent in FIM-250 m and SIM-1000 m respectively. *Methanomassiliicoccus*, *Halobacteriales*, and *Nitrososphaerales* have formed the minor groups (<2% of the total archaeal sequences). *Methanomassiliicoccus* was found in SIM- 250 m and NEM-250 m whereas *Nitrososphaerales* were found exclusively in NEM-250 m. *Halobacteriales* was detected only during SIM.

Table 4.1. Operational taxonomic unitS (OTU) richness *(S)*, Shannon diversity index (*H*') and Pielou's evenness index (*J*') determined from archaeal clone libraries at the Arabian Sea Time Series (ASTS) station during Spring intermomsson (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM). Depths are 5 m, deep chlorophyll maximum (DCM), 250, 500 and 1000 m.

Parameters	**Seasons**	**Depths**				
		5 m	DCM	250 m	500 m	1000 m
Total OTUs (S)	SIM	-	-	21	-	14
	FIM	-	-	15	14	12
	NEM	-	-	18	-	15
Shannon diversity (*H'*)	SIM	-	-	4.32	-	3.72
	FIM	-	-	3.8	3.73	3.49
	NEM	-	-	4.12	-	3.81
Pielous eveness (*J'*)	SIM	-	-	0.98	-	0.97
	FIM	-	-	0.97	0.98	0.97
	NEM	-	-	0.98	-	0.97

Table 4.2. Phylogenetic affiliation of archaeal OTUs (OMZ) common to all season and two seasons at the Arabian Sea Time Series (ASTS) location.

OTUs	Phylogenetic affiliation	Accession numbers	Environment
OTUs common in all seasons			
OTU-01	*Methanocaldococcus* sp.	AB_235312.1	Hydrothermal Field
OTU-02	*Methanolobus taylorii*	NR_028238.1	Central Indian Ridge
OTU-03	*Methanohalophiluslevi halophilus*	NR_125474.1	Deep Aquifers
OTU-04	*Methanothermococcus* sp.	AB_260046.1	Crustal Fluids
OTUs common in 2 seasons			
OTU-05	*Methanotorris formicicus*	NR_028646.1	Central Indian Ridge
OTU-06	*Methanocaldococcus* sp.	AB_095160.1	Hydrothermal Field
OTU-07	*Methanothermococcus* sp.	KR_023948.1	Deep-Sea
OTU-08	*Methanolobus* sp.	HM_053966.1	HaloalkaliphilicSoda Lakes
OTU-09	*Methanocaldococcus* sp.	AB_095162.1	Hydrothermal Field
OTU-10	*Methanotorris formicicus*	NR_112199.1	Environment
OTU-11	*Methanocaldococcus* sp.	KC_261588.1	Deep-Sea
OTU-12	*Thermogymnomona sacidicola*	AB_269873.1	Solfataric Soil
OTU-13	*Methanosaeta* sp.	KX_156800.1	Hydrothermal Vents
OTU-14	*Methanocaldococcus indicus*	NR_028861.1	Central Indian Ridge
OTU-15	*Methanothermococcus* sp.	LC_025624.1	Central Indian Ridge
OTU-16	*Methanolobus profundi*	NR_041665.1	Deep Subsurface Sedi
OTU-17	*Methanosaeta* sp.	AJ_133791.1	Environment
OTU-18	*Methanomethylovorans* sp.	EU_544305.1	Anaerobic reactor
OTU-19	*Archaeoglobus infectus*	NR_028166.1	Deep-sea rock Pacific Ocean
OTU-20	*Methanoculleus taiwanensis*	NR_134762.1	Deep marine sediment

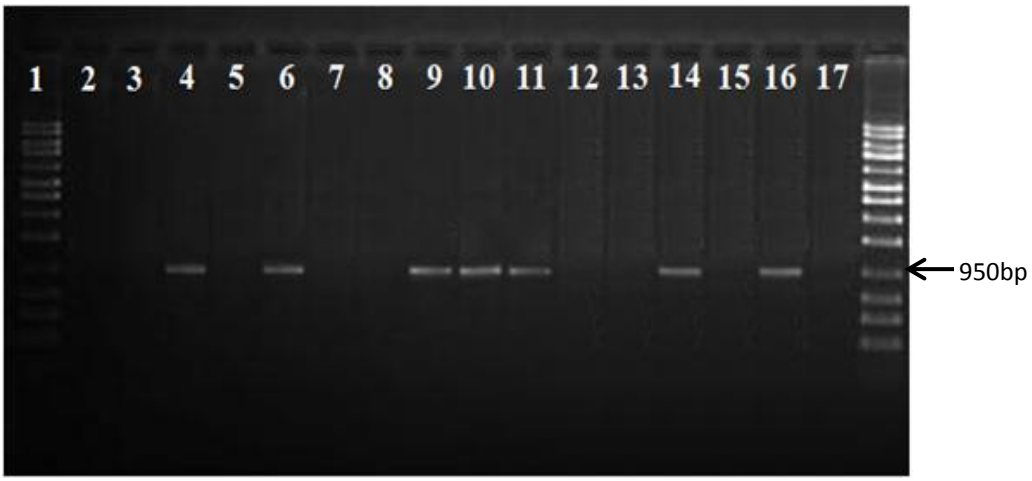


Figure 4.1. PCR amplified product showing *16S rRNA* gene of 15 metagenomic samples from Arabian Sea Time Series (ASTS) station during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM). Depths are 5 m, deep chlorophyll maximum (DCM), 250 m, 500 m and 1000 m. Lane 1 with 1 kb DNA ladder (300 to 10000 bp); lane 2 to 6 (SIM-5 m, SIM-DCM, SIM-250 m, SIM-500 m, SIM-1000 m); lane 7 to 11 (FIM-5 m, FIM-DCM, FIM-250 m, FIM-500 m, FIM-1000 m); lane 12 to 16 (NEM-5 m, NEM-DCM, NEM-250 m, NEM-500 m, NEM-1000 m): lane 17 PCR negative control

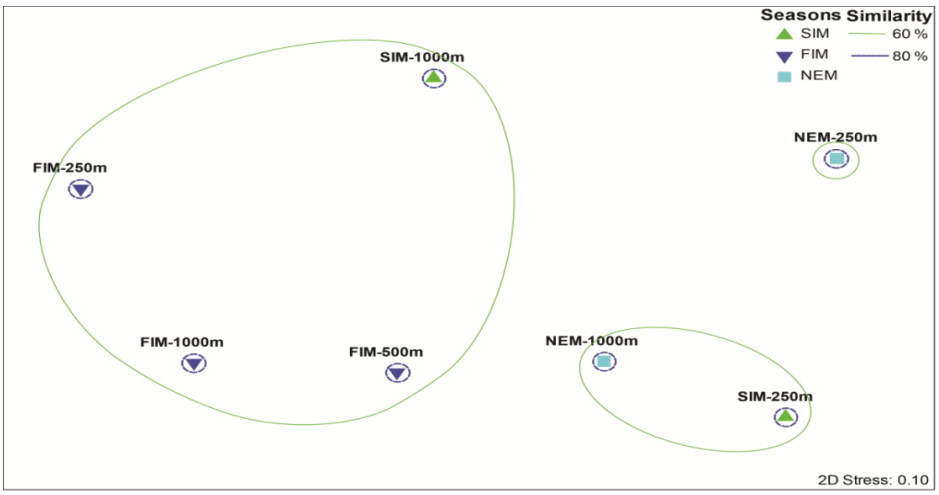


Figure 4.2. Non-metric multidimensional scaling (NMDS) plot of archaeal community profiles was constructed based on the relative abundance of OTUs using Bray–Curtis similarity index. The samples within complete green and dashed Navy blue circles were 60 and 80% similar in archaeal community structure.

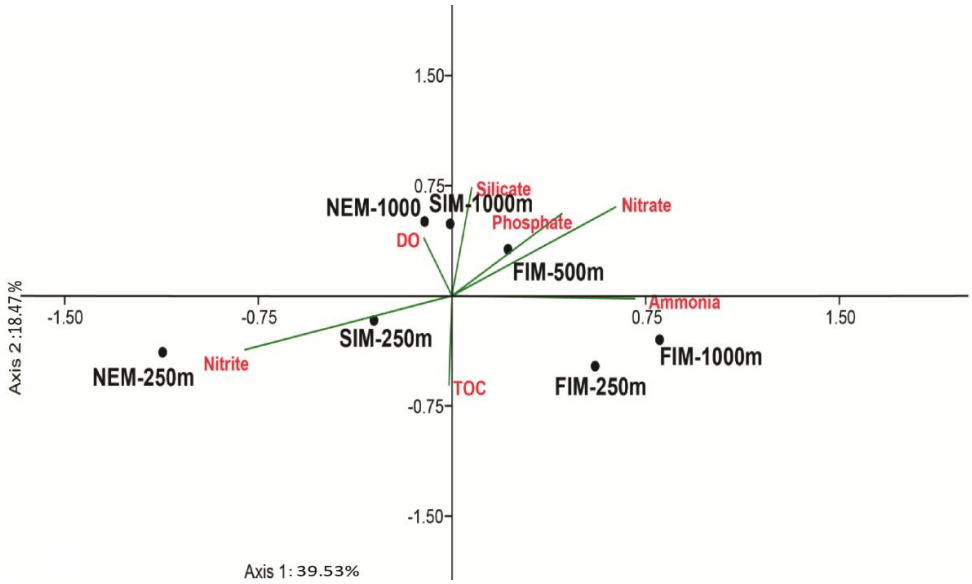


Figure 4.3. Canonical correspondence analysis (CCA) ordination of archaeal communities, associated with environmental variables at the Arabian Sea Time Series (ASTS) location during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM). Red is environmental parameters, black is season and depth.

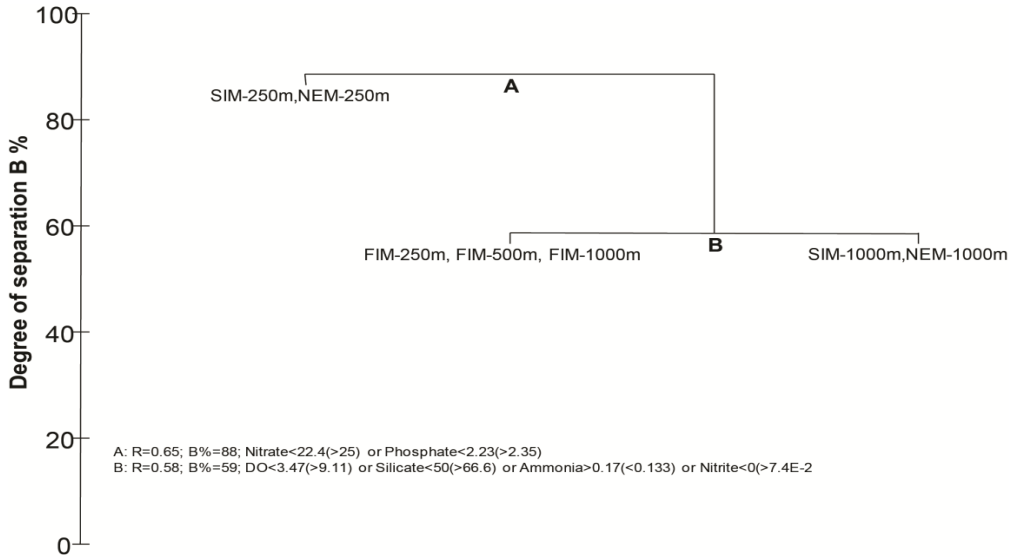


Figure 4.4. Linkage tree analyses (LINKTREE) showing clustering of samples based on environmental variables and archaeal community composition at the Arabian Sea Time Series (ASTS) location during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM).

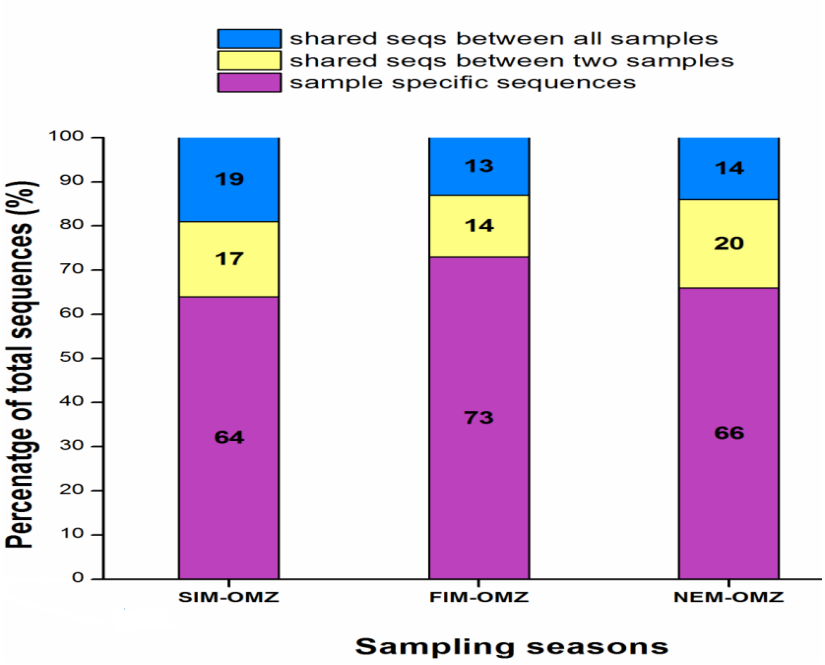


Figure 4.5. Percentage of shared archaeal sequences in the OMZ clone libraries during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM) at the Arabian Sea Time Series (ASTS) location.

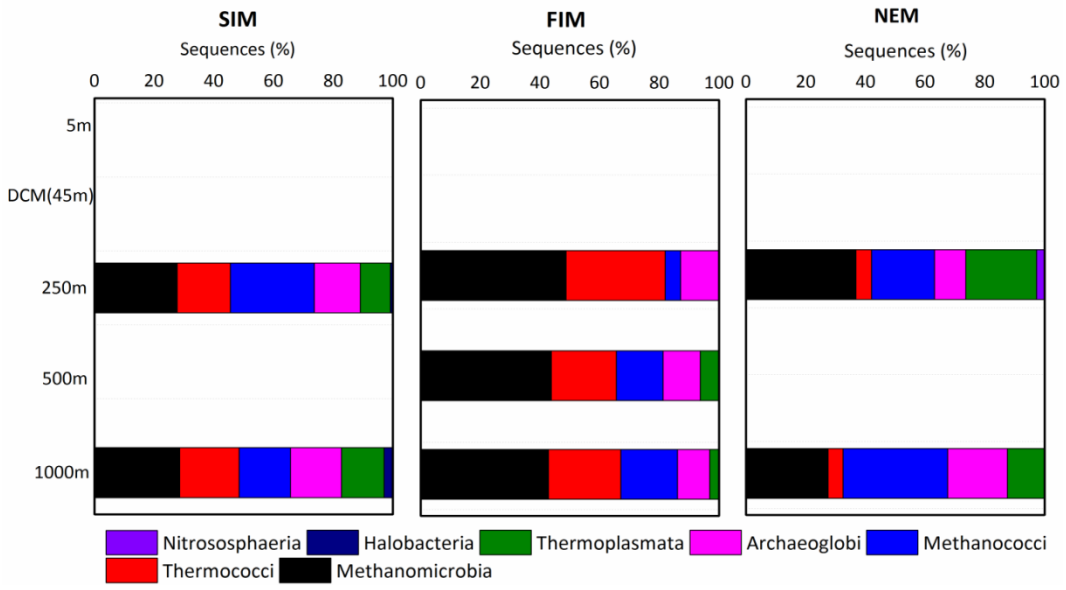


Figure 4.6. Percentages (x-axis) and depth-wise distribution (y-axis) of major archaeal classes at the Arabian Sea Time Series (ASTS) location during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM).

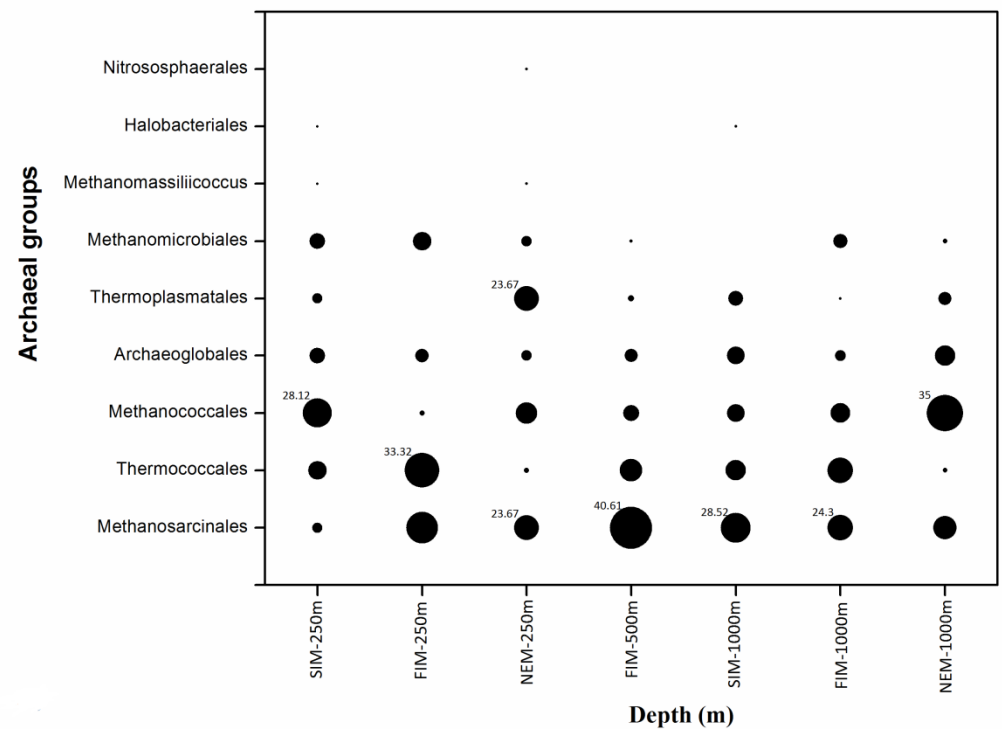


Figure 4.7. Seasonal and depth wise distribution of percentages of archaeal orders at the Arabian Sea Time Series (ASTS) location.

4.4 Discussion

Prevalence of archaea in cold, aerobic marine environments was originally unrecognized. This is because, cultivated members of this domain consisted only of methanogens, extreme halophiles, and sulfur-metabolizing thermophiles (Woese, 1987). However, recent molecular phylogenetic surveys indicated that new types of and yet uncultivated Archaea are common and widely distributed in non-thermophilic marine habitats (Cheng et al. 2008; Cunliffe et al. 2008; DeLong, 1992; Fuhrman et al. 1992; Garcia-Martinez and Rodriguez-Valera, 2000; Massana et al. 1997; Moissl et al. 2008; Steger et al. 2008; Stein and Simon 1996; van der Maarel et al. 1998). The distribution and phylogenetic position of two newly recognized planktonic archaeal groups indicate that they may have phenotypic properties different from those of cultivated Archaea. Unfortunately, very few organisms have been recovered in pure cultures and thus, their specific physiological and metabolic traits remain difficult to be deduced.

Most archaeal studies from the AS-OMZ water column include reports on ammonia-oxidizing archaea (Pitcher et al. 2011). Reports from the Eastern Tropical South Pacific (ETSP) OMZ indicate the presence of MG-II phylotypes throughout the water column (Belmar et al. 2011) which was in agreement with results of this study that showed the predominance of *euryarchaeota* (MG-II) in the OMZ depths. Archaea from G-I.1a are essential components of the meso and bathypelagic microbial communities (Massana et al. 2000, Karner et al. 2001), whereas those from MG-II are predominant within the photic zone (Massana et al. 1997). In contrast to these reports, phylogenetic analyses of archaea from this study showed that MG-II was the most prominent group in the OMZ, with only a few sequences belonging to G-I.1b. Absence of archaea in surface samples and lack of

G-I.1a in AS-OMZ could be due to a bias in the DNA extraction or the PCR reaction. PCR bias and high frequency of mismatches between archaeal primer 958R (used in this study) and G-I.1a SSU-rRNA sequences were reported when applied to deep marine sediments (Teske and Sorensen, 2008). The inadequate-representation of G-I.1a could also be due to an inefficient extraction of its DNA as reported by Feinstein et al. (2009) and Morgan et al. (2010).

Reports speculate the presence of MG II in the surface waters due to higher availability of labile and terrigenous derived organic matter (Galand et al. 2006, 2008; Wells et al. 2006). Teira et al. (2004) reported the presence of MG II in the Atlantic Ocean with little/no variation with depth. The presence of archaeal groups in the OMZ's of the ETSP, northern Chile and other oxygen-deficient ecosystems (Woebken et al. 2007; Quinones et al. 2009; Stewart et al. 2012; Belmar et al. 2011) suggests that archaea are active within the OMZ which is in agreement with the results of the presence of archaea in the AS-OMZ depths. Similar to the results of this study, surveys of SSU-rRNA gene clone libraries from suboxic waters of Cariaco Basin and the Black Sea reveal the dominance of MG-II (Madrid et al. 2001; Vetriani et al. 2003). It is worth noting that although *Crenarchaea* represent an essential group in marine waters (Karner et al. 2001); no sequences are detected in this study.

The archaeal *16S rRNA* sequences from the AS-OMZ suggest that few phylotypes of *Euryarchaeota* obtained bear high similarity to the environmental sequences from the Arctic Ocean and the gas hydrate mound in the Gulf of Mexico (Galand et al. 2009a; Bano et al. 2004; Liu et al. 2009). Most MG II phylotypes, obtained are closely related and

identical to phylotypes reported from deep-ocean, marine sediments, and oceanic hydrothermal vents. The latter two environments have an active sulfur cycle and prominent sulfur-oxidizing bacteria (Sunamura et al. 2004, Park et al. 2010), characteristics reported in the ETSP-OMZ (Stevens and Ulloa, 2008, Canfield et al. 2010). However, number of sequences was similar to those reported in deep-hydrothermal vent environments (Takai and Horikoshi, 1999) or sediments (Vetriani et al. 1999).

Majority of the ASTS sequences affiliated with *Methanosarcinales* (methanogen) were derived from sub-seafloor sediments (Imachi et al. 2011), similar to the findings of the Eastern Tropical North Pacific (ETNP) OMZ sediments (Chronopoulou et al. 2017). Presence of *Methanococcales* at the ASTS could be due to the low oxygen, conditions similar to that of the ETNP-OMZ. Majority of methanogens analyzed from the sediments of the ETNP-OMZ were similar to *Methanococcoides* sp. (Chronopoulou et al. 2017). A few clones from the ASTS location were monophyletic with *Methanomassiliicoccus* indicating the presence of methanotrophic archaea in the AS-OMZ. Similar observations are reported from tropical estuarine sediments of the AS (Singh et al. 2010). Canfield et al. (2010) reported that sequences from ETSP were affiliated to sulfur oxidizing/reducing genes. *Archaeoglobus* and *Thermococcales* harboring the dsr gene were found to be in abundant in AS-OMZ (Kletzin, 2007; Adam et al. 2001). Members of the order *Thermoplasmatales* found in the AS-OMZ are heterotrophic facultative anaerobes, which under anaerobic conditions reduce elemental sulfur to H_2S (Madigan et al. 2006). They are found at oxic-anoxic interfaces (Casamayor and Borrego, 2008) and are reported from diverse environments such as marine waters (Massana et al. 2007), freshwater (Casamayo et al. 2001; Chistoserdova et al. 2005) and hot subsurface waters (Kimura et al. 2005).

Distributions and abundances of MG II phylotypes indicate that planktonic *euryarchaeota* occupy diverse ecological niches (Murray et al. 1999; Hugoni et al. 2013; Lincoln et al. 2014a). Interestingly, *Nitrososphaerales* found in NEM-250 m is affiliated with G1.1b of the archaeal phylum *Thaumarchaeota* (Brochier et al. 2008; Spang et al. 2010). *Nitrososphaera* is reported to grow chemolithoautotrophically under higher ammonia concentrations approaching those tolerated by oligotrophic bacterial ammonia oxidizers (Tourna et al. 2010). The environmental, and biogeochemical functions of MG II are as yet fragmentary and incomplete, mainly due to the lack of pure cultures and whole genomes that would allow us to study the physiology and biochemistry of these organisms better.

