ABUNDANCE AND ROLE OF MARINE BACTERIOPLANKTON IN THE NITROGEN CYCLE OF THE ARABIAN SEA

Thesis submitted for the award of the degree of

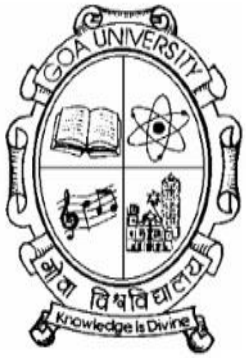
DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY

to the

Goa University



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DECEMBER 2019

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Under the guidance of

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DECEMBER 2019

STATEMENT

As required under the University Ordinance OA 19, I state that the present thesis entitled "**Abundance and role of marine bacterioplankton in the nitrogen cycle of the Arabian Sea**" is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problems investigated has been appropriately cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

Place: Dona Paula, Goa, India. **Amara Begum Mulla**

Date:

CERTIFICATE

This is to certify that the thesis entitled "**Abundance and role of marine bacterioplankton in the nitrogen cycle of the Arabian Sea**" submitted by Ms. Amara Begum Mulla for the award of the degree of Doctor of Philosophy in Department of Microbiology is based on original studies carried out by her under my supervision. The thesis or any part, therefore, has not been previously submitted for any degree or diploma in any universities or institutions.

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Amara Begum Mulla

Dedicated to

Abbu, Ammi, Nisaa and Subhan

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List of abbreviations

°C	Degrees Celsius
°E	Degrees east
°N	Degrees north
°S	Degrees south
µg C L^{-1}	Micrograms carbon per litre
µm	Micrometers
µmol	Micromoles
ABW	Antarctic Bottom Water
Anammox	Anaerobic ammonia oxidation
AS	Arabian Sea
ASHSW	Arabian Sea High Salinity Water
Auto pico	Autotrophic picoplankton
bp	Base pairs
CCA	Canonical correspondence analysis
CTD	Conductivity, Temperature, Depth
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DNRA	Dissimilatory nitrate reduction to ammonia
DO	Dissolved oxygen
DOC	Dissolved organic carbon
ETNP	Eastern Tropical North Pacific
ETSP	Eastern Tropical South Pacific
FALS	Forward angle light scatter
fg C	Femtograms carbon
Hbac	heterotrophic bacteria
IIOE	International Indian Ocean Expedition
JGOFS	Joint Global Ocean Flux studies
MLD	Mixed layer depth
NADW	North Atlantic Deep Water
nar	Nitrate reductase
NCBI	National Center for biotechnology information

NEAS	Northeastern Arabian Sea stations
NEM	North east monsoon
nir	Nitrite reductase
nor	Nitric oxide reductase
nos	Nitrous oxide reductase
OMZ	Oxygen minimum zone
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PGW	Persian Gulf Water
POC	Particulate organic carbon
PP	Primary productivity
PSU	Practical salinity units
RALS	Right angle light scatter
RSW	Red Sea water
SEAS	Southeastern Arabian Sea stations
SNM	Standard nitrite maxima
SSD	Sindhu Sadhana
SSK	Sindhu Sankalp
SSS	Sea surface salinity
SST	Sea surface temperature
SWM	South west monsoon
SYN-PC	*Synechococcus* phycocyanin
SYN-PE	*Synechococcus* phycoerythrin
TAE	Tris-acetate-EDTA
TBC	Total bacterial counts
TOC	Total organic carbon

Chapter 1

Marine nitrogen cycle and its microbial ecology

1.1. General introduction

1.1.1. *The marine nitrogen cycle*

The marine nitrogen cycle, one of the most complicated biogeochemical cycles in the ocean, is of fundamental importance because of the potential for available nitrogen to control the rate or level of primary productivity. Nitrogen covers 70% of earth's atmosphere, yet it is often a limiting nutrient for the growth of living organisms which require fixed nitrogen to biosynthesise their nucleotides and amino acids. Different processes such as nitrogen fixation, ammonification, nitrification, anammox (anaerobic ammonium oxidation), denitrification and dissimilatory nitrate reduction to ammonia (DNRA) govern the transition from one form of nitrogen to another. Nitrate respiration is preferred over other electron acceptors following the order $O_2 > NO_3^- > MnO_2 > FeO(OH) > SO_4^{2-} > CO_2$ (Canfield *et al*., 2005). The reduction of nitrate (NO_3) to nitrous oxide (N_2O) or dinitrogen (N_2) via canonical denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) or to N_2 via anammox leads to a loss of nitrogen from the oceans to the atmosphere. Also, the release of N_2O during these processes has large scale implications as it is a potent greenhouse gas. The biogeochemical cycling of carbon and nitrogen is exemplified by the fundamental significance and interdependence of important processes such as primary production. These elements are important constituents of all living matter and share many common features in terms of biogeochemical cycling. A deeper understanding of individual transformation is illustrated by nitrogen conversions which are influenced by the availability of oxidisable carbon sources.