Archaea are now recognized as equally important as bacteria in the biogeochemical cycles of the global ocean. Recently, the concept of the microbial carbon pump (Jiao et al. 2010) further highlighted the importance of microorganisms (including Archaea) in the long term storage of refractory dissolved organic carbon, which is the largest pool of organic carbon in the ocean. Decade-long and/or long term monitoring of the abundance and elucidation of the functional roles of MG II and their phylotypes in the open ocean and OMZ is essential. This would be of significance from a climate change perspective too. Such efforts would essentially reveal the role of microbial communities in the feedback and/or sinking fluxes of CO_2, CH_4, N_2O, for instance. From the observed prevalence and predominance of *Methanosarcinales* and *Methanococcales*in the AS-OMZ it is likely that the archaeal community role is of great significance in the *insitu* formation of organic components that are mineralized through shorter pathways for ATP generation. Future studies aimed at delineating the functional roles of the major archaeal groups are essential.

Chapter 5

Diversity of denitrifying bacteria

5.1 Introduction

Oxygen minimum zones (OMZs) are critical to the biogeochemical processes in the world oceans (Stramma 2008; Ulloa et al. 2008) accounting for 40% of the oceanic nitrogen loss (Codispoti et al. 2007). Oceanic zones with dissolved oxygen (DO) concentrations below detection limits harbor heterotrophic nitrate (NO_3^-) reducers and chemolithotrophic ammonia oxidizing bacteria which reduce nitrate through denitrification and anaerobic ammonium oxidation (anammox) (Thamdrup et al. 2006).

Denitrification is a process [reduction of NO_3^- to dinitrogen (N_2)] performed by a diverse group of heterotrophic microbes. Reduction of nitrite (Zumft, 1997) is carried out by two enzymes, one of them requiring copper (*nirK*) and the other requiring iron (*nirS*) (Strous et al. 2006). Song and Ward, (2003) suggest that although believed to appear to have spread by horizontal gene transfer, the phylogeny of nitrite reductase is incompatible with *16S rRNA* gene sequencing.

Reduction of nitrous oxide is the final step in denitrification and is carried out by the enzyme nitrous oxide reductase. The gene specific to denitrifying bacteria is used as marker gene for identification of denitrifier-specific bacteria in environmental samples (Scala and Kerkhof, 1998). Beaulieu et al. (2011) indicated that denitrification is a large source of N_2O (potent greenhouse gas), contributing to climate change and ozone destruction. Bange et al. (2005) and Naqvi et al. (2005) report that approximately half of the annual emissions of N_2O ocean come from the three major OMZs located in the Arabian Sea (AS), Eastern Tropical South Pacific (ETSP) and Eastern Tropical North Pacific (ETNP).

The AS is a region of intense upwelling and high productivity (Wyrtki, 1973; Madhupartap et al. 1996; Naqvi and Jayakumar, 2000) with low/no exchange/replenishment of intermediate waters (150-1000 m). Denitrification and anammox dominate the biogeochemistry of this intermediate oxygen-depleted water column. Ward et al. (1998); Jayakumar et al. (2004, 2013) and Castro-Gonzalez et al. (2005) have reported higher abundance and diverse assemblages of denitrifying bacteria in the suboxic layers of the AS-OMZ. Most of the reported studies are carried out using cultured bacterial isolates. In this regard, efforts to look for metagenomic DNA-based diversity and phylogeny of heterotrophic denitrifiers would prove worthy.

In this chapter, phylogenetic analyses and diversity of the denitrifying bacteria from the Arabian Sea Time Series (ASTS) was carried out using *nirS* and *nosZ* marker genes. Observation from this study is important for comparison of the microbial assemblages with different OMZs and also to delineate the true denitrifying bacterial composition of AS-OMZ which will lend insight into the influence of community composition on nitrogen transformation and fluxes.

5.2 Materials and methods

5.2.1 Sample collection and DNA extraction

To differentiate the spatio-temporal variation of denitrifying bacteria, water samples from five depths were collected during three different seasons. Details of the collection of water samples are explained earlier in subsection 3.2.1. In brief, water samples were passed through sterivex filters and stored at -80°C until nucleic acid extraction was performed in the laboratory. Details of DNA extraction is mentioned earlier in subsection 3.2.4.

5.2.2 PCR amplification

The precipitated DNA was hydrated in 50 µl sterile deionised water. All DNA extracts were subjected to PCR amplification for detection of *nirS* and *nosZ* genes using the primer set and annealing temperatures listed in **Table 1.** The PCR reaction (50 µl) contained 3 µl of extracted DNA (5 to 50 ng μl^{-1}), 1 µl of each primer at a concentration of 0.5 µM, 25 µl of ReadyMix *Taq* PCR mix (Sigma Aldrich) (1.5 U *Taq* DNA polymerase; 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM deoxynucleoside triphosphate [dNTP], stabilizers) and 20 µl of milliQ water. The PCR was performed at 95°C for 90s for initial denaturation, followed by 35 cycles at 95ºC for 24s, and a final extension step at 72ºC for 5 min). Nuclease-free water was used as negative control in each reaction. To minimize the PCR errors, more than 3 reactions for each sample with various amount of DNA template were pooled together. The positive amplicons from each DNA sample were confirmed by electrophoresis on a 1% agarose gel.

5.2.3 Purification of PCR products

To ensure good quality DNA for cloning, MOBIO (Qiagen, Carlsbad, USA) PCR purification kit was used. Five volumes of SpinBind buffer was added to one volume of PCR product and vortexed. The above solution was transferred to a Spin Filter unit with a column and centrifuged at 13000 rpm (30 seconds). On discarding the filtrate, 300 µl of SpinClean buffer was added into the column and centrifuged for 30 seconds (13000 rpm). The liquid flow through was discarded and column was centrifuged for one min (13000 rpm). The column was transferred into a fresh 2 ml tube and 25-30µl of the eluent was carefully added. The tubes were allowed to stand at RT for 5 min, and then centrifuged for at 13000 rpm (one min) for collecting purified PCR product.

5.2.4 Cloning and sequencing

Each gene was cloned using pGEM-T Easy Vector System I cloning kit (Promega, Madison, California, USA) and transformed by chemical transformation into high efficiency JM109 cells and grown overnight at 37°C on LB/X-gal/IPTG plates. A minimum of 40 positive clones of each gene and from each sample were picked out, and subjected to the colony-PCR. PCR-temperature conditions are as follows: initial denaturation step of 10 min at 94°C, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min (annealing), elongation (72°C for 1 min) and final extension at 72^{o}C for10 mins. The PCR products were purified using MOBIO PCR purification kit and sequencing was performed with 15-50 ng of the PCR amplicons adding one pmol each forward and reverse primer of pucM13F / pucM13Rin an ABI 3130 Genetic Analyzer following dideoxy chain termination technique. Resulting sequences were compared to those in NCBI database for their matches and their identification confirmed. Sequencing protocolis detailed in subsection 3.2.8.

5.2.5 Phylogenetic analysis of clone libraries

Phylogenetic analyses of clone libraries were prepared following the standard procedures mentioned in subsection 3.2.9. In brief, the sequences were assembled into contigs using DNA Baser sequence assembly software version 2. VecScreen tool (http:// www. ncbi. nlm. nih. gov /tools/vecscreen/) was used to remove vector contamination. The sequences were compared with the database of NCBI (BLASTn and BLASTp programs), using MOTHUR and assigned to a Phylum if their identity was 95% in the databases searched. Alignments were automatically trimmed using Gblocks (Castresana, 2000). All gene sequences with NCBI accession numbers were aligned using ClustalX and distance

matrices using Phylip 3.66 Dnadist. The sequences were assigned into phylotypes (OTUs) with MOTHUR (by applying the average neighbor rule (Schloss and Westcott, 2011). A representative sequence from each OTU and the top-hit sequences from the GenBank identified via BLASTn search were used to build phylogenetic tree by MEGA 6.0 (neighbor-joining method) with 500 replicate bootstrap analyses.

5.2.6 Statistical analysis and rarefaction curves

To determine the significance of temporal variation in diversity, one-way analysis of variance (ANOVA) was done. The diversity indices (Simpson's and Shannon's indices), richness estimators (Chao 1 and ACE), and rarefaction curve were derived using MOTHUR (Schloss et al. 2009). The three nonparametric richness estimators were used to extrapolate the total richness of the clone libraries from the observed number of OTUs.

5.2.7 Accession numbers

The *nirS* sequences obtained and described in this study were submitted to the NCBI GenBank database and allotted accession numbers KP153530 to KP153534, KP317841 to KP317905 and KX078645 to KX078768. The NCBI accession numbers for *nosZ* gene sequences from this study are KX784867 to KX784885 and KX911214 to KX911243, KY065372 to KY065445 and KY100043 to KY100090.

5.3 Results

In spite of many repeated attempts of PCR, no amplification was seen from the surface, DCM and 1000 m. Thus it is clear that the bacteria possessing these genes are sparse in the well-oxygenated waters. Whereas amplification of *nirS* and *nosZ* genes from all 250 m and 500 m samples suggests that denitrifying bacteria are limited to core OMZ

depths. From a total of 365 sequences, 194 were of *nirS* and 171 of *nosZ* genes.

Results from *nir*S and *nos*Z sequencing efforts affirm that the *Proteobacteria* were the predominant denitrifiers in the AS-OMZ as was also ascertained from the *16S rRNA* gene sequencing (detailed in chapter 3). A few *16S rRNA* sequences belonging to Nitratireductor. Sp, a known denitrifying bacterium of the order *Rhizobiales* and of class *Alphaproteobacteria* were detected exclusively in the OMZ depths (details in chapter 3). Similarly, during all three seasons, the OTUs affiliated to *Rhodobacter* and *Burkholderia* bearing *nirS* and *nos*Z sequences were particularly abundant in 250 m and 500 m (core OMZ) samples.

The amino acid sequences of *nirS* gene obtained in this study show 44%-99% identity with previously published sequences from the AS. They were >75% identical to each other and 75–85% similar to the closest match in GeneBank sequences. Most of the present sequences matched the closest with those reported from the deep sea sediments. Phylogenetic analysis of the 194 non-chimeric *nirS* sequences reveals that all the clones match uncultured sequences of the enzyme nitrite reductase. Sequence similarity of the clones from the present study with those in NCBI database showed that all the OTUs belonged to the phylum *Proteobacteria*. The OTUs from 250 m and 500 m were similar to the *nirS* sequence of the cultivated denitrifiers of *Halomonas, Marinobacter, Rhodanobacter, Ideonella, Herbaspirillum, Thauera, Pseudogulbenkiania,* and *Azospirillum.* The closest matches were those of clone sequences obtained from the suboxic zone of Black Sea, the marine sediment of the Northwest Pacific, hyper saline habitats, sub-surface agricultural upland sediment and rice paddy soil.

Of the 102 clones sequenced from 250 m samples, a total of 11 OTUs were

generated (**Table 5.2)** and ~72% of these affiliated to the genus *Halomonas*. The other OTUs with lesser number of clones affiliated to the genera *Thauera, Ideonella,* and *Marinobacter*. Similarly, from 500 m samples, 10 OTUs were generated from 92 clones (**Table 5.3).** Of these 40% affiliated to genus *Halomonas,* 20% to *Pseudogulbenkiania* and 15% each to *Herbaspirillum* and *Azospirillum*.

Sequences of *nosZ* clones from all seasons showed 80-97% identity with each other and and 75–95% similar to the closest match in GeneBank sequences. All the OTUs from the present study on checking with the NCBI database matched to the phylum *Proteobacteria*. Majority of the OTUs from 250 m and 500 m were analogous to the cultivated *nosZ* sequences of *Pseudomonas, Azospirillum* and *Rhodopseudomonas.* OTUs present in minor proportions were affiliated to culturable fractions of *Paracoccus, Thalassobaculum, Achromobacter, Herbaspirillum, Cupriavidus, Rhodobacter, Sinorhizobium, Nisaea, Burkholderia, Alcaligenes* and *Halomonas*. Some of the OTUs did not belong to any genus or class and were classified as unidentified bacterium. The closest matches were those of clone sequences obtained from the suboxic zone of Arabian Sea, deep sea waters of the Mediterranean Sea, crop soil and wetland sediment of Mexico, terrestrial subsurface sediments, marine aquaculture biofilter and paddy soil.

Of the 84 clones sequenced from 250 m samples, a total of 33 OTUs were generated (**Table 5.4**). Of these 24% were affiliated to *Pseudomonas* followed by *Rhodopseudomonas* (12.4%) and *Azospirillum* (12.2%). The other OTUs with lesser number of clones were affiliated to the genera *Paracoccus, Achromobacter, Herbaspirillum, Cupriavidus, Rhodobacter, Sinorhizobium, Nisaea,* and *Burkholderia.* Similarly, from 500 m samples (87 sequences), 16 OTUs were generated (**Table 5.3).**

Of this majority were affiliated to *Pseudomonas* (12.5%), *Rhodopseudomonas* (12.5%), *Sinorhizobium* (12.5%) and *Achromobacter* (12.5%). *Thalassobaculum, Herbaspirillum, Burkholderia, Alcaligenes, Azospirillum* and *Halomonas* were the other OTUs present in few numbers.

Rarefaction analysis was performed based on the number of clones and OTUs obtained for both *nirS* and *nosZ* genes to investigate the relationship between sampling effort and diversity. The number of *nirS* based OTUs was the highest during SIM followed by NEM and FIM. Shannon and Simpson's indices signify that the diversity of this gene was the greatest during SIM followed by NEM and FIM. Chao 1 and ACE richness estimators imply that the estimated OTUs are much higher at 500 m with greater diversity at 250 m during NEM (**Table 5.6**). Saturation of rarefaction curve (**Fig. 5.3a**) at the recovered OTU level with an evolutionary distance of 3% except for SIM-500 and NEM-500 implies that our sampling efforts adequately covered the diversity.

As many as 30 *nosZ* gene clones were picked out and sequenced from the core OMZ depth during each sampled season totaling it to 171 *nosZ* clones. The NEM (250 m) had the maximum number of OTUs. Shannon and Simpson's indices indicate that the *nosZ* based diversity during individual season was the highest during SIM followed by FIM and NEM. Interestingly, the diversity was maximum at 250 m during all the three seasons. The nonparametric, Chao 1 and ACE estimators revealed that the estimated OTUs are much higher at 250 m during all the sampling seasons (**Table 5.6**). Saturation of rarefaction curves of the *nosZ* OTUs (**Fig. 5.3b**) is used to infer that our sampling was adequate.

Table 5.1. Primers used in this study

Primer	Primer sequence (5'–3')	Annealing temperature	Product size (bp)	Reference
nosZ1F	WCSYTGTTCMTCGACAGCCAG	54°C	259	Henry et al. (2006)
nosZ1R	ATGTCGATCARCTGVKCRTTYTC			
nirS1F	CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T	63°C	890	Braker et al. (1998)
nirS6R	CGTTGAACTT(A/G)CCGGT			

Table 5.2. Phylogenetic affiliation of *nirS* gene based OTUs from 250 m depth from the Arabian Sea Time Series (ASTS) location during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM).

OTUs	Phylogenetic affiliation	Accession number
OTU-01	*Halomonas nitroreducens*	FJ686148.1
OTU-02	*Rhodanobacter* sp.	AB480490.1
OTU-03	*Halomonas fontilapidosi*	FJ686147.1
OTU-04	*Halomonas koreensis*	FJ686156.1
OTU-05	*Thauera* sp.	EF204941.1
OTU-06	*Ideonella* sp.	AB937701.1
OTU-07	*Halomonas alimentaria*	FJ686149.1
OTU-08	*Marinobacter* sp.	DQ479849.1
OTU-09	*Thiohalomonas denitrificans*	AM492191.1
OTU-10	*Halomonas*	EU035284.1
OTU-11	*Halomonas denitrificans*	GQ384047.1

Table 5.3. Phylogenetic affiliation of *nirS* gene based OTUs from 500 m depth from the Arabian Sea Time Series (ASTS) location during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM).

OTUs	Phylogenetic affiliation	Accession number
OTU-01	*Rhodanobacter* sp.	AB480490.1
OTU-02	*Halomonas ventosae*	FJ686160.1
OTU-03	*Halomonas* sp.	GQ384053.1
OTU-04	*Gamma proteobacterium*	AJ248394.2
OTU-05	*Herbaspirillum* sp.	FN555558.1
OTU-06	*Halomonas koreensis*	FJ686156.1
OTU-07	*Pseudogulbenkiania* sp.	KU175475.1
OTU-08	*Halomonas halodenitrificans*	FJ686155.1
OTU-09	*Pseudogulbenkiania* sp.	KU175473.1
OTU-10	*Azospirillum* sp.	AB545710.1

Table 5.4. Phylogenetic affiliation of *nosZ* gene based OTUs from 250 m depth from the Arabian Sea Time Series (ASTS) location during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM).

OTUs	Phylogenetic affiliation	Accession number
OTU-01	*Nisaea denitrificans*	AM279767.1
OTU-02	*Rhodopseudomonas palustris*	KU351105.1
OTU-03	*Paracoccus* sp.	U192075.1
OTU-04	*Bradyrhizobium* sp.	FN600635.1
OTU-05	*Pseudomonas* sp.	KY555442.1
OTU-06	*Burkholderia* sp.	AB545693.1
OTU-07	*Sinorhizobium fredii*	KU351118.1
OTU-08	*Unidentified bacterium*	DQ010768.1
OTU-09	*Azospirillum* sp.	AB545698.1
OTU-10	*Azospirillum* sp.	AB545691.1
OTU-11	*Achromobacter xylosoxidans*	KP868706.1
OTU-12	*Unidentified bacterium*	DQ010792.1
OTU-13	*Rhodopseudomonas palustris*	KU351108.1
OTU-14	*Rhodobacter sphaeroides*	AF125260.1
OTU-15	*Bradyrhizobium* sp.	FN600633.1
OTU-16	*Azospirillum* sp.	AB542275.1
OTU-17	*Azospirillum* sp.	LT900220.1
OTU-18	*Alpha proteobacterium*	JN850958.1
OTU-19	*Cupriavidus* sp.	KY555439.1
OTU-20	*Pseudomonas stutzeri*	HE814026.1
OTU-21	*Rhodopseudomonas palustris*	KU351103.1
OTU-22	*Rhodopseudomonas palustris*	KU351107.1
OTU-23	*Unidentified bacterium*	DQ010784.1
OTU-24	*Unidentified bacterium*	DQ010765.1
OTU-25	*Pseudomonas* sp.	MF802248.1
OTU-26	*Pseudomonas* sp.	AM422887.1
OTU-27	*Pseudomonas* sp.	MF802247.1
OTU-28	*Pseudomonas stutzeri*	HE814034.1
OTU-29	*Pseudomonas stutzeri*	KP_868697.1
OTU-30	*Pseudomonas lini*	DQ377783.1
OTU-31	*Bradyrhizobium* sp.	AB542263.2
OTU-32	*Herbaspirillum* sp.	AB545677.1
OTU-33	*Alpha proteobacterium*	JN850958.1

Table 5.5. Phylogenetic affiliation of *nosZ* gene based OTUs from 500 m depth from the Arabian Sea Time Series (ASTS) location during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM).

OTUs	Phylogenetic affiliation	Accession number
OTU-01	*Pseudomonas stutzeri*	HE814033.1
OTU-02	*Thalassobaculum fulvum*	KU176096.1
OTU-03	*Rhodopseudomonas palustris*	KU351101.1
OTU-04	*Achromobacter xylosoxidans*	KP868706.1
OTU-05	*Sinorhizobium fredii*	KU351116.1
OTU-06	*Alpha proteobacterium*	JN850958.1
OTU-07	*Burkholderia* sp.	AB545695.1
OTU-08	*Unidentified bacterium*	DQ010768.1
OTU-09	*Alcaligenes faecalis*	AF361795.1
OTU-10	*Azospirillum* sp.	AB542278.1
OTU-11	*Pseudomonas* sp.	MF802247.1
OTU-12	*Rhodopseudomonas palustris*	KU351109.1
OTU-13	*Herbaspirillum* sp.	AB545673.1
OTU-14	*Achromobacter* sp.	DQ377792.1
OTU-15	*Halomonas campaniensis*	FJ686164.1
OTU-16	*Sinorhizobium fredii*	KU351117.1

Table 5.6. Comparison of *nirS* and *nosZ* gene diversity in clone libraries constructed from the OMZ during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM) at the Arabian Sea Time Series (ASTS) location.

Seasons	Depth	No. of Sequences	No. of OTUs	Richness estimation			
				Shannon	Simpsons	Chao	ACE
nirS							
SIM	250	32	9	1.43	0.39	10.50	12.56
	500	33	8	1.17	0.49	13.00	17.44
FIM	250	31	4	0.68	0.44	4.00	4.60
	500	34	5	0.83	0.59	5.50	7.22
NEM	250	34	6	1.20	0.39	9.00	14.60
	500	30	6	0.83	0.63	7.00	9.75
nosZ							
SIM	250	29	15	3.56	0.03	16.8	23.4
	500	28	13	3.49	0.01	28.3	26.28
FIM	250	30	11	3.27	0.02	85.2	107.31
	500	27	10	2.9	0.04	25	28.39
NEM	250	28	21	2.92	0.04	25.5	27.1
	500	29	11	2.27	0.09	11.33	11.69

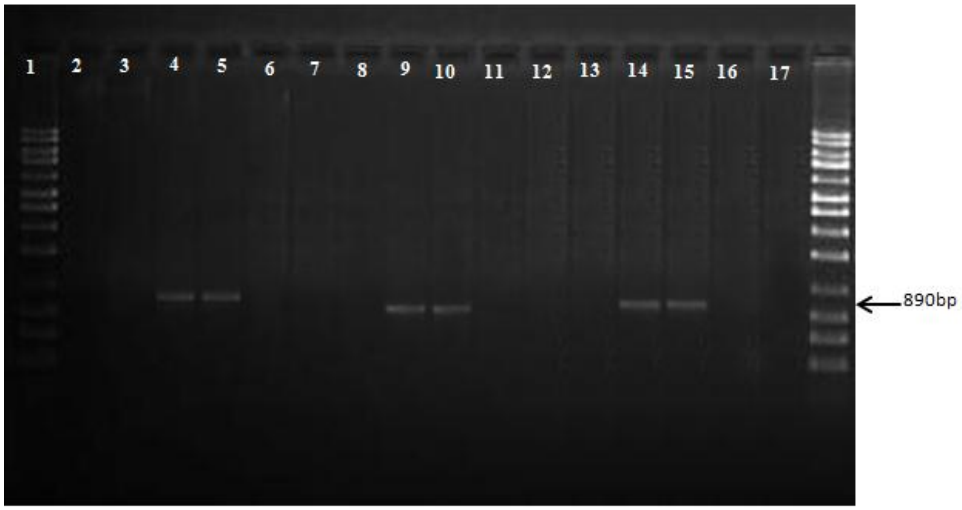


Figure 5.1. PCR amplicons of *nirS* gene 15 DNA extracts of water samples. Lane 1, 1 kb DNA ladder (300 to 10000 bp); lane 2 to 6 (SIM-5 m, SIM-DCM, SIM-250 m, SIM-500 m, SIM-1000 m); lane 7 to 11 (FIM-5 m, FIM-DCM, FIM-250 m, FIM-500 m, FIM-1000 m); lane 12 to 16 (NEM-5 m, NEM-DCM, NEM-250 m, NEM-500 m, NEM-1000 m) depict the PCR amplified at ~890 bp; lane 17 with PCR negative control.

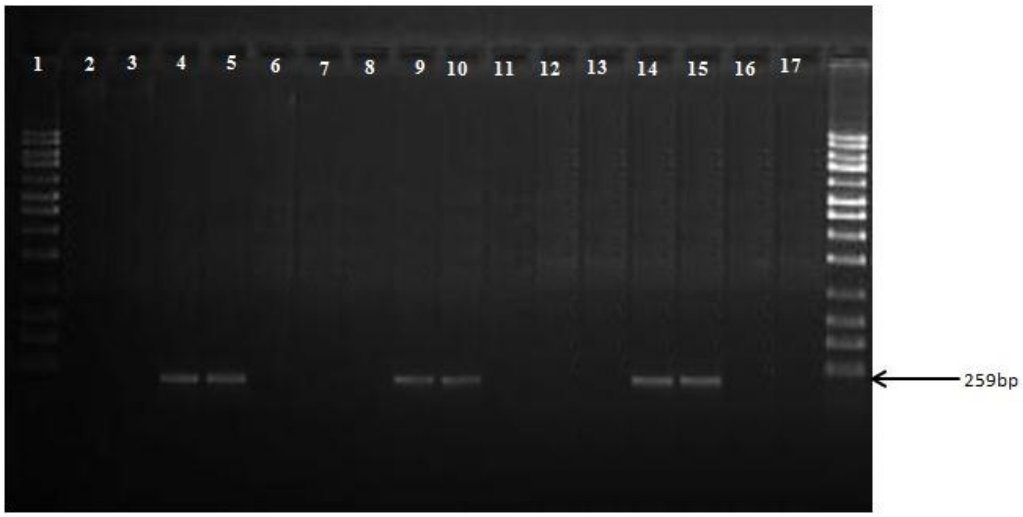


Figure 5.2. PCR amplicons of *nosZ* gene 15 DNA extracts of water samples. Lane 1, 1 kb DNA ladder (300 to 10000 bp); lane 2 to 6 (SIM-5 m, SIM-DCM, SIM-250 m, SIM-500 m, SIM-1000 m); lane 7 to 11 (FIM-5 m, FIM-DCM, FIM-250 m, FIM-500 m, FIM-1000 m); lane 12 to 16 (NEM-5 m, NEM-DCM, NEM-250 m, NEM-500 m, NEM-1000 m) depict the PCR amplified at ~259 bp; lane 17 with PCR negative control.

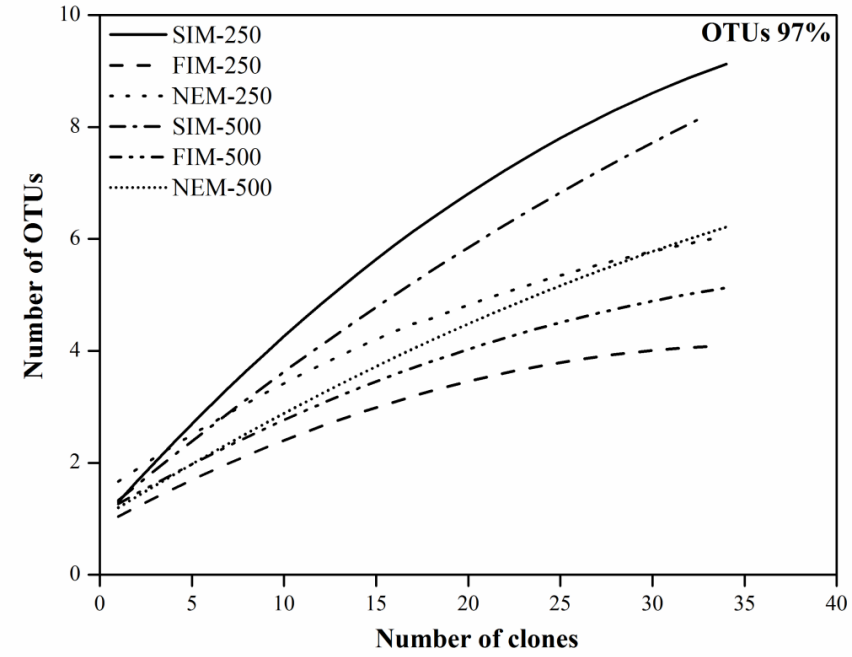


Figure 5.3a. Rarefaction curves of denitrifying bacterial communities based on *nirS* gene sequences during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM) at the Arabian Sea Time Series (ASTS) location.

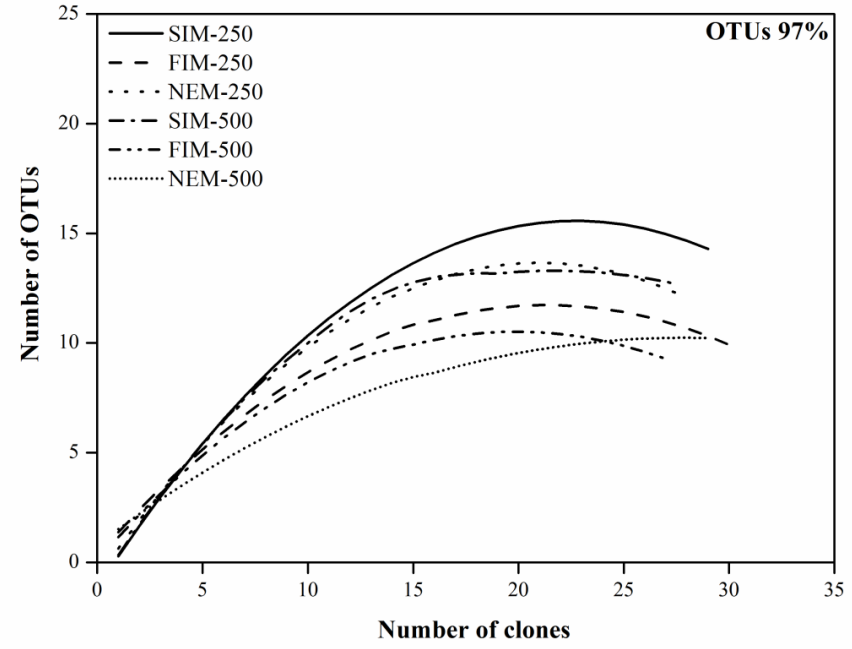


Figure 5.3b. Rarefaction curves of denitrifying bacterial communities based on *nosZ* gene sequences during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM) at the Arabian Sea Time Series (ASTS) location.

5.4 Discussion

The unusual circulation pattern and nutrient-rich waters promote very high primary production in the AS (Madhupratap et al. 1996), decomposition of the organic matter from these highly productive surface waters results in consumption of DO at 150-1200 m leading to denitrification and anammox (Naqvi et al. 1990) in these depths. Denitrification is recognized as an important process in the OMZs of the AS, (Ward et al. 2009, Dalsgaard et al. 2012) and often occurs in habitats where oxygen is restricted, such as suboxic or anoxic water masses in OMZs (Naqvi et al. 2008) or in oxic-anoxic interface of sediments.

Presence of denitrifying bacteria was quite consistent/co varied with the environmental parameters that define the OMZs. PCR amplification of both *nirS* and *nosZ* genes was negative from surface, DCM, and 1000 m samples but invariably positive for samples from 250 m and 500 m during all three seasons. This is in agreement with recent reports by Jain et al. (2014) and Bandekar et al. (2018) where the overall diversity in surface waters of the AS-OMZ is high, but denitrifiers comprise a small part of the community. As the oxygen concentration falls below the threshold for the onset of denitrification (1–4 μM) (Codispoti et al. 2005; Devol, 1978) conditions become more favorable for denitrifiers. Therefore, presence of denitrifying bacteria is limited in the core of the OMZ (250 m-500 m) during all three season.

Stepwise and subsequent reduction of nitrate as an electron acceptor is carried out by denitrifying bacteria. Due to its high redox state, the OMZ is selective for the growth and multiplication of denitrifying bacteria. All *nirS* and *nosZ* gene fragments from AS-OMZ were affiliated with *Proteobacteria* as also reported by Divya et al. (2017) and

Wang et al. (2017). Distribution of ubiquitous *Proteobacteria* is well known and their dominance was reported in AS OMZ depths (Jain et al. 2014; Bandekar et al. 2018).

All ASTS *nirS*-OTUs aligned into three classes: *Gammaproteobacteria, Alphaproteobacteria* and *Deltaproteobacteria,* similar to results reported by Zheng et al. (2014). All sequences from the ASTS matched with uncultured sequences of the *nirS* gene. A few of them are identical to those reported the ETSP-OMZ (Castro-Gonzalez et al. 2005), and from sediment samples of the North West Pacific (Braker et al. 2000).

The *nirS* gene sequences in the AS-OMZ bear striking similarity to those of *Halomonas* (Yan et al. 2003). *Marinobacter* bearing *nirS* gene sequences similar to those in AS-OMZ are reported from the Eastern South Pacific-OMZ and pyrite-fed denitrifying reactor by Castro et al. (2005) and Torrentó et al. (2011) respectively. Sequences of *nirS* were similar to those from *Rhodanobacter* sp. and *Thauera* (Vilar Sanz et al. 2013). These are established denitrifiers reported from wastewater treatment plants (Lui et al. 2006: Zhao et al. 2013). Similarly, denitrifying strains of *Ideonella*, *Azospirillum, Herbaspirillum,* and *Pseudogulbenkiania* recorded from this study from the AS-OMZ are reported from Japanese rice paddy field soils (Tomoyasu et al. 2013). Further, *nirS* sequences from ASTS are 75-85% similar to the cultivated denitrifiers of *Thiohalomonas, Rhodospirillales,* and *Deltaproteobacteria. Chromatiales*as the active sulfide-utilizing denitrifiers (Beller et al. 2006; Park et al. 2011) are widely distributed in OMZs (Beman and Carolan, 2013), and their abundance is tightly coupled to DO concentrations. Dimitry et al. (2007) reported that *Thiohalomonas denitrificans* of the order *Chromatiales* reduced nitrate completely to N_2 with intermediate production of nitrite and N_2O. Detection of *Azospirillum* belonging to *Rhodospirillales* (*Alphaproteobacteria*) in this study is in

agreement with the observation of Luke et al. (2016) wherein *Rhodospirillales* comprised 4.1% of the community in the OMZ core.

All the *nosZ* OTUs obtained from the ASTS aligned into *Alphaproteobacteria*, *Gammaproteobacteria* and *Betaproteobacteria* (Philipott, 2002). Results from this study clearly indicates that *nosZ* based denitrifying community in the AS-OMZ is dominated by the class *Alphaproteobacteria* as also observed by Wyman et al. (2013). Phylogenetic analysis reveals that sequence from this study clustered with *nosZ* sequences from other marine environments such as *Nisaea denitrificans* (class *Alphaproteobacteria*) isolated from the Mediterranean Sea and pure culture of *Alcaligenes faecalis* reported by Urios et al. (2008). Further, *nosZ* bearing sequences from ASTS are 82-85% similar to the cultivated denitrifiers of *Rhodopseudomonas, Rhodobacter, Pseudomonas,* and *Achromobacter* similar to the reports of Stres et al. (2004). Bacteria harboring *nosZ* gene including *Bradyrhizobiaceae, Paracoccus*, *Herbaspirillum*, *Halomonas, Sinorhizobium* and *Azospirillum* are previously reported in paddy soil, hyper saline environment and marine sediments (Hou et al. 2012; Wang et al. 2013; Horn et al. 2006). While some of the sequences from this study were affiliated to culture of *Thalassobaculum*, their role is yet to be understood. *Thalassobaculum* is a facultative anaerobe belonging to the family *Rhodospirillaceae* with *Azospirillum lipoferum* as its closest relative as reported by Zhang et al. (2008).

The two different diversity indices and richness estimators ACE and Chao 1 indicates higher degree of diversity of denitrifying bacteria in the core OMZ depths. Higher diversity of *nosZ* denitrifiers than that of *nirS* based bacteria at ASTS is in agreement with the recent reports of Yang et al. (2018) who suggest that environmental parameters could

shape the denitrifying bacterial community structure. Although the sampling size in this study is not very large, it is evident from the saturation of rarefaction curves that the diversity of denitrifying bacterial communities is adequately captured, and the dominant phylotypes sufficiently represented.

Since denitrification is a major sink for loss of nitrogen from the OMZ regions, knowledge of bacterial assemblages involved in denitrification in OMZ could be linked to loss of fixed nitrogen from these regions. Previous studies have shown that *16S rRNA* based analysis underestimates the diversity of denitrifying bacteria. Thus identification of denitrifiers by their signature genes (*nirS* and *nosZ*) help to identify the different clades that are abundant and possibly active at different stages of denitrification. Jones et al. (2013) suggested that nitrate and nitrous oxide utilizing bacteria difficult to characterize by pure culture. In this regard, use of metagenomic approach, to understand the diversity of denitrifying bacteria using functional marker genes (*nirS* and *nosZ*), as shown in this study will help to determine the different bacteria involved in both nitrate reductions to elemental nitrogen and anaerobic oxidation of ammonia to produce nitrite which goes through further denitrification would prove vital.

Chapter 6

Anammox bacterial assemblages

6.1 Introduction

Anammox bacteria belong to the phylum *Planctomycetes* (Strous et al. 1999). So far five *Candidatus* genera of anammox bacteria are identified (Strouss et al. 1998; Schmid et al. 2003; Quan et al. 2008). Earlier, *Candidatus Scalindua* was the only species detected in the marine environment. Discovery of *Candidatus Kuenenia* by Byrne et al. (2009) and Stewart et al. (2012) in deep-sea hydrothermal vents and in the Eastern Tropical South Pacific (ETSP) further highlights the importance of study on the largely uncharacterized anammox bacterial communities in the Arabian Sea (AS) and other marine ecosystems. As documented by Harhangi et al. (2012) *16S rRNA* gene do not capture the entire diversity of the anammox bacteria. Therefore, molecular detection based on hydrazine oxidoreductase (*hzo*), a key enzyme catalyzing the oxidation of the important intermediate hydrazine (an unstable intermediate in the formation of nitrite from ammonium, N_2H_4) to dinitrogen (N_2) is developed.

Naqvi et al. (1994), Ward et al. (2009) and Dalsgaard et al. (2012) suggest heterotrophic denitrification as the primary process for the loss of fixed nitrogen (N) in the AS and ETSP oxygen minimum zone (OMZ). This view is widely changed owing to the substantiation on anammox as a predominant pathway of N_2 formation in many oxygen-deficient marine systems by Lam and Kuypers, (2011), Kuypers et al. (2005) and Thamdrup et al. (2006). Anammox is a complicated processs in which the concentrations of N compounds do not necessarily correlate with N fluxes or transformations thereby making it difficult to associate ammonium exclusively with either biogeochemical or microbial process (Zehr, 2009). Anammox bacteria oxidize ammonium while simultaneously reducing nitrite as an electron acceptor, which results in the production N_2 gas that is

eventually lost from the ecosystem (van de Graaf et al. 1995). Thus, their role in global nitrogen cycle is vital

As is well known, in the AS the subsurface, intermediate layers is among the very intensely suboxic OMZs. Nitrogenous nutrients are often in limiting concentrations affecting biological processes in the marine ecosystems. Recycling of these nutrients within ecosystems mainly occurs through N fixation or land discharges. Microbially catalyzed reductive processes are responsible for ~40% of the marine-fixed N loss from the world oceans (Codispoti, 2007). Due to profound nitrogen transformation in the suboxic 150-1000 m of the water column in the AS-OMZ, there is a removal of up to 60 Tg nitrogen annually (Codispoti, 2007).

Anammox bacterial communities in the AS-OMZ remain to be described in greater details although Woebken et al. (2008) and Jain et al. (2014) detected the rare presence of anammox *16S rRNA* gene fragments in the open AS-OMZ waters. In order to obtain detailed account of the presence and possible diversity, phylogenetic analysis of *hzo* and *16S rRNA* genes was carried out in the AS-OMZ. This is because, knowledge of the diversity, of *hzo*-producing anammox bacteria along with *16S rRNA* related anammox bacteria will help in assessment of some mechanisms responsible, in particular for anammox. To achieve this PCR amplification of *16S rRNA* gene fragments was carried using the two commonly used primer sets, Amx368F/Amx820R and Brod541F/Amx820R.

6.2 Materials and methods

6.2.1 Sampling and DNA extraction

Water samples were collected under the SIBER program during May 2012 (representing Spring intermonsoon [SIM]), September 2012 (Fall intermonsoon [FIM]), and February 2013 (Northeast monsoon [NEM]), from the Arabian Sea Time Series (ASTS) station (17°0.126' N, 67°59.772'E) as described earlier in subsection 3.2.1. Extraction of DNA was carried out using modified method of Ferrari and Hollibaugh, (1999), explained in subsection 3.2.4.

6.2.2 PCR amplification and purification

Oligonucleotide primers, Brod541F /Amx820R, and Amx368F /Amx820Rwere used to PCR-amplify *16S rRNA* genes from all the DNA extracts from different water samples. Gene fragments of *hzo* were amplified using the primer pairhzoF1and hzoR1Li et al. (2010). PCR reactions were performed in a final volume of 50µl using a thermal cycler Veriti (Applied Biosystem, USA). The PCR mixture (50 µl) contained 1 µl of extracted DNA (5 to 50 ng μl^{-1}), 1 µl of each primer at a concentration of 0.5 µM, 25 µl of ReadyMix *Taq* PCR mix (Sigma Aldrich) (1.5 U *Taq*DNA polymerase; 10 Mm Tris-HCl, 50 Mm KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM deoxynucleoside triphosphate [dNTP], stabilizers) and 22 µl of milliQ water. The PCR parameters for *16S rRNA* gene amplification were: 95°C for3 min for initial denaturation followed by 35 cycles each at 95°C for 45 seconds denaturation, 1 min of annealing at 59°C and one min of extension at 72°C. Thermal cycling for *hzo* gene was as follows: 94°C for5 min of initial denaturation followed by 95°C for 1 min, 1 min of annealing at 60°C and two min of extension at72°C for a total of 30 cycles. Nuclease-free water was used in all negative controls run along. Positive amplicons from each DNA sample were confirmed by electrophoresis on a 1% agarose gel.

To ensure good quality DNA for cloning, MOBIO PCR purification kit was used. In brief, mixture of PCR product and Spinbind buffer was centrifuged at 13000 rpm. SpinClean buffer was added to column and centrifuged at 13000 rpm followed by a dry spin at the same rpm. 25-30 µl of elution buffer was added, centrifuged and purified PCR product collected. Detailed protocol is provided in subsection 5.2.3.

6.2.3 Cloning and sequencing

Amplicons were cloned into pGEM-T Easy Vector System I and transformed into JM109 competent cells grown overnight on LB/X-gal/IPTG plates at 37°C. Detailed cloning protocol is explained in subsection5.2.4. As many as 45 clones of *hzo* and 31 of *16S rRNA* gene from each sample were randomly picked out for colony-PCR. The PCR products were purified using MOBIO PCR purification kit (Qiagen, Carlsbad, USA) and sequencing was performed with 15-50 ng of the PCR amplicons adding one pmol each forward and reverse primer using an ABI 3130 Genetic Analyzer following dideoxy chain termination technique. Resulting sequences were compared to those in NCBI database for their matches identification confirmed. Temperature conditions for colony-PCR and sequencing is described in subsection 3.2.7 and 3.2.8 respectively.

6.2.4 Phylogenetic analyses

Phylogenetic analyses of clone libraries were prepared following the standard procedures mentioned in subsection 3.2.9. In brief, the sequences were assembled into contigs using DNA Baser sequence assembly software version 2.VecScreen tool (http:// www. ncbi. nlm. nih. gov /tools/VecScreen/) was used to remove vector contamination. The sequences were compared with the database of NCBI (BLASTn and BLASTp programs), using MOTHUR and assigned to a Phylum if their identity was 97% in the

databases searched. Alignments were automatically trimmed using Gblocks (Castresana, 2000). All gene sequences with NCBI accession numbers were aligned using ClustalX and distance matrices using Phylip 3.66 Dnadist. The sequences were assigned into phylotypes (OTUs) with MOTHUR (by applying the average neighbor rule (Schloss and Westcott, 2011). Phylogenetic trees were constructed using MEGA 6.0.

6.2.5 Statistical analysis

To compare the *hzo* and *16SrRNA* gene-based richness within each of the investigated depth, MOTHUR was used. The indices for diversity (Simpson's and Shannon's indices) and richness estimates (Chao 1 and ACE, Schloss et al. 2009) were calculated. Also, rarefaction analyses were performed using MOTHUR.

6.2.6 Accession numbers

The *hzo* sequences obtained and described in this study were submitted to the NCBI GenBank database and allotted accession numbers KT211367-KT211397 and KU572781-KU573035. The NCBI accession numbers for *16S rRNA* gene sequences from this study are KY650836 to KY651019.

6.3 Results

In the AS-OMZ region, the surface and DCM depths were well oxygenated. A steep oxycline between DCM and 250 m was evident in **Figure 6.1**. Average DO concentration ranged from 185.24 ± 31.1 μM in the surface to 5.56 ± 5.5 μM in the OMZ depths. A subsurface peak of ammonium and nitrite concentrations in the euphotic zone was observed during all three seasons. Highest concentration of nitrite (2.57 μM) was observed during NEM. Concentration of ammonia (0.27 μM) was at peak during FIM. Maximum

concentration of both nitrite and ammonia was noted within the core OMZ depths. As explained in Table 3.1 of chapter 3, DO in the core OMZ depths were ≤ 1.0 μM.

Equivalent concentrations of DNA extracts from all five depths were used for PCR amplification. Unlike Amx368F/Amx820R, primer setBrod541F/Amx820R of the *16S rRNA* gene of anammox bacteria and primer hzoF1/hzoR1 yielded positive amplification at ~279 bp (**Figure 6.2**) and ~740 bp (**Figure 6.3**) respectively. PCR amplification was positive in the DNA extracts from 250 m and 500 m. DNA extracts from surface, DCM, and 1000 m did not show amplification even with 3-5 repeated attempts with increased combinations of template DNA and/or primer concentration. Samples from 250 m and 500 m were replete with *hzo* and *16S rRNA* genes during all three seasons. A total of 285 *hzo* and 184 *16S rRNA* gene positive clones were randomly picked out and sequenced for clone library construction.

All the *hzo* OTU sequences from 250 m and 500 m samples during all three seasons were well aligned. The deduced *hzo* amino acid sequences were 84–97% identical to each other and 74–97% identical to the closest-match GenBank sequences retrieved from engineered and natural habitats. At 97%sequence similarity all 285 non-chimeric *hzo* sequences match with hydrazine oxidoreductase sequence of the anaerobic ammonia oxidizing bacteria.

Of the 142 sequences from 250 m and 143 sequences from 500 m a total of 17 and 22 OTUs were obtained respectively. The phylogenetic trees from the alignment of experimental and achieved deduced *hzo* OTU sequences from 250 m (**Figure 6.4a**) and 500 m samples (**Figure 6.4b**) during all three seasons were constructed. All the OTUs at

250 m and 500 m were affiliated to *Candidatus Scalindua*. The known GenBank sequences obtained were from sediments of the South China Sea, Okhotsk Sea, Bohai Sea and Jiaozhou Bay.

The *16S rRNA* gene sequences were 95-100% identical to each other and 95-97% similar to the closest-match in GenBank sequence database. All of the OTU sequences were related to the genus *Scalindua* of the phylum *Planctomycetes*. *S.brodae, S.marina* and *S.wagneri* were found to be the predominant species during all three seasons. *S.brodae* was the most dominant species followed by *S. Marina* and *S. wagneri*.

Of the 7 OTUs obtained from 250 m (**Figure 6.5a**), 57% belonged to *S.brodae* (4 OTUs), and 43% to *S.wagneri* (3 OTUs). Similarly, the *16S rRNA* gene sequencing from the DNA extracts of 500 m ((**Figure 6.5b**) yielded 6 OTUs with most of them aligning with *S. marina* followed by *S.brodae* and *S. wagneri*.

The number of *hzo* OTUs for individual season was highest in 500 m depth during all seasons. Maximum number of OTUs was during FIM followed by NEM and SIM. Shannon and Simpson indices clearly indicate that the diversity was the highest during NEM followed by FIM and SIM (**Table. 6.1**). However, the nonparametric estimators, Chao 1 and ACE revealed that the estimated OTUs are higher in 500 m (SIM and FIM) with the exception of NEM (250 m). Notably, saturation of rarefaction curve (**Figure 6.6a**) at the recovered OTU level with an evolutionary distance of just 3% implies that our sampling efforts adequately covered the diversity.

As many as 30 positive clones of *16S rRNA* gene were selected per season per depth (250 m and 500 m) for clone library construction and phylogenetic analysis. The

number of OTUs were the highest during SIM (500 m) and FIM (250 m) followed by NEM (500 m). Shannon and Simpson indices indicate that the diversity was the highest during FIM in 250 m followed by SIM and NEM at 500 m (**Table. 6.1**). Chao 1 and ACE revealed that the highest species richness is 500 m (SIM) followed by FIM (250 m) and NEM (500 m). Saturation of rarefaction curve (**Figure 6.6b**) confirms diversity was sufficiently covered.