Biological nitrogen fixation and denitrification are the most important natural processes that could influence the amount of reactive nitrogen, and hence alter the global carbon cycle and climate, without changing the C/N ratio of autotrophs. In the ocean, denitrification primarily influences the marine nitrogen budget. The dynamic marine nitrogen cycle consists of reactive nitrogen that has a residence time of fewer than 3,000 years (Gruber & Galloway, 2008). Canonical denitrification was considered to be the primary pathway by which fixed nitrogen was removed in these regions. However, the recent discovery of the anaerobic ammonia oxidation (anammox) in these regions led to the suggestion that it might be an important, or even dominant, pathway of N_2 production (Kuypers *et al*., 2005; Thamdrup *et al*., 2006). The relationship between important

biogeochemical cycles of nitrogen, carbon, phosphorous is quite conspicuous through biological stoichiometric requirements and any alteration in these cycles including those due to human activities is likely to have consequences for ecosystem functioning.

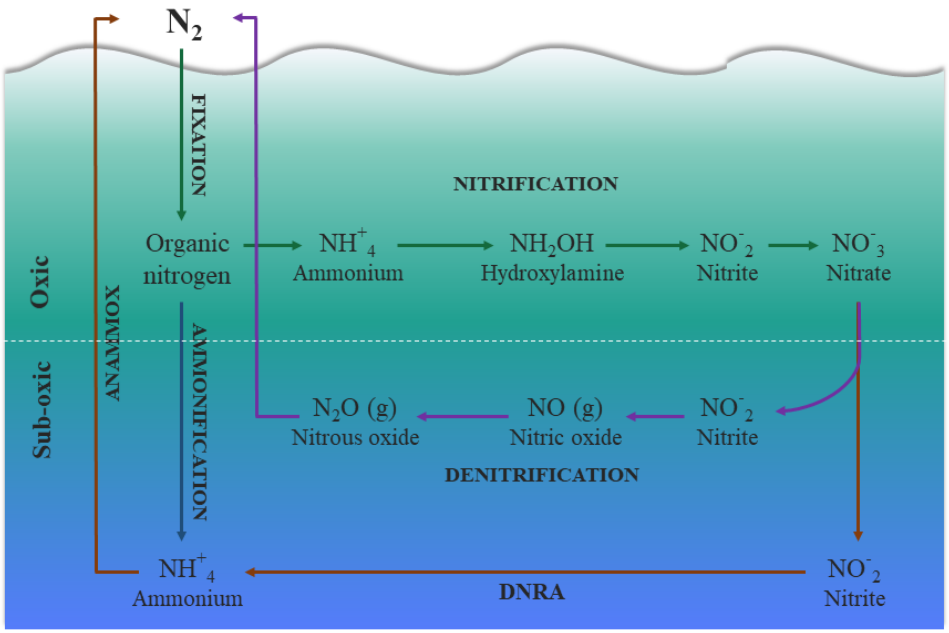

Figure 1.1. Schematic diagram of the marine nitrogen cycle in the oxygen minimum zone, modified from Francis *et al*., 2007.

1.1.2. *Physical processes in the Arabian Sea*

The Arabian Sea (AS), situated in the northwest region of the Indian Ocean, is surrounded by the Asian continent of northeast Africa, the Arabian Peninsula, and India. This semi-enclosed sea is distinct from the other low-latitude seas and has a wide continental shelf along the Indian west coast. Although AS receives about 350 km^3 y^{-1} river runoff, it exhibits a net water loss annually due to more substantial evaporation rates than combined precipitation and riverine input. The thermohaline structure of the AS is driven by a combination of multiple physical processes occurring in this region. A well-structured circulation in terms of water masses having characteristic temperature and salinity was also observed in the AS. Five water masses have been identified in the upper 1000 m of the northern Indian Ocean. Out of which, three have been distinguished as

originating in the Red Sea, the Persian Gulf, and the Arabian Sea respectively. The very high rate of evaporation makes the Arabian Sea a negative water body leading to high salinity at the surface. The water mass found close to the surface, and the thermocline is the Arabian Sea High Salinity Water (ASHSW), between 200-300 m is the Persian Gulf Water (PGW) and the Red Sea water at 600-800 m. In the deeper layers, the North Indian Deep Water (NIDW), North Atlantic Deep Water (NADW) and Antarctic Bottom Water (ABW) occur in that order with the increasing depth (Kumar, 2006).