Table 6.1. Comparison of *hzo* and *16S rRNA* gene diversity in clone libraries constructed from the OMZ during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM) at the Arabian Sea Time Series (ASTS) location

Seasons	Depth (m)	No. of sequences	No. of OTUs	Richness estimation			
				Shannon	Simpson	Chao	ACE
***Hzo* gene**							
SIM	250	47	10	1.81	0.21	11	12.13
	500	49	15	2.2	0.15	16.42	19.01
FIM	250	47	16	2.36	0.11	18.5	28.99
	500	47	19	2.52	0.1	23.5	31.39
NEM	250	48	14	2.22	0.13	22	50.39
	500	47	18	2.6	0.08	22.2	22.72
***16S rRNA* gene**							
SIM	250	31	8	1.46	0.30	18	40
	500	30	14	2.3	0.10	26	50.26
FIM	250	30	14	2.37	0.08	23.33	34.44
	500	31	6	1.04	0.50	6.33	7.4
NEM	250	31	8	1.06	0.55	15.5	29
	500	31	10	1.67	0.29	15	31.30

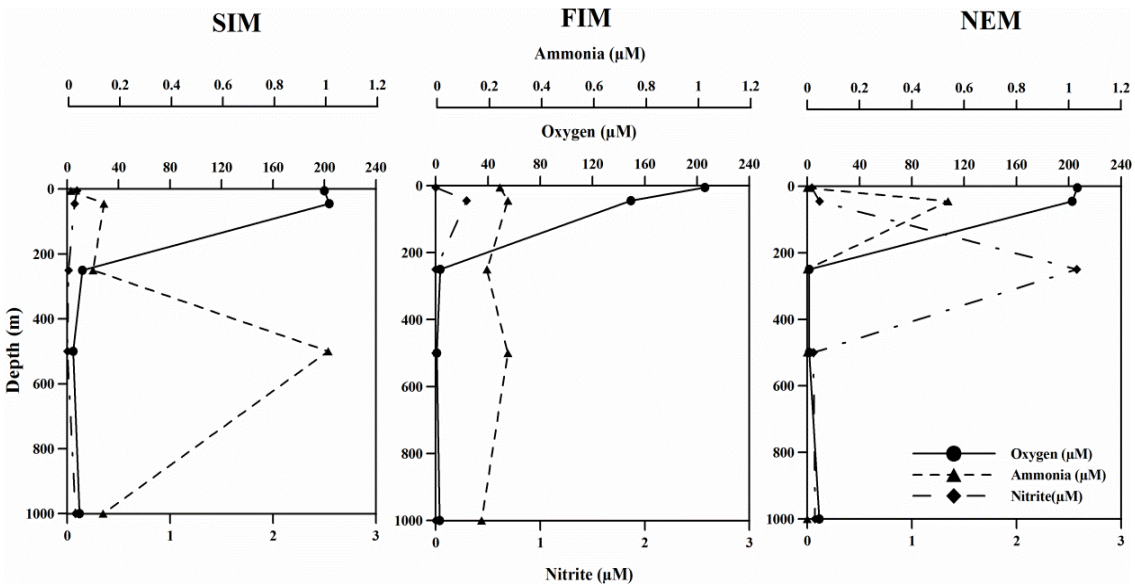


Figure 6.1. Vertical distribution of concentrations of dissolved oxygen (DO), ammonium (NH_4) and nitrite (NO_2) during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM) at the Arabian Sea Time Series (ASTS) location.

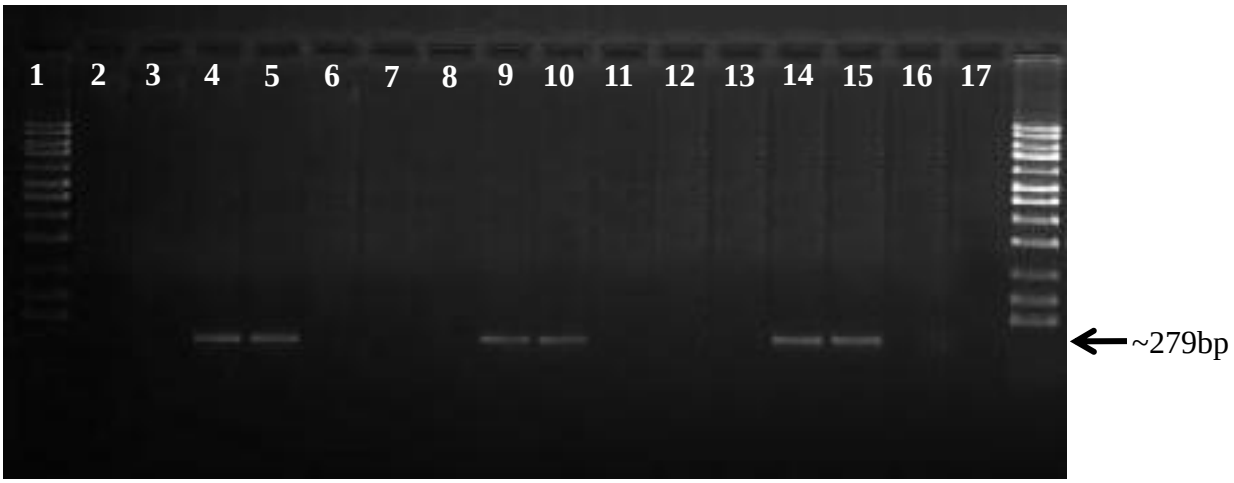


Figure 6.2. PCR amplicons of *16S rRNA* gene anammox bacteria from 15 DNA extracts of water samples. Lane 1, 1 kb DNA ladder (300 to 10000 bp); lane 2 to 6 (SIM-5 m, SIM-DCM, SIM-250 m, SIM-500 m, SIM-1000 m); lane 7 to 11 (FIM-5 m, FIM-DCM, FIM-250 m, FIM-500 m, FIM-1000 m); lane 12 to 16 (NEM-5 m, NEM-DCM, NEM-250 m, NEM-500 m, NEM-1000 m) depict the PCR amplified at ~259 bp; lane 17 PCR negative control.

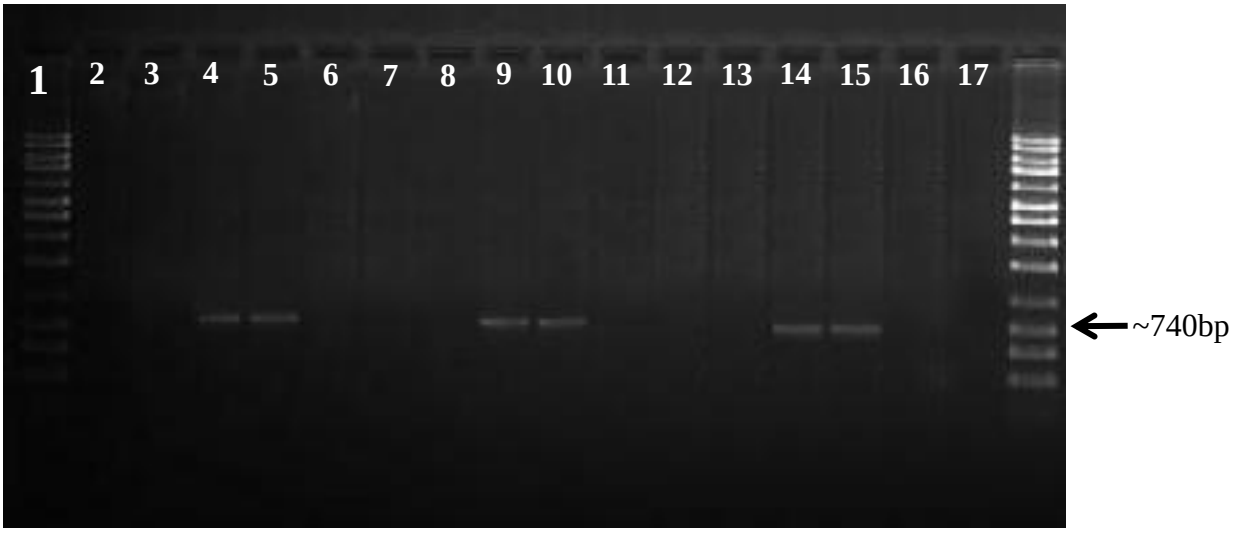


Figure 6.3. PCR amplicons of *hzo* gene of 15 metagenomic samples. Lane 1, 1 kb DNA ladder (300 to 10000 bp); lane 2 to 6 (SIM-5 m, SIM-DCM, SIM-250 m, SIM-500 m, SIM-1000 m); lane 7 to 11 (FIM-5 m, FIM-DCM, FIM-250 m, FIM-500 m, FIM-1000 m); lane 12 to 16 (NEM-5 m, NEM-DCM, NEM-250 m, NEM-500 m, NEM-1000 m) depict the PCR amplified at ~740 bp; lane 17 PCR negative control.

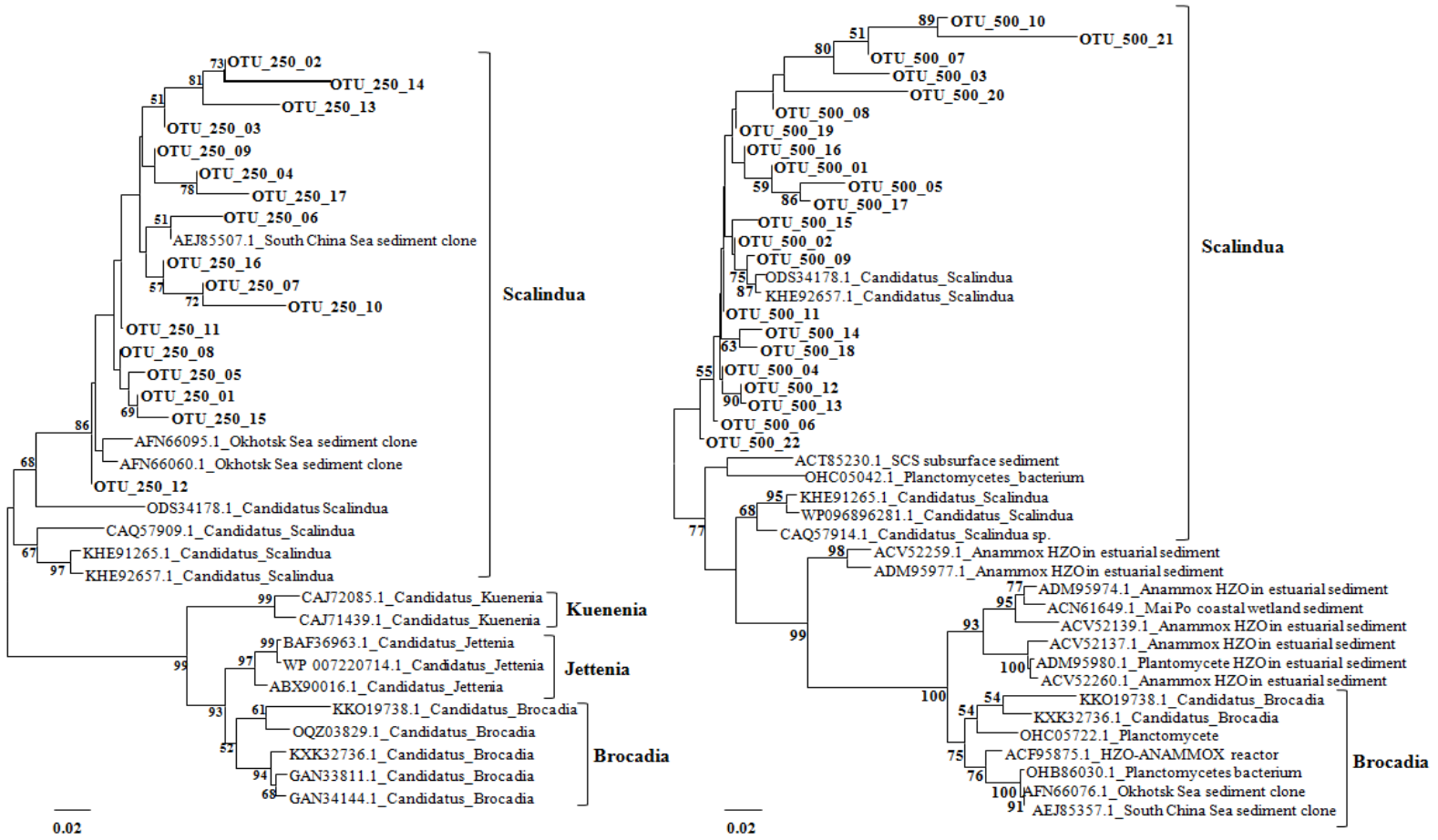


Figure 6.4. Phylogenetic trees of *hzo*-amino acid OTU sequences at 250 m (a) and 500 m (b) at the Arabian Sea Time Series (ASTS) location during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM). The sequences in bold are from the present study.

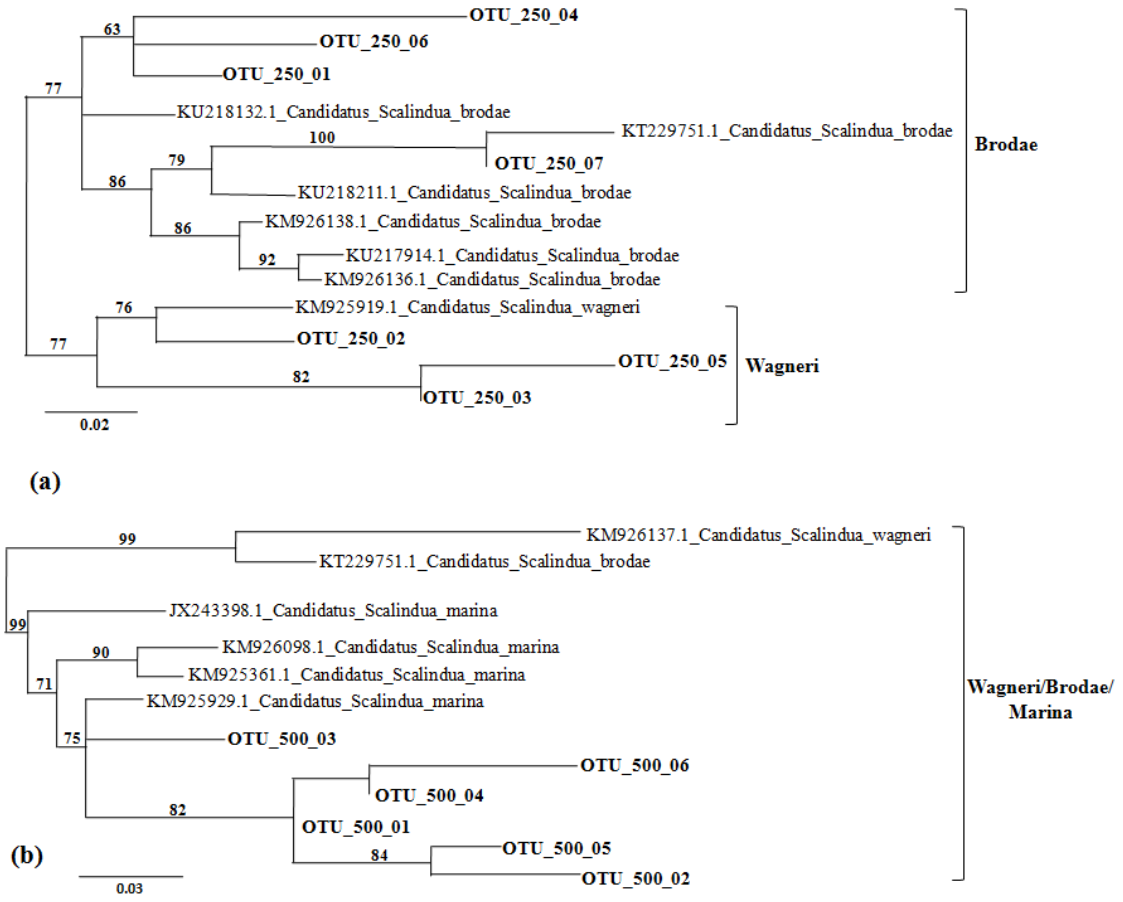


Figure 6.5. Phylogenetic trees of *16S rRNA* gene nucleotide OTU sequences at 250 m (a) and 500 m (b) at the Arabian Sea Time Series (ASTS) location during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM). The sequences in bold are from the present study.

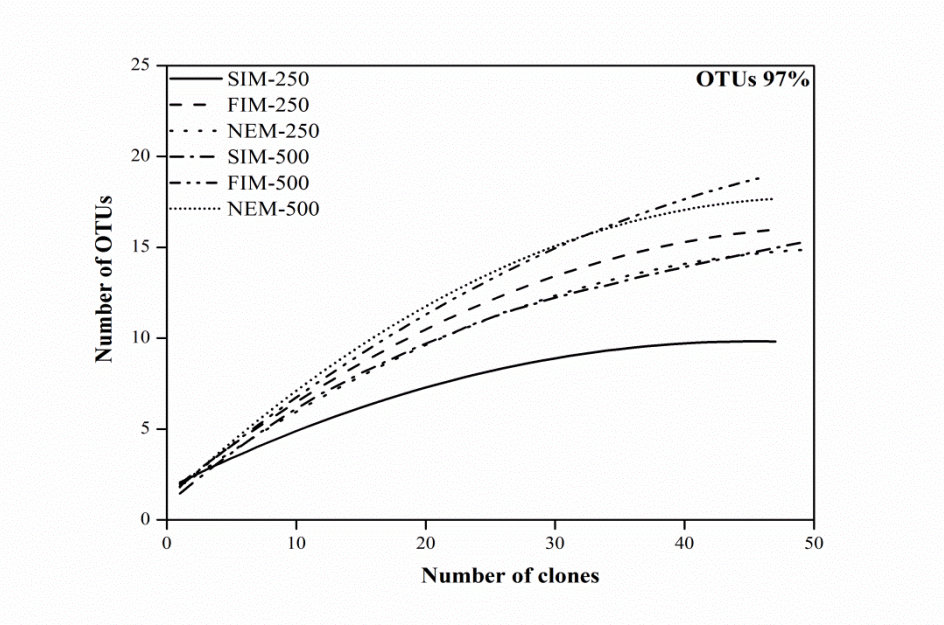


Figure 6.6a. Rarefaction curves of anammox bacterial communities based on *hzo* gene sequences during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM) at the Arabian Sea Time Series (ASTS) location.

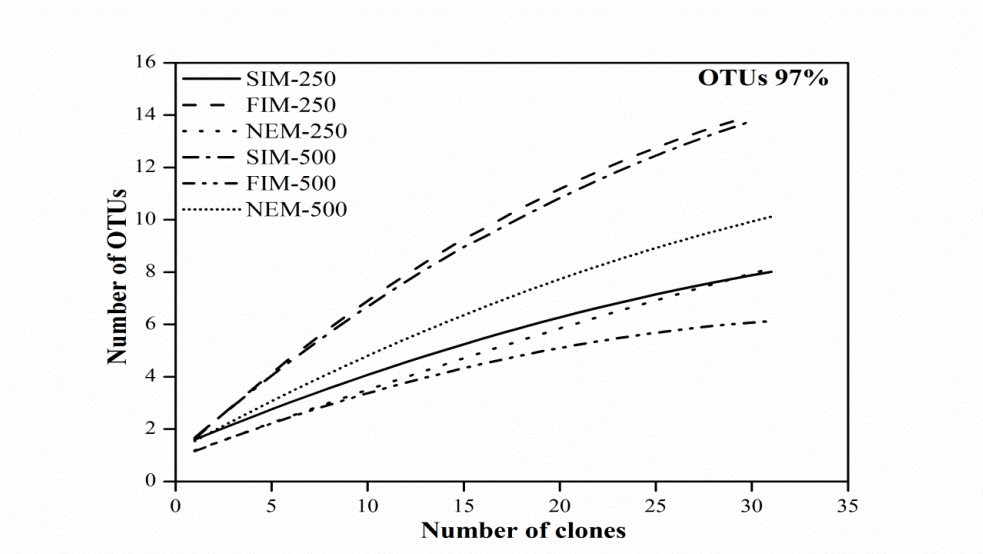


Figure 6.6b. Rarefaction curves of anammox bacterial communities based on *16S rRNA* gene sequences during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM) at the Arabian Sea Time Series (ASTS) location.

6.4 Discussion

Traditionally the OMZs were believed as regions dominated by heterotrophic denitrification. This view has changed drastically, in part, because anammox has now been recognized as a significant and even, the dominant pathway of N_2 formation in many oxygen-deficient marine systems as elucidated by Kuypers et al. (2005), Thamdrup et al. (2006) and Lam and Kuypers, (2011). The discovery of anammox has changed our paradigm on marine nitrogen cycle in the last decade, which reveals a previously unrecognized key route for nitrogen removal from marine ecosystems (Devol, 2003). However, Ward et al. (2009) and Dalsgaard et al. (2012) have recently reported denitrification to be important for the removal of fixed nitrogen in the OMZs of the AS and ETSP.

In the AS-OMZ, highest microbial richness is at the base of the euphotic zone or the upper oxycline (Jain et al. 2014; Bandekar et al. 2018). The sinking particles ought to promote a diverse community of heterotrophs that get specialized to inhabit the OMZ depths. Anammox bacteria might be in sparse fraction but ought to contribute significantly in the elemental cycling in AS-OMZs akin to observations of Beman and Carolan, (2013) and Pedros-Alio, (2012) from the OMZs off Chile and ESTP respectively. Anammox bacteria represent less than 4 % of the microbial community (Kuypers et al. 2005; Hamersley et al. 2007) exhibiting a wider tolerance for O_2 concentrations (10–20 μmol L^{-1}) in the environment (Hamersley et al. 2007; Jensen et al. 2011; Kuypers et al. 2005) such as the ones in the AS-OMZ.

As stated earlier (section 6.3) PCR amplification of both *hzo* and *16S rRNA* genes were unsuccessful from surface (5 m), DCM (50 m), and 1000 m samples but invariably positive for samples from 250 m and 500 m during all three seasons. Similar observation for anammox (*hzo*) gene was reported in the Chilean open ocean OMZ by Chang et al. (2010) where the surface waters revealed fewer transcripts of anammox bacteria compared to the prominent anammox gene transcripts in the core OMZ. These results are consistent with predicted distributions of N_2 production by anammox bacteria in OMZ settings (Chang et al. 2010). Results of chapter 3, delineates the presence of *Planctomycetes* (anammox bacteria) exclusively in the core OMZ depths few along with few of the common denitrifiers such as *Thiobacillus denitrificans* and *Roseobacter*.

Recent metagenomic analyses have helped identify five genera of anammox. They are *Brocadia, Kuenenia*, *Anammoxoglobus*, *Jettenia* and *Scalindua*. Although metabolism and competitive fitness of *Scalindua* is yet to be known (Van de Vossenberg, 2013), *Scalindua* is by far the only genus reported from marine environment. Previous studies on anammox bacteria, report that *16S rRNA* gene sequences obtained from Black Sea, AS, Namibian, and the Peruvian OMZs, are closely related to *Candidatus Scalindua*. *16S rRNA* gene sequences from the ASTS were related to *Candidatus Scalindua brodae, Candidatus Scalindua marina* and *Candidatus Scalindua wagneri*. Similar results were reported by Woebken et al. (2008), wherein, the *16S rRNA* gene sequences from the AS were all closely related to *Candidatus Scalindua sorokinii* and *Candidatus Scalindua brodae*. Results of ASTS are in agreement with observations of Penton et al. (2006) and Schmid et al. (2007) that, only *Scalindua* is found in marine ecosystems.

Wang and Gu, (2013) suggests that the use of *hzo* gene is a highly reliable marker for detecting anammox bacteria from environmental samples. Being a functional gene unique to anammox bacteria (Schmid et al. 2008; Quan et al. 2008; Li et al. 2010) thus an appropriate biomarker for their phylogenetic analyses. From the very high similarity percentages, it is suggested that the AS-OMZ harbors anammox bacteria exclusively similar to uncultured *Planctomycete* sequences obtained previously from the AS and Chilean OMZs by Woebken et al. (2008) and Galán et al. (2009) respectively. The ASTS sequences show 98 to 100% similarity to the *Planctomycetes* sequences recovered by Hong et al. (2011) from marine sediments. Further, Jayakumar et al. (2009) and Bao-lan et al. (2011) also reported *Planctomycete* as the dominant anammox bacteria in the AS-OMZ.

Anammox gene surveys in the global ocean OMZs have recognized very less diversity comprising mostly of "*Candidatus Scalindua*" (Chang et al. 2010). The *hzo* sequences obtained in this study are 74–93% similar to sequences reported from marine sediments. All *hzo*-OTUs from 250 m and 500 m showed affiliation to the genus *Candidatus Scalindua*. The phylogenetic analysis of *hzo* protein sequences implies that *Candidatus Scalindua* is the dominant anammox bacteria in the water samples from the ASTS location which is in agreement with the previous finding that most of the *16S rRNA* gene sequences obtained from AS-OMZ is closely related to *Candidatus Scalindua* (Pitcher et al. 2011; Luke et al. 2016). These observations are useful to suggest that these bacteria contribute to the anammox process in the AS-OMZ. It is likely that depending on the hydro chemical features distinct to each OMZ, specific groups of anammox or any other functional group may be the dominant ones.

Sonthiphand et al. (2014) proposed that the unique characteristics of *Candidatus Scalindua* could be the reason for their adaptations to marine environment. Compared with other genera of anammox bacteria, their genes of high affinity ammonium transport (*amtB*) and formate/nitrite transport (*focA*) proteins for *Candidatus Scalindua* are highly expressed enhancing their ability to utilize oligo peptides and small organic matters (Lam and Kuypers 2011; van de Vossenberg et al. 2013). Moreover, anammox bacteria from Black Sea had unique structure of the ladderane lipid, which might be specific to *Candidatus Scalindua* (Kuypers et al. 2003, 2005; van de Vossenberg et al. 2008, 2013).

In conclusion, it is apparent that oxygen depletion with high nitrate concentrations provides an ideal environment for anammox bacteria (Codispoti et al. 2001). It is worthwhile to note that in this study 500 m samples were more diverse than 250 m as indicated by the non-parametric indices (Shannon, Simpson, Chao and ACE). This was in agreement with the observation of Kong et al. (2013) where *Planctomycetes w*ere detected only in 500 m depth of the Costa Rica Dome. Increased abundances of bacteria in surface and OMZ depths is linked to higher rates of decomposition of organic matter which is sinking from the productive surface waters of the AS (Ramaiah et al. 2009). Given that most anammox bacteria are unculturable, metagenomic approach, along with specific marker genes done in this study, is useful in recognizing greater fractions of ammonia oxidizers. The predominance of *Candidatus Scalindua* is indeed useful to suggest that the process of anammox does add to N_2 formation and helps the nitrite utilizing communities to perform their role in the ongoing and hitherto understood biogeochemical process in the OMZ depths.

Chapter 7

Quantification (qPCR) of denitrifying and anammox bacteria

7.1 Introduction

Denitrification and anaerobic ammonium oxidation (anammox) are the two key microbial processes responsible for the loss of fixed marine nitrogen to the atmosphere as dinitrogen gas (N_2) (Payne 1973; Arrigo 2005; Brandes et al. 2007). Metabolic pathway intermediates and an array of enzymes of denitrification are well known, whereas molecular mechanism of anammox is largely understudied and it's many details yet to be known of this equally important biogeochemical process. Van de Graaf et al. (1997) and Strous et al. (2006) proposed anammox to proceed through a hydrazine (N_2H_4) intermediate. Naqvi et al. (2003) and Codispoti et al. (2005) suggest that the oxygen minimum zones (OMZs) enclose water masses with oxygen levels below 0.5 ml L^1 this is thought to induce nitrate reduction via denitrification and anammox processes.

Takaya et al. (2003) reports that only a few denitrifiers produce N_2 in the complete absence of oxygen but, nonetheless, are capable of partial denitrification and production of nitrous oxide (N_2O) under suboxic conditions. The only known metabolic pathway for the consumption of N_2O is through nitrous oxide reductase (*nosZ*) enzyme. Thus, their abundance in the OMZ is important in understanding the N_2O flux. Harhangi et al. (2012) reported that *16S rRNA* gene do not capture the entire diversity of the anammox bacteria. Therefore, hydrazine oxidoreductase (*hzo*) gene is believed to be more specific and a good marker for identification and abundance analysis of anammox bacteria in the marine environments.

Previous studies by Jayakumar et al. (2009); Ward et al. (2009) and Pitcher et al. (2011) in the Arabian Sea (AS) OMZ have examined the distribution and abundance of

denitrifiers and anammox assemblages using *16S rRNA* gene, with limited studies using the specific marker genes. Knowledge over the abundance of denitrifying and anammox bacteria of the AS-OMZ is limited in comparison to the OMZs of the Eastern Tropical North Pacific (ETNP) and Eastern Tropical North Pacific (ETSP). There have been no systematic studies in the AS-OMZ targeting the seasonal variation, distribution and abundance of the genes involved in denitrification and anammox processes.

The purpose of this study was to understand the seasonal distributions and relative abundance/copy numbers of denitrifying (*nirS* and *nosZ*) and anammox genes (*hzo*) through quantitative polymerase-chain-reaction (qPCR) analyses. Real-time polymerase-chain-reaction has come to play a prominent role in the field of life sciences since its introduction almost two decades ago. It is the gold standard for study of copy number of genes and its expression. It is a molecular biology technique, which allows amplification and simultaneous quantification of a targeted DNA molecule. The improvement compared to the traditional PCR method is its sensitivity and the ability to measure the amplification of DNA in real time as the reaction progresses.

7.2 Materials and methods

7.2.1 Sampling and extraction of DNA

Details on sampling and extraction of DNA are explained in subsection 3.2.4. In brief, DNA was extracted using modified method of Ferrari and Hollibaugh, (1999) from water samples collected from the Arabian Sea Time Series (ASTS)during May 2012 (spring intermonsoon, SIM), September 2012 (fall intermonsoon, FIM) and February 2013 (northeast monsoon, NEM).

7.2.2 Amplification of denitrifying and anammox genes

All DNA extracts were subjected to PCR amplification. Primer set nirS1F (CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T) and nirS6R (CGTTGAACTT(A/G)CCGGT) and nosZ1F (WCSYTGTTCMTCGACAGCCAG) and nosZ1R (ATGTCGATCARCTGVKCRTTYTC) were used for amplification of *nirS* and *nosZ* respectively. PCR-temperature conditions for both *nirS* and *nosZ* gene is detailed in subsection 5.2.2. PCR-amplification of *hzo* gene fragments was carried using primer pairs, hzoF1 (TGTGCATGGTCAATTGAAAG) and hzoR1 (CAACCTCTTCWGCAGGTGCATG). Thermal cycling parameters for *hzo* gene are described in subsection 6.2.2. Negative controls were run along during all PCR reactions. Positive amplicons from each DNA extract were confirmed by electrophoresis on a 1% agarose gel.

7.2.3 Purification and cloning

Positive PCR-products were purified using MOBIO PCR purification kit. Details of purification protocol are explained in subsection 5.2.3. Purified PCR products of *nirS, nosZ* and *hzo* genes were cloned into pCR4-TOPO vector using a TOPO-TA cloning kit. Cloning details are described in subsection 3.2.7.

7.2.4 Plasmid extraction

The plasmid from every clone of each gene was extracted using AxyPrep TM Plasmid Miniprep Kit (Axygene BioScience; U.S.A). Ten white clones of each target gene were grown individually overnight in a 5 ml LB medium containing 100 µg $m1^{-1}$ of ampicillin for 16 hrs at 37°C. 1-4 ml of overnight culture was centrifuged at 12000 rpm. The pellet was resuspended in 250 µl of S1 and vortexed. 250 µl of buffer S2 and 350

µl of buffer S3 was added and centrifuged for 10 mins (12000 rpm) to clarify the lysate. Clarified supernatant was transferred in a fresh 2 ml column placed in a microfuge tube and centrifuged at 12000 rpm (1 min). Buffer W2 (700 µl) was added to the column and centrifuged for 1 min (12000 rpm). The filtrate was discarded and 60-80 µl of eluent was added to the column and centrifuged at 12000 rpm for 2 mins.

7.2.5 Plasmid sequencing

All the extracted plasmids were sequenced in both the directions using Ml3F and M13R sequencing primers in an ABI3130 Genetic Analyzer system (Applied BioSystems, USA) to confirm positive insert of the target gene. Temperature details and sequencing protocol is detailed in subsection 3.2.7.

7.2.6 Construction of standard curve and quantitative PCR assay

The plasmid DNA concentration was determined on a Nanodrop (Thermo Scientific, USA) and the copy number of each target gene was calculated directly from the concentration of the extracted plasmid DNA. Ten-fold serial dilutions of a known copy number of the plasmid DNA were subjected to qPCR assay in triplicate to generate an external standard curve. Separate standard curve was generated for each of the target gene. Each reaction was performed in a 20 µl volume containing 4 µl of plasmid, 0.5 µl of each primer (20 µM), and 12.5 µl of 5x qARTA Green qPCR Mix (AXYGEN, USA).

The copy numbers of *nirS, nosZ* and *hzo* gene in all samples were determined in triplicate using the ABI 7500 Real-Time PCR system (Applied Biosystems, USA). Each reaction for every target gene was performed in a 20 µl volume containing 4 µl of DNA template, 0.5 µl of each primer (20 µM), and 12.5 µl of 5x qARTA Green qPCR Mix

(AXYGEN, USA). PCR was carried out using modified method of Levy-Booth and Winder, (2010), PCR conditions are as follows: 15 min at 95°C, followed by 40 cycles of 15s at 95°C, and 1 min at 60°C.

7.3 Results

Temporal distribution patterns of *nirS, nosZ* and *hzo* genes were estimated using qPCR. Based on their respective amplification profiles/curves (**Figure 7.1a, 7.2a**and **7.3a)** an independent standard curve was constructed for *nirS* (**Figure 7.1b)**, *nosZ* (**Figure 7.2b)** and *hzo* (**Figure 7.3b)** genes. Regression slope of -3.94, -3.75 and -4.63 and regression coefficient (R^2) of ≥0.99, ≥0.95 and ≥ 0.97 were obtained for *nirS, nosZ* and *hzo* gene respectively. Non-template control had C_t values at least ≤ 9 cycles higher than the most diluted plasmid containing the target gene. The post-amplification melting curve analysis clearly showed that there was no target gene contamination in reagents for any of the three genes. Primers used to determine copy numbers of the different genes in each sample is mentioned in **Table 7.1**.

At surface, distribution pattern of *nirS* genes (**Figure 7.4**) were analogous during all three seasons with copy number ranging from 0.01 to 0.02 $x10^6$ L^{-1}. No significant values of copy numbers were observed at DCM and showed a constant value of 0.02 x 10^6 L^{-1}. Distribution of *nirS* was highest in the core OMZ depths (250-500 m) during all three seasons. Highest copy number during three seasons was recorded during the NEM (0.35 x 10^6 L^{-1}) followed by SIM (0.2 x 10^6 L^{-1}) and FIM (0.16 x 10^6 L^{-1}). At 250 m, the copy numbers ranged from 0.09 to 0.35 x 10^6 L^{-1}, whereas at 500 m it was 0.1 to 0.16 x 10^6 L^{-1}. Copy number at 1000 m was in the range of 0.02 to 0. 4 x 10^6 L^{-1}.

At surface, copy numbers of *nosZ* gene was higher in comparison to *nirS* ranging from 0.08 to 0.42 $x10^6$ L^{-1}. Distribution pattern of *nosZ* gene (**Figure 7.5**) was similar at DCM depth during all the three seasons with copy numbers ranging between 0.08 to 0.15 $x10^6$ L^{-1}. These values were much higher than those observed for *nirS* at DCM. Abundance of *nosZ* was highest in 250 m during FIM and NEM seasons except during SIM where the abundance was maximum at 500 m. At 250 m, the copy numbers ranged from 0.49 to 1.32 $x10^6$ L^{-1}, as at 500 m it was 0.34 to 1.5 $x10^6$ L^{-1}. The highest copy number during all three season was recorded at 500 m (1.5 $x10^6$ L^{-1}) during the SIM season. Copy number at 1000 m ranged from 0.15 to 0.18 $x10^6$ L^{-1}.

The *hzo* gene copy numbers ranged from 0.04 to 0.05 10^6 L^{-1} in the surface samples during all seasons. No significant values of copy numbers were observed in the DCM either. The *hzo* copy numbers were the highest in the core OMZ depth ranging from 0.1 $x10^6$ L^{-1} to 1.5 x 10^6 L^{-1}. During SIM, the highest copy numbers was recorded at 500 m (1.5 x 10^6 L^{-1}) followed by FIM (0.5 $x10^6$ L^{-1}) whereas during NEM (0.8 x 10^6 L^{-1}) the highest was recorded at 250 m. Copy numbers at 1000 m were quite small during all three seasons (**Figure 7.6**).

Overall distribution of *nirS, nosZ* and *hzo* genes observed a similar trend during all three seasons with all the three genes having highest copy number in core OMZ (250-500 m). Interestingly, during NEM abundance of all genes was maximum at 250 m.

Vertical distribution of *nirS, nosZ* and *hzo* gene copy number with respect to dissolved oxygen (DO), ammonia (NH_4) and nitrite (NO_2) is shown in **Figure 7.7.** Highest copy numbers of *nirS* (0.35$x10^6$ L^{-1}), *nosZ* (1.5 $x10^6$ L^{-1}) and *hzo* (1.5 $x10^6$ L^{-1}) coincided

with the lowest oxygen values in the core OMZ depths. The average DO concentration at 250 m and 500 m was 5.55 μmol L^{-1}) and 2.28 μmol L^{-1} respectively. Average ammonia concentration increased gradually from surface (0.08 μmol L^{-1}) to DCM (0.31 μmol L^{-1}) with no significant change in the copy number of any of the genes. The upper thermocline region (50 m) noted the highest nitrite concentration of 0.29 μmol L^{-1} with low copy numbers of *nirS* (0.02 x 10^6 L^{-1}), *nosZ* (0.13 x 10^6 L^{-1}) and *hzo* (0.04 x 10^6 L^{-1}) genes. Interestingly, highest copy number of *nirS* (0.35 x 10^6 L^{-1}) was obtained during NEM season at 250 m which coincided with the highest nitrite value of 2.5 μmol L^{-1} and no detectable ammonia.

Table 7.1. Primers used in this study

Primers	Primer sequence (5'–3')	Annealing temperature	Base pairs	Reference
nirS1F	CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T	63°C	890	Braker et al.(1998)
nirS6R	CGTTGAACTT(A/G)CCGGT			
nosZ1F	WCSYTGTTCMTCGACAGCCAG	54°C	259	Henry et al. (2006)
nosZ1R	ATGTCGATCARCTGVKCRTTYTC			
hzoF1	TGTGCATGGTCAATTGAAAG	60°C	740	Li et al. (2010)
hzoR1	CAACCTCTTCWGCAGGTGCATG			

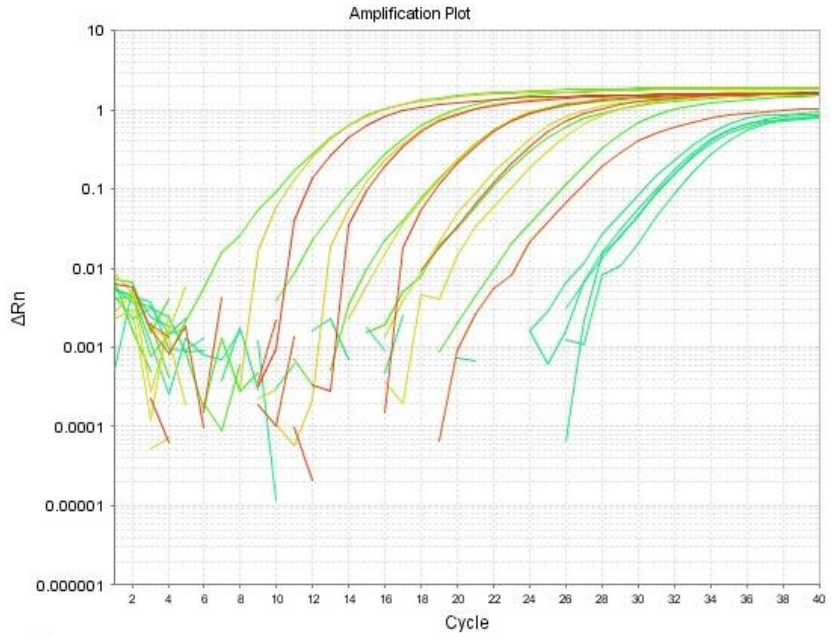


Figure 7.1a. Amplification curves for 5 different amounts of plasmid of *nirS* gene. It is to be noted that each one reaches the threshold at a different cycle. The largest concentration samples (sample 1 corresponding to 10^5copies/ml) reach the threshold sooner (at cycle 10) while sample 5 (10^1copies/ml) reaches the threshold at cycle 28.

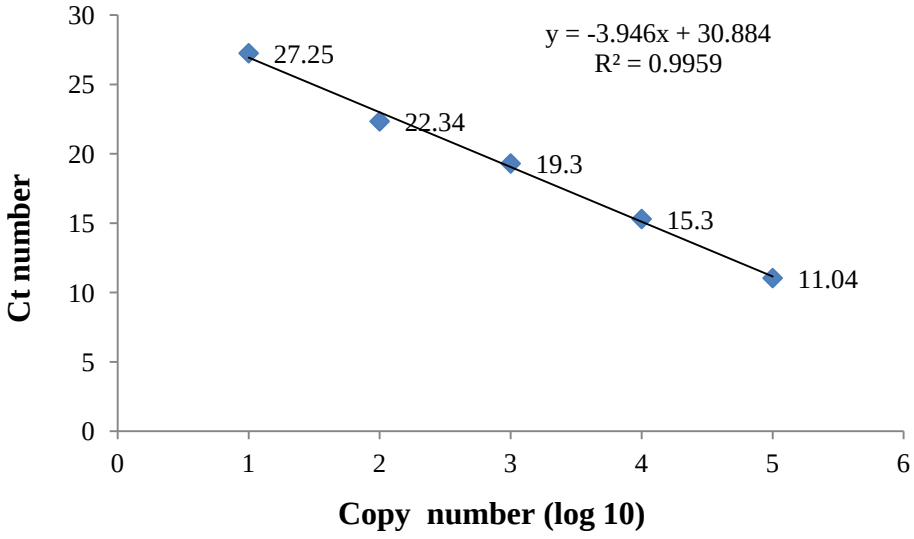


Figure 7.1b. A standard curve for *nirS* generated plotting each pair of data (C_t *vs* initial concentration, C_0). By linear regression, coefficient of regression calculated using the slope generated by joining the initial (known) copy numbers.

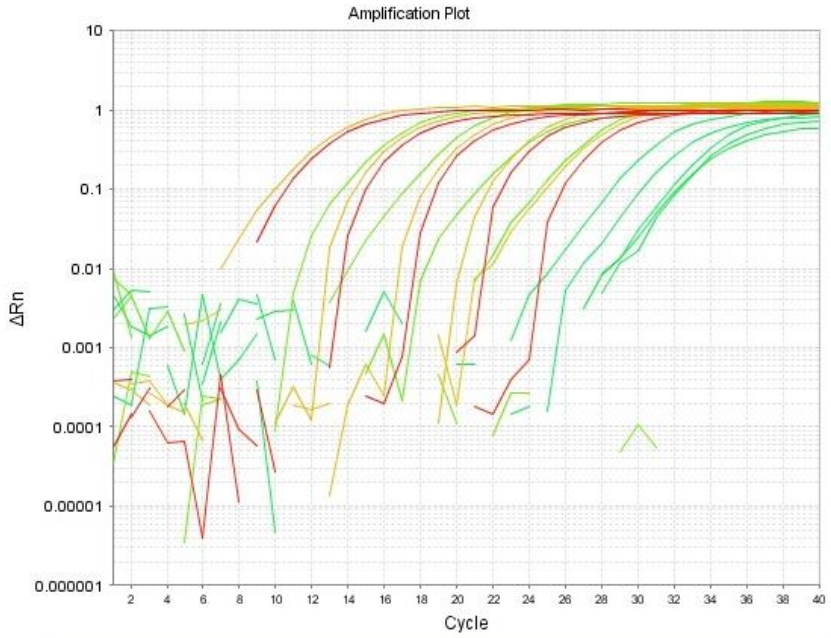


Figure 7.2a. Amplification curves for 5 different amounts of plasmid of *nosZ* gene. It is noted that each one reaches the threshold at a different cycle. The largest concentration samples (sample 1 corresponding to 10^5copies/ml) reach the threshold sooner (cycle 5) while sample 5 (10^1copies/ml) reaches the threshold at cycle 25.

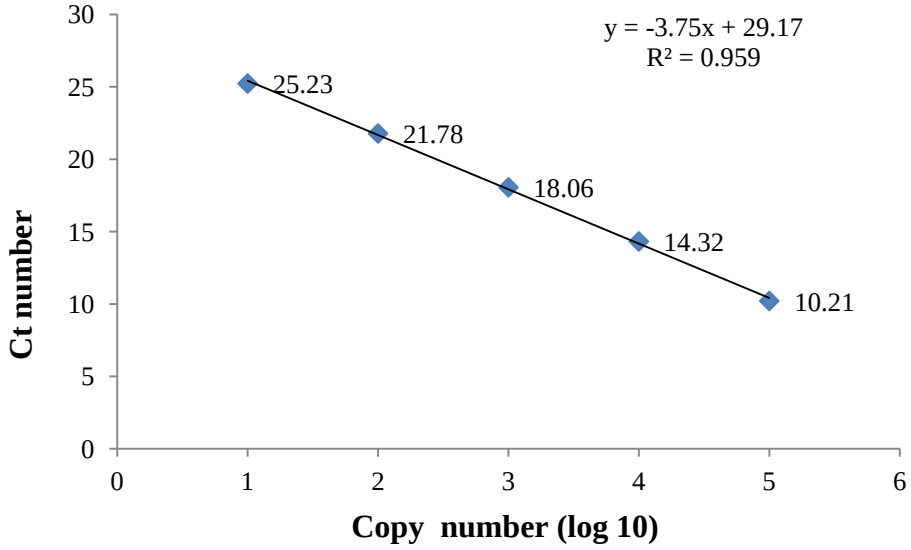


Figure 7.2b. A standard curve for *nosZ* was generated plotting each pair of data (C_t *vs* initial concentration, C_0). By linear regression, coefficient of regression calculated using the slope generated by joining the initial (known) copy numbers.

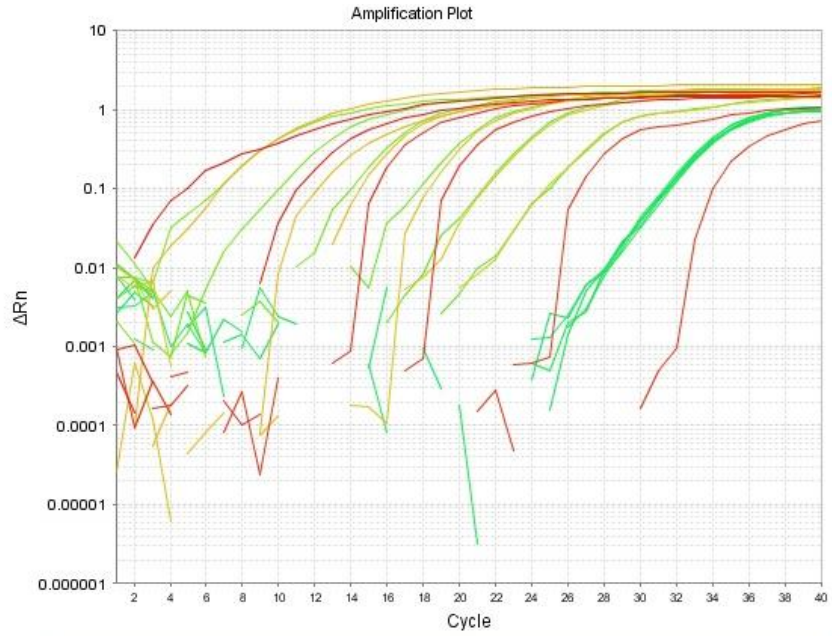


Figure 7.3a. Amplification curves for 5 different amounts of plasmid of *hzo* gene. It is noted that each one reaches the threshold at a different cycle. The largest concentration samples (sample 1 corresponding to 10^5copies/ml) reach the threshold sooner (cycle 5) while sample 5 (10^1copies/ml) reaches the threshold at cycle 25.

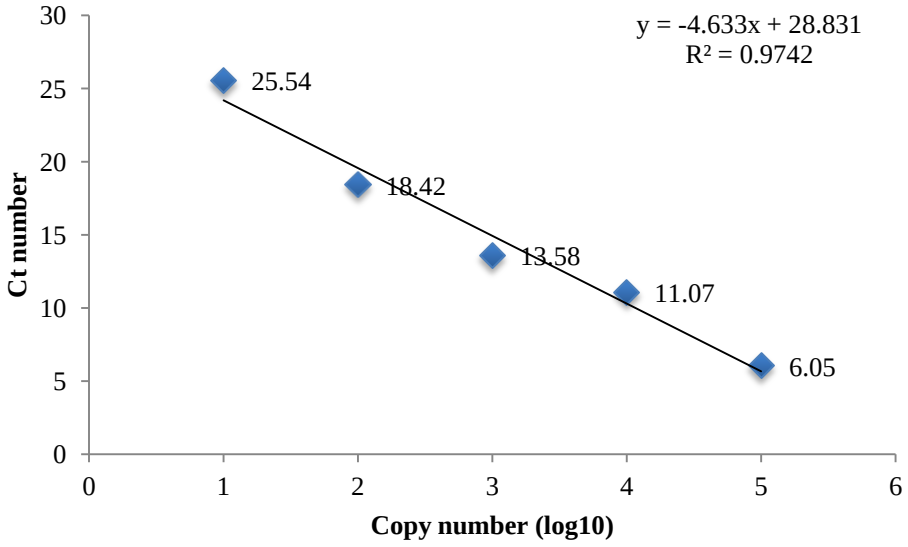


Figure 7.3b. A standard curve for *hzo* gene was generated plotting each pair of data (C_t *vs* initial concentration, C_0). By linear regression, coefficient of regression calculated using the slope generated by joining the initial (known) copy numbers.

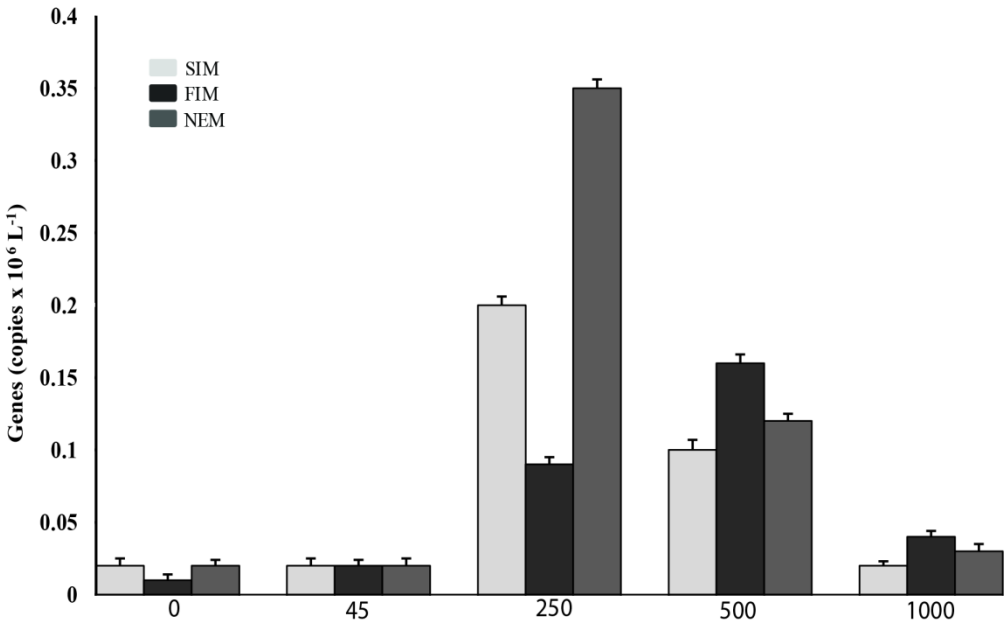


Figure 7.4. Seasonal and depth wise distribution of copy numbers of *nirS* gene at the Arabian Sea Time Series (ASTS) location during Spring intermonsoon (SIM), Fall intermonsoon (FIM) and Northeast monsoon (NEM). Sampling depths are surface (5 m), Deep chlorophyll maxima (DCM), 250 m, 500 m and 1000 m.

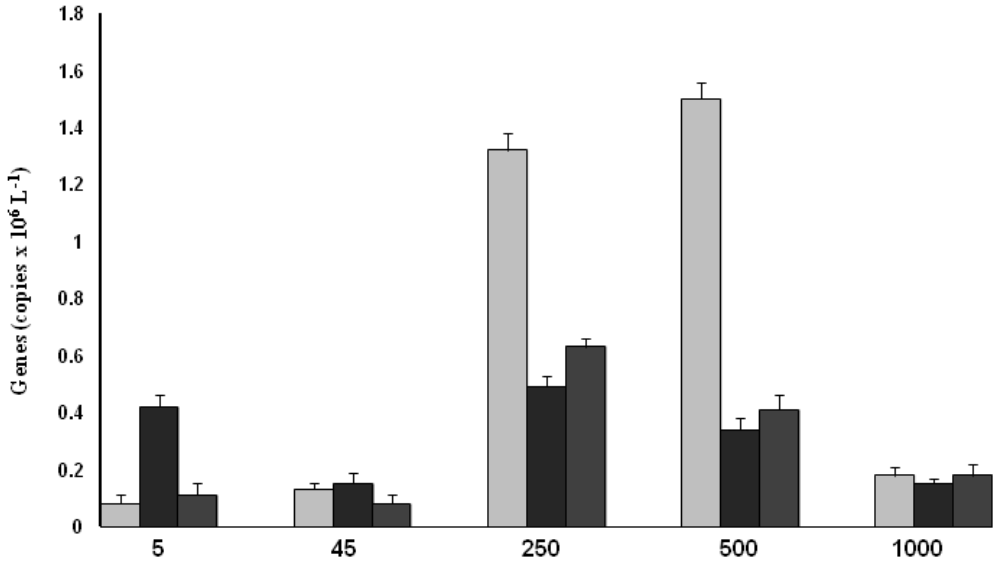


Figure 7.5. Seasonal and depth wise distribution of copy numbers of *nosZ* gene at the Arabian Sea Time Series (ASTS) location during Spring intermonsoon (SIM), Fall intermonsoon (FIM) and Northeast monsoon (NEM).

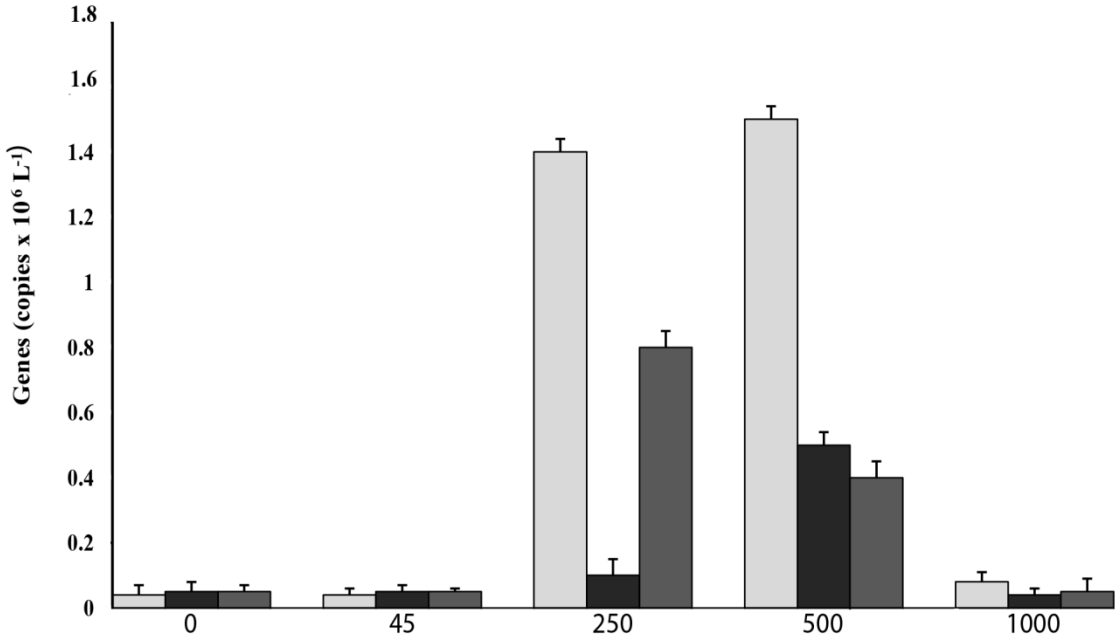


Figure 7.6. Seasonal and depth wise distribution of copy numbers of *hzo* gene at the Arabian Sea Time Series (ASTS) location during Spring intermonsoon (SIM), Fall intermonsoon (FIM) and Northeast monsoon (NEM).

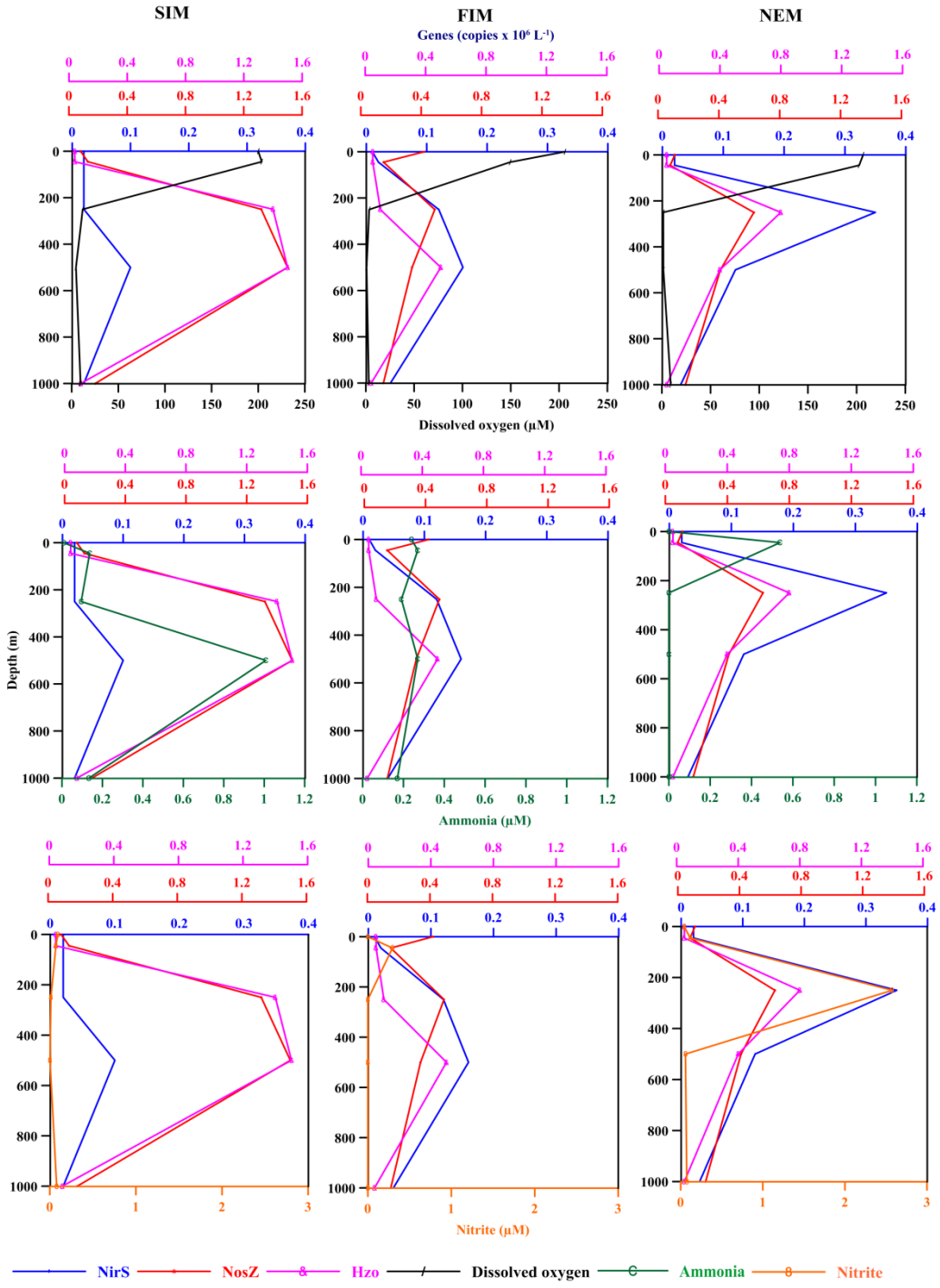


Figure 7.7. Vertical distribution profiles of *nirS*, *nosZ* and *hzo* copy numbers, and concentrations of dissolved oxygen (DO), ammonia (NH_4) and nitrite (NO_2) during Spring intermonsoon (SIM), Fall intermonsoon (FIM) and Northeast monsoon (NEM) at the Arabian Sea Time Series (ASTS) location.

7.4 Discussion

Although denitrification is important in OMZs of the AS, (Ward et al. 2009; Dalsgaard et al. 2012), the discovery of anammox (Mulder et al. 1995) reveals additional pathway for loss of fixed nitrogen from the marine ecosystem. Since the unusual circulation pattern and nutrient-rich waters promote very high primary production in the AS (Madhupratap et al. 1996), decomposition of the organic matter from these highly productive surface waters culminates in total consumption of DO. This can be evidenced by its near exhaustion at 150-1200 m leading to denitrification and anammox (Naqvi et al. 1990) in these depths.

As explained in chapters 5 and 6, repeated attempts of PCR in the surface, DCM and 1000 m did not show the presence of *nirS*, *nosZ* and *hzo* genes. Unambiguously, the bacteria possessing these genes are relatively rare in the well-oxygenated waters. Even increased template DNA concentrations did not yield positive PCR for any genes. The Ct values of these samples (5 m, DCM, and 1000 m) were very high than those recorded from the core OMZ. Thus, it is assumed that there are fewer *nirS*, *nosZ* and *hzo* gene bearing bacteria at these depths and hard to detect via conventional PCR. Although qPCR results show the signatures of the presence of both these groups of bacteria, their abundances are quite small. However, I acknowledge detection of these genes in water samples from 5 m; DCM and 1000 m may be due non- specific amplicons even though the melt curves, the melting temperature, and all other protocol were verified to confirm that amplification in our qPCR was not an artefact. Also, as reported in several studies (Staggemeier et al. 2015; Nagdev et al. 2015; Zemtsova et al. 2015; Paiva-Cavalcanti et al. 2010) the sensitivity of qPCR is far higher than the conventional PCR.

Distribution pattern of both *nirS* and *hzo* genes was consistent with the environmental settings that define the OMZs. The highest abundance of *nirS* gene copy numbers was in the core OMZ depths (250 m-500 m) of the AS. Although different species of denitrifiers are present in a few numbers, the population is dominated by organisms that do not possess *nirS* genes. At the ASTS, the oxygen concentration is at threshold (1–4 μM) level, ideal enough to favor denitrifiers and denitrification to happen as suggested by Codispoti et al. (2005) and, Devol, (1978). At this location, the abundance of *nirS* gene is significantly lower than that reported by Jayakumar et al. (2013) from other sites in the AS-OMZ swath. This abundance suggests the existence of perennially active nitrite reducing populations.

The distribution of the three genes was consistent with the environmental conditions that define the OMZs. Although the abundances of these genes differed, maxima in all three marker genes coincided with the core OMZ depth (250 m-500 m) of the ASTS location. Different species of denitrifiers though present in a few numbers, the population was dominated by organisms that did not possess *nirS* genes. At the ASTS, the oxygen concentration is at threshold (1–4 μM) level, ideal enough to favor denitrifiers and denitrification as suggested by Codispoti et al. (2005) and Devol, (1978). At the ASTS location, the abundance of *nirS* gene is significantly lower than that reported by Jayakumar et al. (2013) from other sites in the AS-OMZ swath. This abundance suggests the existence of perennially active nitrite reducing populations. The abundance of *nirS* gene was the highest during NEM season. Primary productivity in the AS is at the peak during the NEM as reported by Barber et al. (2001). Naqvi et al. (2003) suggests that sinking and decomposition of the organic matter generated due to high primary production culminates

in excessive consumption of dissolved oxygen leading to denitrification.

Higher number of *nosZ* gene can be attributed to the fact that not all bacteria consuming nitrous oxide can carry out total denitrification and hence may lack the other genes required for the process (Sanford et al. 2012). Graf et al. (2014) reports that *nirS* gene sequences are represented by bacteria that have the complete denitrification genes. Another possible reason could be the primers used during this study which represent *nosZ* sequences of the year 2006. Thus these primers represent larger database of *nosZ* sequences (terrestrial and marine sequences). Whereas, the *nirS* primers used are more specific to marine cultures (Braker et al. 1998) and hence may be underestimated. One possible reason for detection of low concentrations of *nirS* in this study may be due to the length of the primers used for their detection. Earlier reports on *nirS* copy numbers from the AS-OMZ use primers which produce a much shorter fragment (Ward et al. 2009; Jayakumar et al. 2009) in comparison to the longer fragment used in this study which could probably lead to an underestimate of the actual copy number. Quantification of *nirS* gene was carried out using primer pair nirS1F/nirS6R. This is because primer pair, nirS1F/nirS3R failed to show positive amplification.

Copy numbers of *hzo* genes reported by Ward et al. (2009) and Pitcher et al. (2011) from the AS-OMZ are much higher than that of *hzo* observed in this study. It is to be noted however that reports on the distribution of anammox gene by Ward et al. (2009) are limited to the secondary nitrite maxima (SNM) depths whereas samples analyzed for this study were from the core OMZ depths. The highest copy number of the *hzo* gene during SIM may be because the organic carbon is a significant substrate supporting the anammox bacterial communities in the core OMZ depths (Dang et al. 2010). As suggested by

Ramaiah et al. (2000) it is apparent that bacterial communities are sustained by the slow-to-degrade dissolved organic carbon (DOC) during SIM which is because; SIM is a transitional period where primary production is low (Madhupratap et al. 1996) mainly due to stratification and prevalence of oligotrophic conditions.

Richards, (1965) and Richards et al. (1965) state that ammonium should accumulate in waters if nitrogen loss is solely due to heterotrophic denitrification. No accumulation of NH_4 was evident at the ASTS. Low concentrations of oxygen and ammonia and increased nitrite concentration (Dalsgaard et al. 2003) appear to provide a perfect condition in the ASTS for the presence of anammox bacteria.

Results from this study may be taken to denote that anammox might be playing an equal role at the ASTS leading to such high percentage of fixed nitrogen loss. Incubation experiments by Kuypers et al. (2005), in the oxygen-deficient waters off Namibian Shelf suggest higher rates of anammox with no detection of denitrification. One of the most striking features at the ASTS location is the greater abundance of *hzo* over *nirS* and *nosZ* gene copy numbers. Earlier reports on denitrification and anammox in the AS-OMZ and ETSP have suggested denitrification as a dominant pathway in the removal of fixed nitrogen (Ward et al. 2009; Dalsgaard et al. 2012). Information of the distribution and relative abundance of denitrifiers and anammox functional genes gathered during this study is hoped to prove essential for understanding the dynamics of the microbial communities involved as well as their response to changing environmental parameters.

Chapter 8

Next generation sequencing based bacterial community structure

8.1 Introduction

The next-generation sequencing (NGS) technique facilitates high throughput sequencing of microbial communities, thus covering a broader spectrum of biodiversity which is not possible through conventional methods. Hundreds to thousands of samples may be sequenced using short DNA sequence (Hamady et al. 2008), to characterize the top 99.99% of the micro biota. This has facilitated comparative ecological analysis on a large scale and, with sensitivity well beyond that of first-generation profiling technologies—provides relatively quantitative comparisons of microbial communities across ecosystems at depths previously unattainable (Caporaso et al. 2011).

Notwithstanding the *16S rRNA* gene-based community structure details described in the previous chapters, this additional work of NGS was included in this study. The rationale for this add on study was to describe as much bacterial community/diversity extant in the oxygen minimum zone (OMZ) depths of the AS. To achieve this, DNA extracts from different sampled depth (described in Chapter 3) were used to obtain the vertical and seasonal profile of the bacterial community structure in the core OMZ depths (250 m and 500 m) of the AS-OMZ. Notably this new NGS data from this study would prove useful to compare microbial diversity from diverse oceanic regions.

8.2 Materials and methods

8.2.1 Sampling

Water samples from two depths (250 m and 500 m) were collected during three different seasons. Details of the collection of water samples and seasons are explained earlier in subsection 3.2.1.

8.2.2 DNA extraction

Sterivexed water samples were immediately stored at -80°C until nucleic acid extraction was performed in the laboratory at the NIO. Details of DNA extraction is mentioned earlier in subsection 3.2.4.

8.2.3 Library Preparation and Sequencing

16S V3-V6 metagenome libraries were prepared using 16S Bacteria V3-V6 region-specific targeting proprietary primers at the Genotypic Technology Pvt. Ltd., Bangalore, India. Briefly, 50 ng of genomic DNA (Quantification method: Nanodrop spectrophotometer) was amplified for 26 cycles using KAPA HiFi Hot Start PCR Kit (Boston, MA USA). The forward and reverse primer concentration is kept at 0.2uM each. A positive control and non-template control samples were run to validate PCR. The amplicons were analyzed on 1.2% agarose gel (Round 1 PCR). 1 ul of 1:10 diluted round 1 PCR amplicons were used for indexing PCR (round 2). Here, the round 1 PCR amplicons were amplified for 8-10 cycles to add Illumina Sequencing barcoded adaptors (Nextera XT v2 Index Kit, Illumina, and U.S.A). The Illumina Adapter Sequences are: 5'-AATGATACGGCGACCACCGAGATCTACAC [i5] TCGTCGGCAGCGTC and 5'-CAAGCAGAAGACGGCATACGAGAT [i7] GTCTCGTGGGCTCGG. Round 2 PCR amplicons were analyzed on 1.2% agarose gel.

8.2.4 Sequence data statistics and quality filtering

The Illumina paired end reads for v3 (150*2) and v6 (75*2) was demultiplexed using bcl2fastq1 tool. The paired end reads were quality checked using FastQC2. The raw reads having primer sequence and high quality bases were selected. The reads were further

stitched using Fastq-join3.These stitched reads were considered for further analysis using QIIME4 pipeline. The query sequences were clustered using UCLUST5 method. The taxonomy of these clusters were assigned based on >=97% sequence similarity against the curated chimera free *16S rRNA* database (Greengenes6 v 13.8). This results in the generation of a biom file. The biom was taken ahead for further advance analysis and visualization.

8.2.5 Stacked bar and heat map

Stacked bar plots were generated using the normalized read count values. The plot has been generated based on the top 20 organisms across all the samples among 18 groups. Heat map represents the values in color codes. The cells in the matrix with high relative values are colored differently from those with low relative values. The color slab has been generated based on the maximum and minimum values in the matrix. The heat map has been clustered row-wise and has been generated using R package NMF9.

8.2.6 Alpha diversity

Alpha diversity is the microbial diversity assessed within a sample. The rarefaction tables are the basis for calculating diversity metrics, which reflect the diversity within the sample based on the abundance of various taxonomy within a community. We have calculated the diversity using different matrices i.e Shannon, Simpson, Chao1 and observed species. Shannon diversity index (H): It is used to characterize species diversity in a community (http://www.tiem.utk.edu/~gross/bioed/bealsmodules/shannonDI.html). Simpson index: The proportion of species *I* relative to the total number of species (pi) is calculated and squared. Chao1 index is commonly used, and is based upon the number of rare classes (i.e operational taxonomic units (OTUs)) found in a sample

(http://www.coloss.org/beebook/I/gut-symbionts/2/2/4).

8.2.7 Rarefaction curves

Rarefaction plot shows the rarefaction curve of annotated species richness in v3 and v6 samples. This curve is a plot of the total number of distinct species annotation as a function of the number of sequences sampled. On the left, a steep slope indicates that a large fraction of the species diversity remains to be discovered. If the curve becomes flatter to the right, a reasonable number of individuals are sampled. Sampling curves generally rise very quickly at first and then level off towards an asymptote as fewer new species are found per unit of individuals collected.

8.3 Results

The v3-v6 hyper variable region of the *16S rRNA* gene was sequenced using Illumina Miseq to obtain a near-real diversity of the OMZ-inhabiting bacterial community and its seasonal variation. Libraries were constructed for both v3 and v6 regions for samples collected during the seasons mentioned in subsections 3.2.1. Sequencing of community DNA and RNA across 250 m and 500 m depths for both v3 and v6 region during three different seasons generated a total of 3.01 and 4.04 million sequence reads, respectively. The NGS effort is useful to recognize that bacterial diversity was more or less unchanged temporally whereas; there were distinct depth wise differences in the OTUs recorded. At 250 m, *Gammaproteobacteria, Alphaproteobacteria, Synechococcophycideae, Actinobacteria, Flavobacteriia, Deltaproteobacteria*, AB16 and TM7-3 (**Figure 8.1**) was the most important groups and accounted for 96.74% of the reads across all three seasons. The 500 m library was dominated by *Gammaproteobacteria, Alphaproteobacteria, Flavobacteriia, Deltaproteobacteria*, and AB16 bacteria, and these

represented 97.26% of the reads (**Figure 8.2**). The overall diversity of bacterial community was higher at 250 m than at to 500 m depth during all three seasons. All the OTUs were identified at a 97% sequence identity.

At 250 m, the v3-region sequence based OTUs totaled to 5617. Of these, 1780, 1943 and 1894 OTUs **(Table 8.1)** respectively were from FIM, SIM and NEM. In the overall, the 250 m-OTUs clustered into 30 phyla, 87 classes, and 159 different bacterial orders. Majority of the identified OTUs in 250 m belonged to phylum *Proteobacteria,* Cyanobacteria and *Actinobacteria*. *Proteobacteria* was dominated by *Gammaproteobacteria* and *Alphaproteobacteria* (**Figure 8.3a**). *Gammaproteobacteria* made up 44.14% of the total OTUs. The orders of *Gammaproteobacteria* found at higher proportions include *Alteromonadales* (36%), *Oceanospirillales* (4.1%) and *Vibrionales* (2.7%). *Alphaproteobacteri*a made up 29% of the community with majority affiliating to *Rickettsiales* (15%), *Rhodobacterales* (5.3%) and *Sphingomonadales* (3.6%). The second dominant phylum affiliated to Cyanobacteria in the order *Synechococcales* contributed to 9% of the total bacterial community. *Actinobacteria, Bacteroidetes, and Deltaproteobacteria,* contributed to about 2% whereas AB16 (phylum SAR406) and TM7 contributed to ~1% of the total bacterial community. Some of the other minor groups include *Firmicutes, Acidobacteria, Verrucomicrobia, Planctomycetes, Gemmatimonadetes, Nitrospirae,* SBR1093, Chloroflexi and *Betaproteobacteria*.

At 500 m the OTUs formed were less number. A total of 3770 OTUs were detected. They aligned into 28 phyla, 76 classes, and 141 orders. Of these 1337, 1239 and 1194 **(Table 8.1)** were found during FIM, SIM and NEM respectively. Majority of the OTUs identified from 500 m were affiliated to three major phyla: *Proteobacteria,*

Bacteroidetes and SAR406 (**Figure 8.3b**). *Gammaproteobacteria* contribute to 74% of the total bacterial community. *Alteromonadales* (70.4%) of *Gammaproteobacteria* are found in higher proportion as also noted in 250 m depth. *Alphaproteobacteri*a makes up 15% of the community, with *Rickettsiales* (6.4%) and *Sphingomonadales* (4.4%) found in maximum proportion. *Bacteroidetes* (4.5%) the second dominant phyla were dominated by *Flavobacteria*. AB16 of SAR406 contributes to more than 2% of the microbial community. *Actinobacteria, Deltaproteobacteria, Betaproteobacteria, Synechococcophycideae, Verrucomicrobia, Firmicutes, Planctomycetes, Acidobacteria, Gemmatimonadetes, Nitrospirae,* TM7, GN02 and Chloroflexi are the other minor groups found in the 500 m depth.

A total of 6031 OTUs were discerned from 250 m in the v6-regionduring the three seasons, and grouped into 36 phyla, 109 classes and 207 orders. Among these, 1724, 2081 and 2226 OTUs **(Table 8.1)** were recorded for FIM, SIM and NEM respectively. *Gammaproteobacteria* (59%) *Alphaproteobacteria* (15%) and *Deltaproteobacteria* (7%) (**Figure 8.4a**) were the most dominant. *Alteromonadales* (40.4%) and *Vibrionales* (12%) of *Gammaproteobacteria* and *Rickettsiales* (8%) of *Alphaproteobacteria* were in higher proportions. *Bdellovibrionales* (6%) of *Deltaproteobacteria* was also found. *Synechococcophycideae* (6.4%) affiliated to Phylum Cyanobacteria was the second dominant group followed by *Flavobacteria* (5.4%). *Actinobacteria* and AB16 (SAR406) constituted 4% and 1.6% to the total bacterial community at 500 m. Other minor groups include *Verrucomicrobia, Acidobacteria, Planctomycetes, Firmicutes,* TM7, *Chloroflexi,* SBR1093, *Gemmatimonadetes,* and *Betaproteobacteria*.

The numbers of OTUs observed from 500 m samples on the basis of v6-region

sequences were just about half of the OTUs recorded from 250 m. In all 3783 OTUs were obtained of which 1254, 1277 and 1252 OTUs **(Table 8.1)** were found respectively during FIM, SIM and NEM. *Proteobacteria, Bacteroidetes* and *Actinobacteria* were the three most dominant phyla (**Figure 8.4b**). *Gamma-* and *Alpha-proteobacteria* made up 87% and 5% of the total bacterial community. *Alteromonadales* (70%) and *Vibrionales* (15%) of *Gammaproteobacteria* and *Rickettsiales* (2.5%), *Rhodobacterales* (1.8%) and *Sphingomonadales* (1.6%) of *Alphaproteobacteria* were higher in numbers. *Falvobacteria* (phylum *Bacteroidetes)* constituted 5.5% whereas *Actinobacteria* contributed 0.6% of the overall community. Minor fractions in the total community were: *Verrucomicrobia, Acidobacteria, Planctomycetes, Firmicutes,* TM7, *Deltaproteobacteria, Chloroflexi,* SBR1093, SAR406, Cyanobacteria, *Gemmatimonadetes, Fusobacteria,* OP3, *Fibrobacteres,* and *Spirochaete*.

To get an insight in sample level similarity a hierarchical clustered heat map was performed. Heat maps were generated using relative abundance values of top 20 groups across samples for both v3 (**Figure 8.5**) and v6 (**Figure 8.6**) regions. The relative abundance for each bacterial genus was depicted by color intensity with; Red = least abundant and Blue= highly abundant. A row-based scaling method in heat map function auto generates the scale from negative to positive based on the z-score calculated across the row. The - to + scale represents lower to higher abundance profiling for the taxa's represented.

Statistical analysis of v3 and v6 region sequence-based diversity analyses also indicated little or no difference between seasons (**Table 8.2**). Shannon and Simpson diversity indices clearly indicated that at an evolutionary distance of 3%, the bacterial

diversity is significantly higher at 250 m during all three seasons **(Table 8.2)**. Higher values of Chao1 richness estimator are useful to indicate that bacterial communities at 250 m are considerably richer than those at 500 m and the estimated OTUs during all the seasons are much higher than the actual number of OTUs generated by both v3 and v6 region sequences **(Table 8.2)**. However, during individual season, both the highest diversity and richness were evident in the NEM-250 samples. The lowest was observed in NEM-500 (v3- region) and in SIM-500 (v6-region) samples. Rarefaction curves of OTUs from 250 m samples imply higher diversity owing to much higher number of OTUs than that of 500 m samples during all three seasons. Saturation of rarefaction curve for both v3 (**Figure8.7a**) and v6 (**Figure8.7b)** region at the recovered OTU level with an evolutionary distance of 3% implies that diversity was adequately covered.

Table 8.1. Read count statistics of V3 and V6 regions

SampleID	Total Paired End Reads	Processed Reads	Total identified rRNA sequences	Total OTUs Picked
V3-Region				
F-250	482796	270064	199297	1780
S-250	491330	286762	214275	1943
N-250	327125	191896	141424	1894
F-500	549802	320746	248820	1337
S-500	444546	251175	196251	1239
N-500	399874	223781	173423	1194
V6-Region				
F-250	568104	426886	369052	1724
S-250	874628	660783	566577	2081
N-250	788808	600632	501881	2226
F-500	813704	632570	549220	1254
S-500	974941	746331	644044	1277
N-500	863552	651388	556855	1252

Table 8.2. Alpha diversity indices across various matrices for V3 and V6 region

Sample ID	Shannon	Simpson	Chao1
V3-Region			
F-250	5.77	0.88	2538.71
S-250	5.70	0.88	2640.27
N-250	5.99	0.90	2777.78
F-500	3.17	0.55	1834.25
S-500	3.06	0.53	1767.95
N-500	3.00	0.51	1678.18
V6-Region			
F-250	5.51	0.90	2267.84
S-250	5.47	0.90	2695.70
N-250	5.54	0.91	2945.88
F-500	2.83	0.61	1763.52
S-500	2.78	0.61	1666.37
N-500	2.79	0.61	1789.66

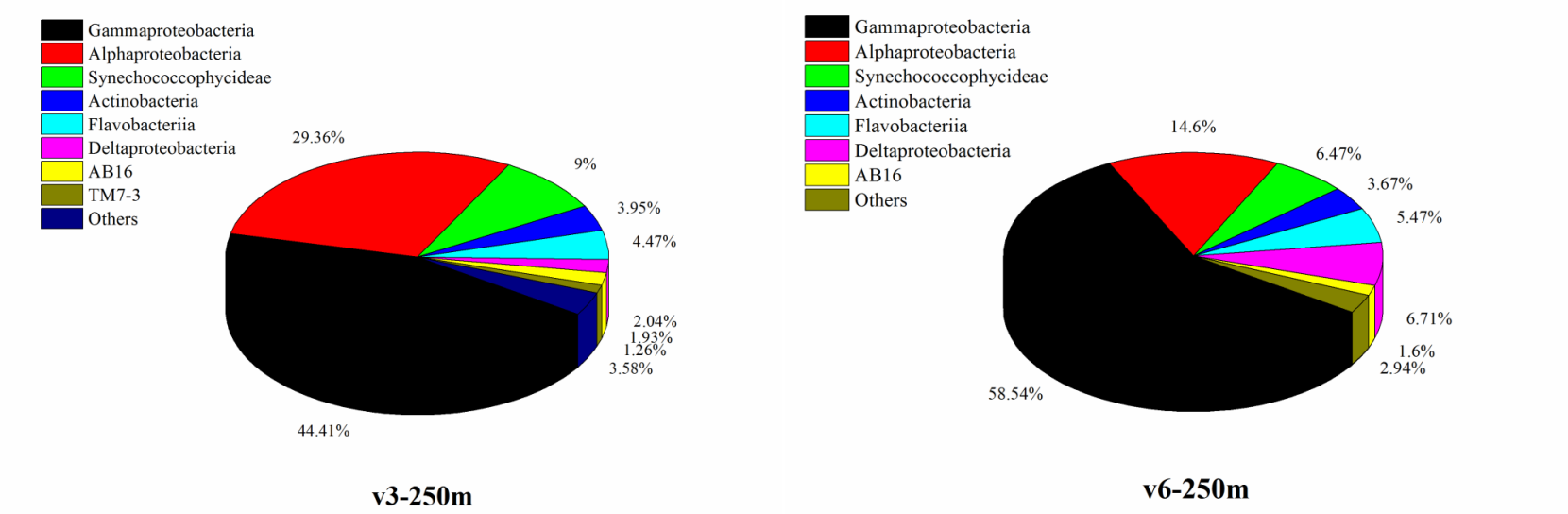


Figure 8.1. Distribution of major bacterial groups in 250 m depth in the Arabian Sea Time Series (ASTS) station during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM).

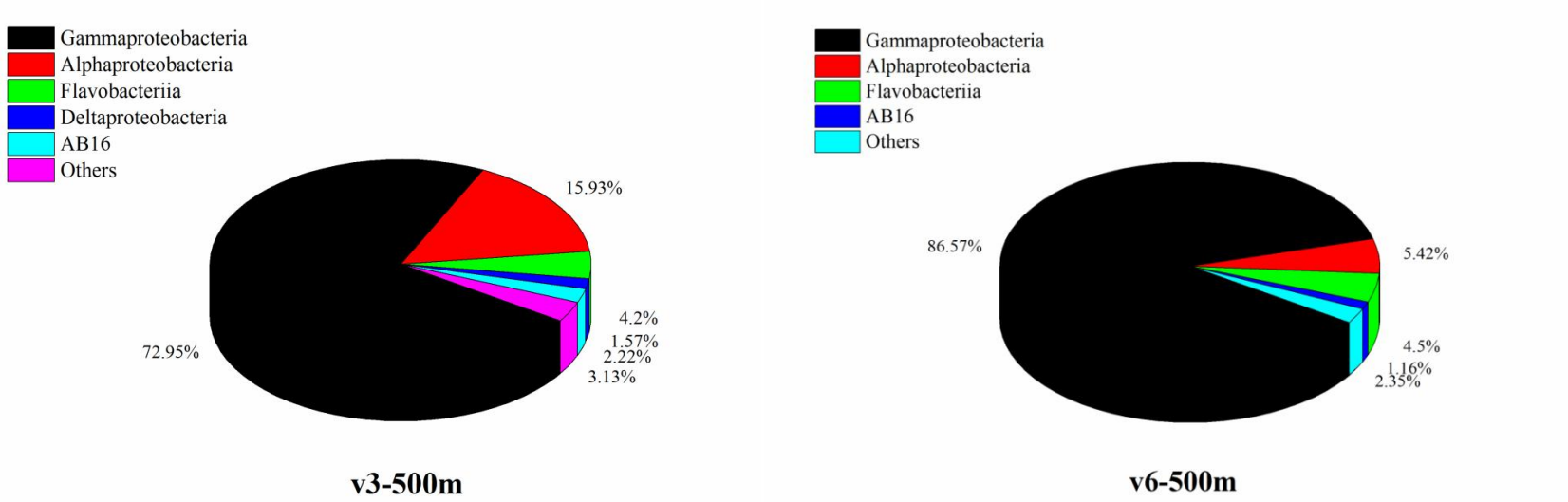
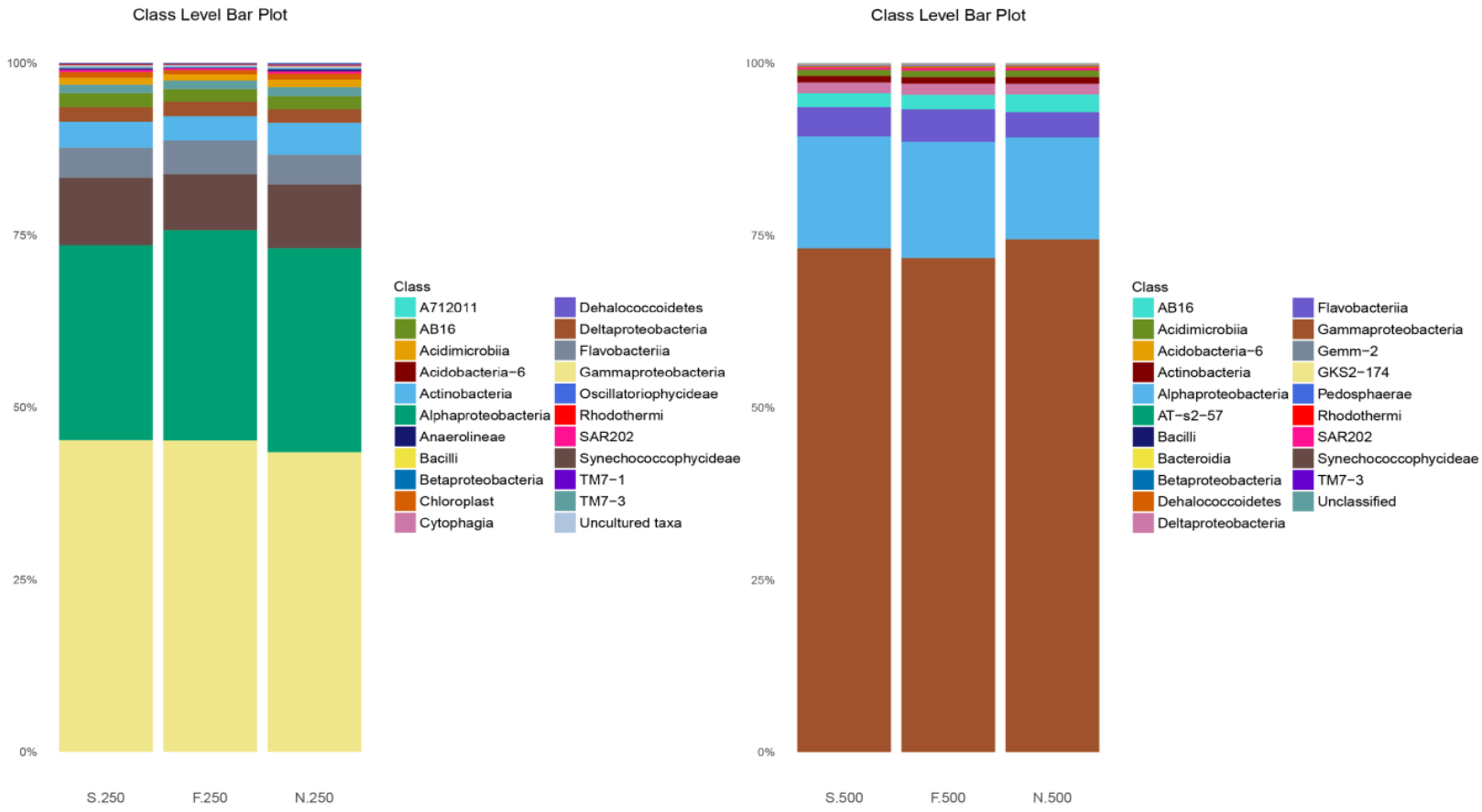
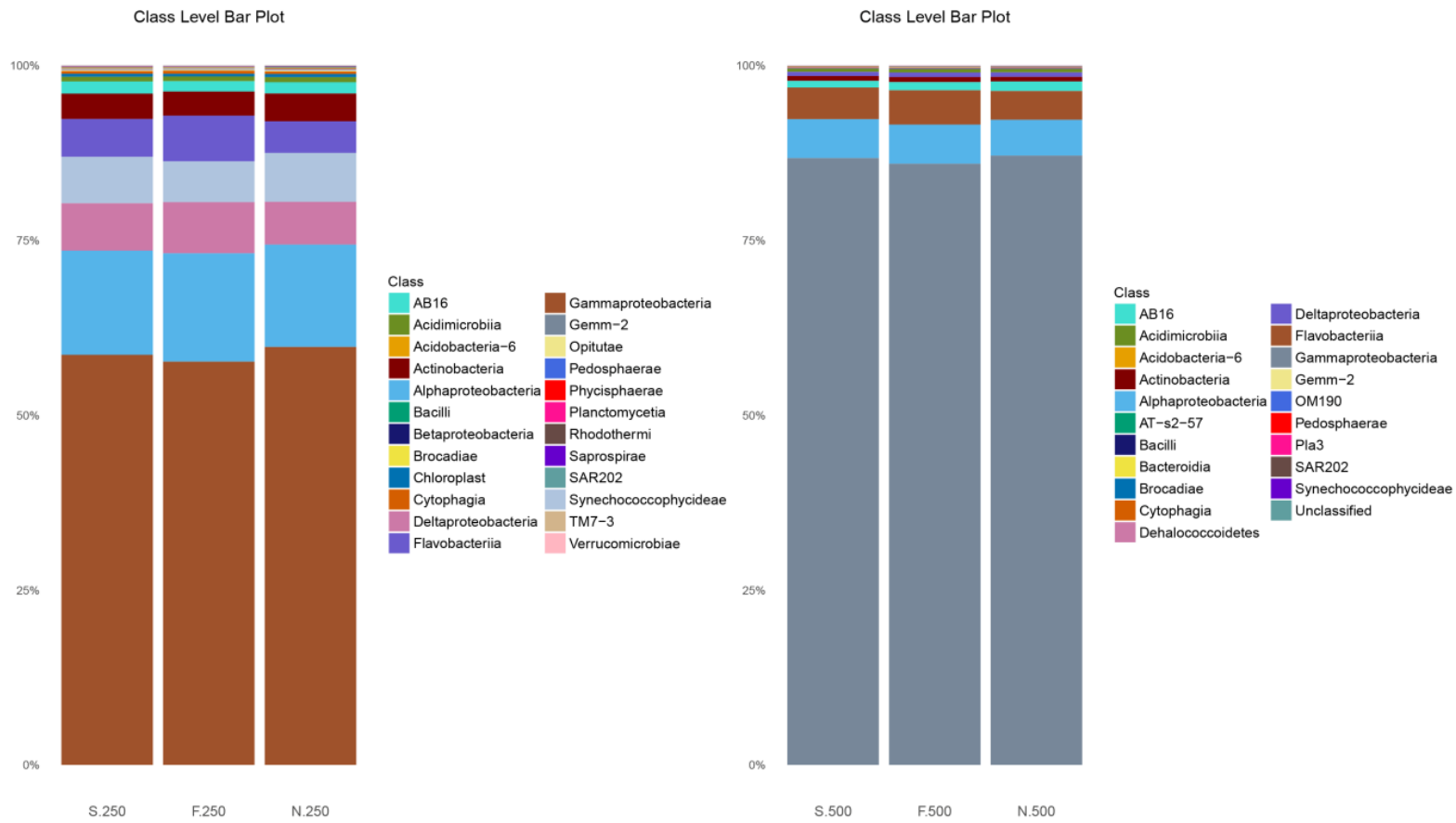


Figure 8.2. Distribution of major bacterial groups in 500 m depth in the Arabian Sea Time Series (ASTS) station during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM).



(a) **(b)**

Figure 8.3. Distribution of major classes of bacteria based on v3 region sequences from 250 m (a) and 500 m (b) samples from Arabian Sea Time Series (ASTS) location during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM).



(a) **(b)**

Figure 8.4. Distribution of major classes of bacteria based on v6 region sequences from 250 m (a) and 500 m (b) samples from Arabian Sea Time Series (ASTS) location during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM).

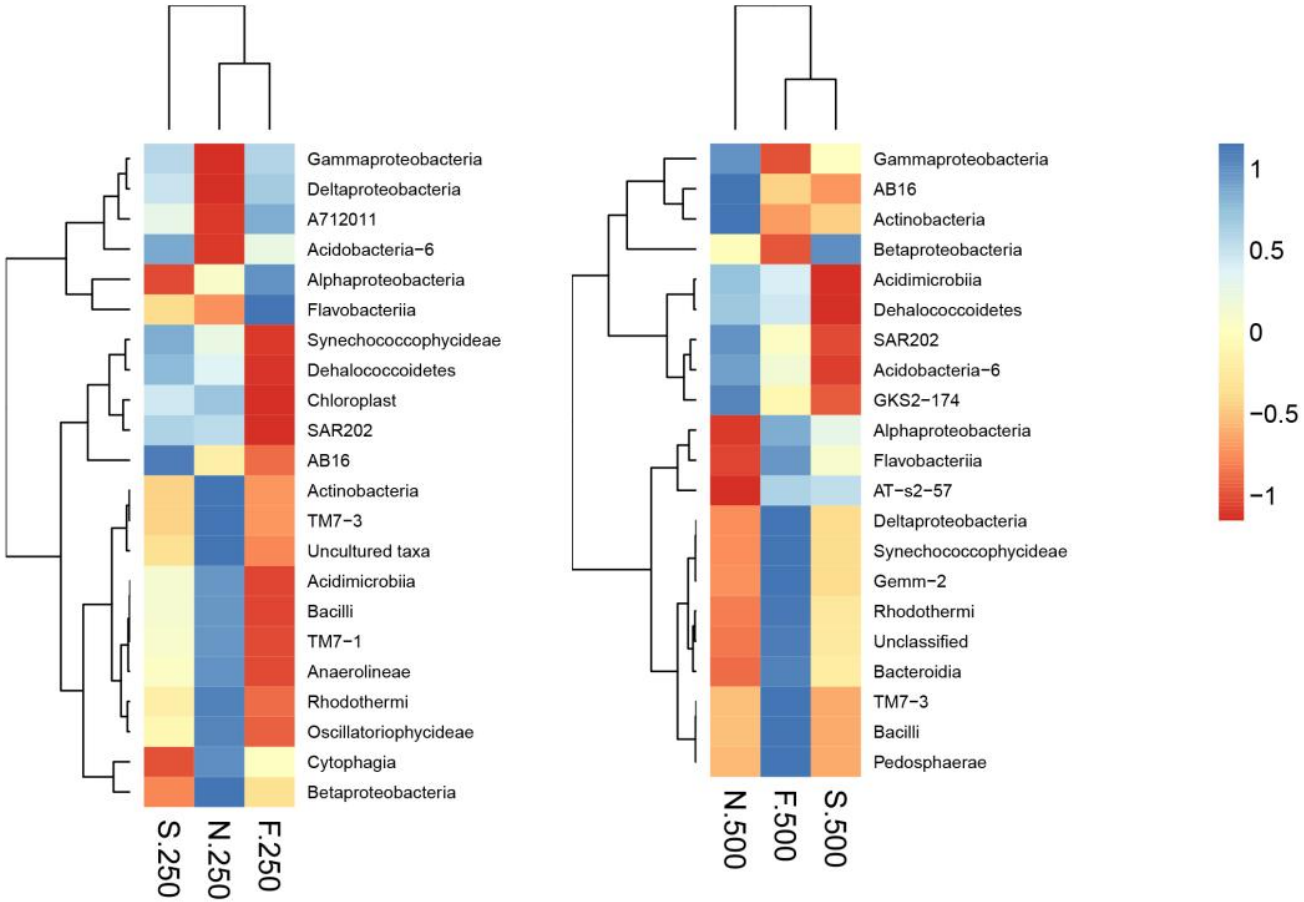


Figure 8.5. Hierarchically clustered heat map of the major bacterial communities from v3 region sequences in the Arabian Sea Time Series (ASTS) location during Spring intermonsoon (S), Fall intermonsoon (F), and Northeast monsoon (N).

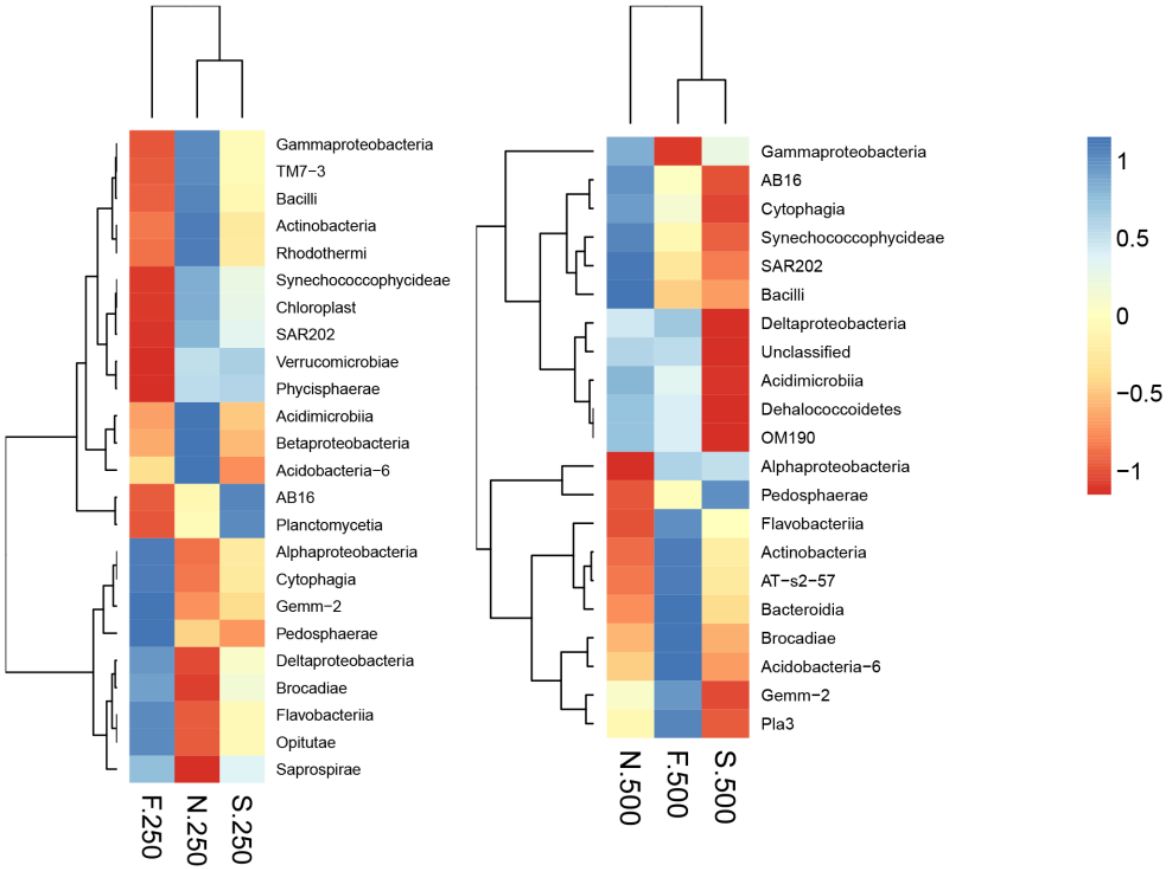
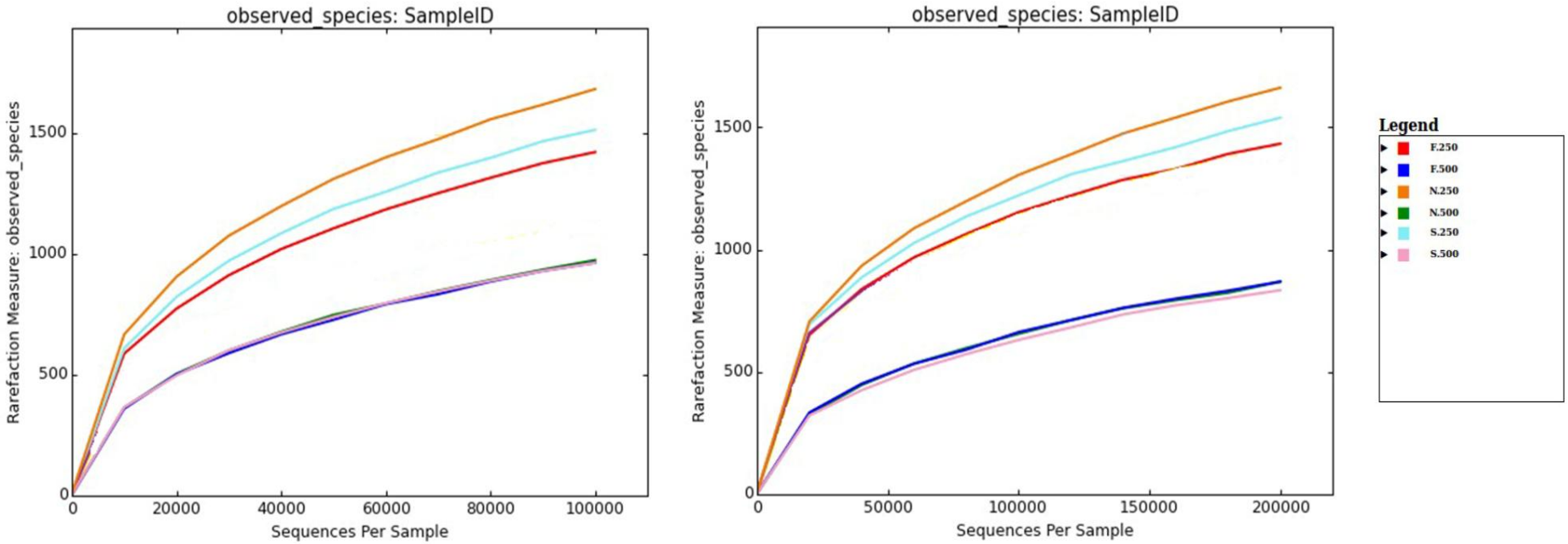


Figure 8.6. Hierarchically clustered heat map of distribution of major bacterial communities from v6 region at the class level in the Arabian Sea Time Series (ASTS) location during Spring intermonsoon (S), Fall intermonsoon (F), and Northeast monsoon (N).



(a) **(b)**

Figure 8.7. Rarefaction analysis of 16S rRNA gene bacterial communities based on v3 (a) and v6 (b) region sequences during Spring intermonsoon (SIM), Fall intermonsoon (FIM), and Northeast monsoon (NEM) from the Arabian Sea Time Series (ASTS) location.

8.4 Discussion

As is made clear in earlier desscriptions, the microbial communities within OMZs run the vital biogeochemical processes. Yet, there is a minimal understanding of the distribution microbial communities across most OMZs of the world oceans. Metagenomic analysis (Venter et al. 2004; DeLong et al. 2006) and NGS strategies (Sogin et al. 2006; Huber et al. 2007) have previously identified 'unexpectedly' high bacterial and archaeal phylogenetic and functional diversity. To date, research on microbial diversity in the AS-OMZ is focused mainly on culturable fractions of bacteria (Divya et al. 2010, 2011) or through construction of clone libraries of *16S rRNA* gene fragments (Jain et al. 2014; Fuchs et al. 2005). In this regard, efforts to implement NGS technology to delineate the bacterial community structure from metagenomic samples are useful in providing novel insights.

Distribution of bacterial communities found in this study resembled those routinely found in several pelagic marine environments using culture independent methods as reported by Giovannoni and Rappé, (2000). *Gammaproteobacteria* and *Alphaproteobacteria* were the two foremost groups at the ASTS location during all the sampled seasons. Variety of pelagic marine environments (Giovannoni, and Rappe, 2000), have documented similar results including the ETSP-OMZ by Stevens and Ulloa, (2008) and the Southern AS by Fuchs et al. (2005). The most common *Gammaproteobacteria* dominating the marine habitats as reported by Fuhrman et al. (1993) and Giovannoni and Rappé, (2000) is *Alteromonas*, as also found in this study. Kirchman et al. (2005) and Zhang et al. (2007) suggest that *Alphaproteobacteria* are generally abundant in marine waters and orders *Sphingomonadales, Rickettsiales* and *Rhodobacterales* (Campbell and Kirchman 2013; Miller et al. 2010; Fredrickson et al. 1995) are the most dominant of the class *Alphaproteobacteria*, similar to the findings of this study.

Substantial number of OTUs in the ASTS was affiliated with *Alteromonas.* Of the two major clusters of *Alteromonas,* Martinez et al. (2008) reported that the deep ecotype is better suited to micro-aerophilic conditions and degrade recalcitrant compounds and colonize relatively large particles, which sink rapidly to meso and bathypelagic depths. Whereas, Perez et al. (2012) suggests that the surface ecotype is a typical r-strategist and is specialized in colonizing smaller particulate organic matter with much slower sinking rates. The ability of *Alteromonas* to degrade recalcitrant compounds and sinking particulate matter which are abundant in the northern AS as documented by Kumar et al. (1998) and Ramaiah et al. (2005) could be the possible reason for the perennial persistence of *Alteromonas* at the ASTS location.

Presence of *Sphingomonas* in the AS-OMZ is reported earlier by Riemann et al. (1999). Although their ecological significance in OMZ is not clear, Miller et al. (2010) report them to be metabolically versatile and, Fredrickson et al. (1995) suggests they can degrade aromatic compounds. Therefore, their abundance in the AS-OMZ may be driven by high concentrations of phosphate and ammonia as postulated by Liu et al. (2015). Hansell and Peltzer, (1998) suggest that the organic carbon concentration and changes in primary productivity patterns in the surface layers may be responsible for variation in the abundance of *Alteromonas* and *Sphingomonas*. Higher abundance of these two genera in the ASTS libraries might be supported by their ability to use the abundantly available nitrate (Martinez et al. 2008) as their terminal electron acceptor for energy generation. Given the complexities and diversity of genes for metabolic pathways and the lack of experimental data, it is likely that *Rickettsiales* fall into multiple functional groups.

In marine waters, *Bacteroidetes* are commonly found with a relative abundance of

2.4–5.5% as reported by Kirchman, (2002), Abell and Bowman, (2005) and DeLong et al. (2006). However, within the *Bacteroidetes*, one family that seems to predominate is *Flavobacteria* and it appears that their presence is governed by several environmental factors. In this study as well as that of Kirchman, (2002) and Abelland Bowman, (2005), *Flavobacteria* was found to be predominant. Surprisingly, sequences affiliating to *Synechococcus* (phylum Cyanobacteria) were found in 250 m of the ASTS location, although their phylotypes are often reported to be present in the upper part lit part of the OMZs (Johnson et al. 1999, Goericke et al. 2000, Ulloa et al. 2008, Galán et al. 2009, Ulloa et al. 2012). However, phylotypes of *Synechococcus* are ubiquitously found in the marine pelagos, including ASTS and they do not show a clear vertical partitioning with depth.

The deep-branching bacterial phylum *Marinimicrobia,* formerly known as SAR406 (Fuhrman, McCallum & Davis 1993; Gordon and Giovannoni, 1996; Rinke et al. 2013) were also present at the ASTS location. A recent transcriptome study by Nobu et al. (2015) indicates the involvement of these bacteria in extracellular proteolysis and fermentative amino acid degradation in a methanogenic environment. These findings of an anaerobic lifestyle agree well with their presence in a low oxygen environment and especially in the core OMZ depths. However, in contrast to the findings of this study, Fuchs et al. (2005) did not detect SAR406 below the chlorophyll maximum layer in the AS. Whereas, Agogue et al. (2011) report that SAR406 increases in relative abundance with depth. Another bacterium whose abundance increases with depth is SAR202. Reports from the Atlantic by Varela et al. (2008b) and Agogue et al. (2011) suggest that the contributions of SAR202 to total bacterial abundance increases with increasing depth. This supports the presence of SAR202 in both 250 m and 500 m depth at the ASTS location.

Sequences affiliating to anammox-group of the phylum *Planctomycetes* were found

in both depths during all three seasons. Sequences affiliating to class, *Brocadiae*, OM190, Pla3, *Phycisphaerae*, BD7-11, *Planctomycetia*, ODP123 and C6 in the core OMZ depths were consistent with geochemical results that show the anammox pathway to be the main sink for fixed nitrogen at the same sampling location. Similar results by Thamdrup et al. (2006) and Stevens and Ulloa, (2008) were reported from the ETSP. In contrast, no known denitrifying bacteria were obtained in our samples. This was in agreement with the results of chapter 3. However, denitrifying bacteria are widely distributed in the phylogenetic tree and they appear to be present in the study area as reported in chapter 5.

Higher bacterial diversity is reported from transition zones, as in the Cariaco Basin (Madrid et al. 2001) and from anoxic environments rather than in stable habitats (Araujo, 2002; Daffonchio et al. 2006; Kirkpatrick et al. 2006). Consequently, one would anticipate the core of the AS-OMZ to have lower diversity, because of physico-chemical characteristics in this layer of the water column appear to be more stable. In contrast, the diversity observed was much higher, which could be ascribed to the versatility of the *insitu* microbial communities in suboxic/anoxic environment to utilize more than one type of electron acceptors for metabolic activities.

Predominance of sequences affiliating to bacteria that are involved in the sulfur cycle in both the investigated depths was clearly evident from the NGS analyses. This is fact that wasn't clearly evident from the *16S rDNA* based results, described earlier. Sequences affiliating to *Rhodobacteraceae* of the class *Alphaproteobacteria* (Moran et al. 2003) and *Desulfarculales* of the class *Deltaproteobacteria* were far higher in abundance than could be noted through phylogenetic analyses of *16S rRNA* gene. The community of *Rhodobacteraceae* in the OMZ depths can oxidize inorganic, but also organic sulfur

compounds, such as dimethylsulfoniopropionate (DMSP). Whereas, *Desulfarculales* bacteria, is a chemo-organotroph and reduces sulfate, sulfite or thiosulfate to hydrogen sulfide. The other OMZ-OTUs were *Verrucomicrobia, Acidobacteria, Firmicutes,* TM7, *Betaproteobacteria, Chloroflexi*, SBR1093, *Gemmatimonadetes, Fusobacteria,* OP3, *Fibrobacteres,* and *Spirochaete*. Notably, none of the OTUs were affiliated to known denitrifiers, probably implying that denitrification is spatially heterogeneous.

It is important to note that the rarefaction curve of all libraries reached saturation, indicating that the diversity was adequately captured from all evaluated depths. The rarefaction analysis of both v3 and v6 region showed that diversity was higher in 250 m during all three seasons. The species richness estimator Chao1 and two different diversity indices, which take the abundance of a population and its evenness in account, supported this finding, showing a higher diversity in the 250 m than at the other investigated depth (500 m).

Chapter 9
Summary

Oxygen minimum zones (OMZ) play a vital role in the biogeochemical cycles of the world oceans through denitrification and anaerobic ammonium oxidation (anammox). The Arabian Sea (AS) OMZ is among the largest suboxic regions in the ocean with DO levels as low as 0.1 μM in a vertical depth of 150 to 1000 m water column. Many studies, whose main findings have been described and compared with the present study explicitly suggest that the AS-OMZ is responsible for loss of nitrogen and accounts for 20% of oceanic denitrification, thus this region of ~ 1.2 million Sq Km area does a disproportionate contribution to the marine nitrogen budget.

The hypothesis put forward was to explore the diversity of unculturable bacterial community in the AS-OMZ and to examine its seasonal and vertical variations. In addition to measuring bacterial biomass this study also explored the seasonal distribution of a few functional marker genes necessary to appreciate bacteria mediated denitrification, nitrous oxide reductase, and anammox genes.

Systematic sampling was carried out during May 2012 (representing Spring Intermonsoon [SIM]), September 2012 (Fall Intermonsoon [FIM]), and February 2013 (Northeast monsoon [NEM]), from the Arabian Sea Time Series (ASTS) station (17°0.126' N, 67°59.772'E). Water samples were collected from five depths [surface (5 m), deep chlorophyll maxima (DCM), upper OMZ (250 m, 500 m) and lower OMZ (1000 m) (1000 m)]. Significance of seasonal variation, and changing chemical parameters on the overall diversity were evaluated for samples from all depths. Further, estimation of bacterial biomass and distribution of marker genes involved in denitrification and anammox were also analyzed.

Following are the salient observation of this study

- Bacterial biomass estimation showed highest numbers, ranging from 1.4 x 10^9 L^{-1} (in surface waters) to 0.6 x 10^9 L^{-1} (below 250 m) during the FIM whereas the lowest number of bacteria was recorded during SIM (0.53 - 0.36 x 10^9 L^{-1}).
- The sequencing of 743 bacterial clones, from 15 *16S rRNA* gene libraries, from different seasons, revealed the presence of 177 operational taxonomic units (OTUs) in the surface samples and 217 OTUs in the OMZ samples at 97% sequence similarity.
- Maximum number of OTUs (14) was recorded from 500 m sample during SIM and minimum (5) were from 5 m during NEM.
- Depth wise profiles reveal clear patterns of vertical partitioning of the bacterial communities during all 3 sampled seasons.
- Temporal variations among surface (5 m and DCM) bacterial communities were evident whereas OMZ (250 m, 500 m and 1000 m) bacterial communities showed no variation.
- Majority of bacterial *16S rRNA* gene sequences in the surface are affiliated to Cyanobacteria, *Alphaproteobacteria* and *Gammaproteobacteria.*
- Sequencing of bacterial *16S rRNA* gene sequences from OMZ depths highlights the preponderance of *Gammaproteobacteria* and *Alphaproteobacteria*.
- The three OTUs shared among surface groups during all seasons were affiliated to genus *Synechococcus*. The OTUs shared between any two seasons were affiliated to *Alteromonas, Burkholderia, Synechococcus, Acidobacterium*, and uncultured_*Alphaproteobacteria*.

- In the OMZ samples, OTUs shared between all three sampling seasons were related to *Alteromonas, Rhodobacter, Sphingomonas,* and *Burkholderia*. The OMZ OTUs common to any two seasons were affiliated to *Sphingomonas, Nitratireductor, Acidimicrobium,* uncultured_*Gammaproteobacteria*, and Marine group A.
- Shannon diversity index (H') is higher in the OMZ depths than in surface depths during all three seasons.
- Higher bacterial diversity in OMZ depths was predominant of *Alteromonadales, Sphingomonadales, Rhodobacterales, Burkholderiales* and *Acidimicrobiales*
- Canonical correspondence analysis (CCA) indicates differences in the concentrations of DO and total organic carbon to cause the depth-wise separation among bacterial communities.
- Next Gen sequencing revealed dominance of *Gammaproteobacteria* and *Alphaproteobacteria* at depths 250 m and 500 m during all seasons.
- Shannon and Simpson's index suggest higher diversity of bacteria at 250 m than that at 500 m depth during all seasons.
- No seasonal variation evidenced in bacteria communities at 250 m and 500 m depths.
- Amplification for archaeal *16S rRNA* genes was not evidenced from the DNA extracts of samples from surface, DCM and 500 m. As an exception, the 500 m FIM sample was positive for archaeal amplification.
- At 97% sequence similarity as many as 182 OTUs were formed from sequencing of 254 archaeal clones.
- The highest number of archaeal OTUs was observed at 250 m during SIM and, the lowest numbers at 1000 m during FIM.

- Archaeal diversity was found to be higher in 250 m than in 500 m and 1000 m.
- No seasonal or vertical variation in the archaeal communities was observed and majority of the sequences were affiliated to Marine Group II.
- CCA analyses indicated that DO and nutrient have a considerable effect on the archaeal community during different seasons.
- Statistical analyses showed no significant seasonal variation between the archaeal clone libraries.
- Functional gene based denitrifiers and anammox bacteria were exclusively limited to upper OMZ (250 m and 500 m) depths.
- Shannon diversity index show minimal seasonal variation among denitrifiers and anammox bacterial community with higher diversity of denitrifying bacteria at 250 m, whereas diversity of anammox bacteria was higher at 500 m depth.
- Phylogenetic analysis revealed that *nirS* gene was present in bacteria mostly belonging to *Gammaproteobacteria, Alphaproteobacteria* and *Deltaproteobacteria.*
- *NirS* gene based bacterial community is dominated by *Halomonas* followed by *Thauera, Ideonella, Marinobacter, Pseudogulbenkiania, Rhodanobacter, Herbaspirillum* and *Azospirillum.*
- Majority of *nosZ* gene were affiliated to *Alphaproteobacteria, Gammaproteobacteria* and *Betaproteobacteria.*
- Majority of *nosZ* gene based bacterial community were affiliated to *Rhizobiales* followed by *Pseudomonas*, *Rhodopseudomonas*, *Sinorhizobium*, *Azospirillum* and *Achromobacter*.

- The entire *hzo* gene based anammox bacterial sequences obtained in this study aligned with *Candidatus Scalindua*.
- *16S rRNA* gene sequences of anammox bacteria were affiliated with *Scalindua brodae, Scalindua marina* and *Scalindua wagneri.*
- Overall diversity of anammox bacteria is higher than that of denitrifying bacteria.
- Quantitative analyses shows copy numbers of both anammox and denitrifying genes had their maxima in the upper OMZ depths.
- Abundance of anammox gene was higher than of denitrifying genes during all three seasons implying probable dominance of anammox process at the ASTS.
- Copy numbers of *hzo* gene (1.5-0.04 x 10^6 L^{-1}) and *nosZ* (1.5-0.08 x 10^6 L^{-1}) was higher than *nirS* (0.35-0.02 x 10^6 L^{-1}) gene.

Future prospects

Most previous studies in the AS-OMZ focused on characterization of heterotrophic bacteria, and taxonomic identification of phytoplankton and zooplankton. Present study involving metagenomic approach is the first report on the overall bacterial community structure including their response to seasonality in the AS-OMZ water column which is hoped to bring out information that will bridge gaps existing in our knowledge of AS-OMZ microbial community. Long term monitoring of the microbial communities in the AS-OMZ and their role in biogeochemical cycles should be the focus for future studies. Continued application of molecular techniques [next gen sequencing, gene expression] and isotopic investigation would help in assessing the kinetics of denitrification and anammox. In this regard, studies connecting biogeochemistry of the OMZs as well as microbial metagenomic, phylogenetics and their functional processes are contextual.

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