The weather conditions in the AS are strongly influenced by the surrounding landmasses, especially the Tibetan plateau to the north of India and result in the formation of powerful monsoon systems. Seasonal reversal of monsoonal winds imparts distinct seasons; summer monsoon or the southwest (SWM), winter monsoon or the northeast (NEM), Fall intermonsoon and Spring intermonsoon. Associated with seasonal changes of the wind field, surface circulation of the system also reverses thoroughly and produces dramatic physical, chemical and biological changes in the upper layers of the water column. The summer monsoon (june to september) is characterised by the northward movement of warm air over the Arabian Sea, producing heavy rains over some areas of Africa and India. A seasonal low-pressure area developing over central Asia during this period causes the wind system to blow persistently from the southwest. During the winter monsoon period (november to february), the cold, dry winds blow from a high-pressure source forming over the Tibetan plateau moving towards low-pressure belt in the equatorial Indian Ocean. The winds during southwest monsoon are in general stronger and steadier than those during the northeast monsoon, while they are weaker during the transition (intermonsoon) periods.

Another important characteristic of the AS is the upwelling phenomenon, which occurs due to the seasonal reversal of winds and increases the productivity of the region. Upwelling occurs along the continental margins due to southwest monsoon winds. The Somalia and Oman upwelling system in the Arabian Sea is one of the five major coastal upwelling systems in the world and unique as it occurs along a western boundary of an ocean basin. Strong winds during the southwest monsoon force surface waters along the coast to move away due to Ekman circulation. An intense upwelling between 5°N and 11°N is quite evident, with the replacement of warmer surface water by cooler water

(14°C)**.** The west coast of India experiences a similar phenomenon which causes coastal upwelling and is quite essential in coastal productivity.

These factors are critical in the dynamics of the upper-ocean and influence the biogeochemistry of the region (Prasanna Kumar *et al*., 2001). An important observation of this confluence is the apparent high biological productivity in the coast as well as open ocean regions of this basin. The JGOFS (Joint Global Ocean Flux studies) programme carried out by Indian researchers in the eastern Arabian Sea found primary productivity (PP) values ranging between 440-1760 mg C m^{-2} d^{-1}. These values were consistent with PP values of 792-1782 mg C m^{-2} d^{-1} stated by Bhattathiri *et al*., 1996 during the southwest monsoon. Naqvi *et al*., 2003 reported that along the eastern Arabian Sea where the cold, nutrient-rich (> 20 µM NO_3) waters are brought close to the surface, the PP could be as high as 6 g C m^{-2} d^{-1}. Convective mixing and upwelling processes inject copious amounts of nutrients (nitrate, silicate, phosphate) to the euphotic zone, which are utilised by phytoplankton thereby enhancing the primary productivity. This high biological productivity of 1.03 to1.64 C m^{-2} d^{-1} (US JGOFS) has led to an increased flux of organic matter to the ocean floor. Microbial communities can remineralise this organic matter travelling between the surface to 1000 m depth, thereby creating an oxygen minimum in the intermediate ocean waters.

1.1.3. *Arabian Sea oxygen minimum zone*

Oxygen minimum zones (OMZ) play an essential role in the global nitrogen cycle. These are regions of the world ocean in which dissolved oxygen in the water column is reduced or absent. Oxygen deficient marine systems are diverse (e.g., permanently or seasonally anoxic/sub-oxic/hypoxic; open-ocean or coastal) and are sensitive to perturbations. The Arabian Sea harbours one of the three main oxygen minimum zones in the world oceans, besides ETNP-Eastern Tropical North Pacific and ETSP-Eastern Tropical South Pacific (Wrytki, 1966). The open ocean deep water OMZ in the AS occurs permanently between 150-1500 m (Wyrtki, 1971; Von Stackelberg, 1972). Low oxygen conditions also develop along the eastern boundary of the Arabian Sea, on the west coast of India. This seasonally influenced oxygen deficient zone has an

impact on the biogeochemistry of the region (Naqvi *et al*., 2000). Almost half of the global mesopelagic fixed N loss occurs in the Arabian Sea which forms a significant site of enhanced denitrification in the water column (Bange *et al*., 2005). The widespread open-ocean oxygen deficiency results when the respiratory O_2 demand during the degradation of organic matter exceeds oxygen availability in this poorly ventilated region. Although OMZ in the Pacific is larger and voluminous than those in the Atlantic and the Arabian Sea, oxygen deficiency is often intense in the AS due to large amounts of carbon export and subsurface respiration. These naturally euphotic waters harbour a diverse microbial community which thrives on the carbon produced in the upper layers of the water column. The OMZs are principally associated with denitrification, which is a bacterial process occurring only in O_2-deficient regions (e.g., Codispoti *et al*., 2001, Paulmier and Ruiz-Pino, 2009).

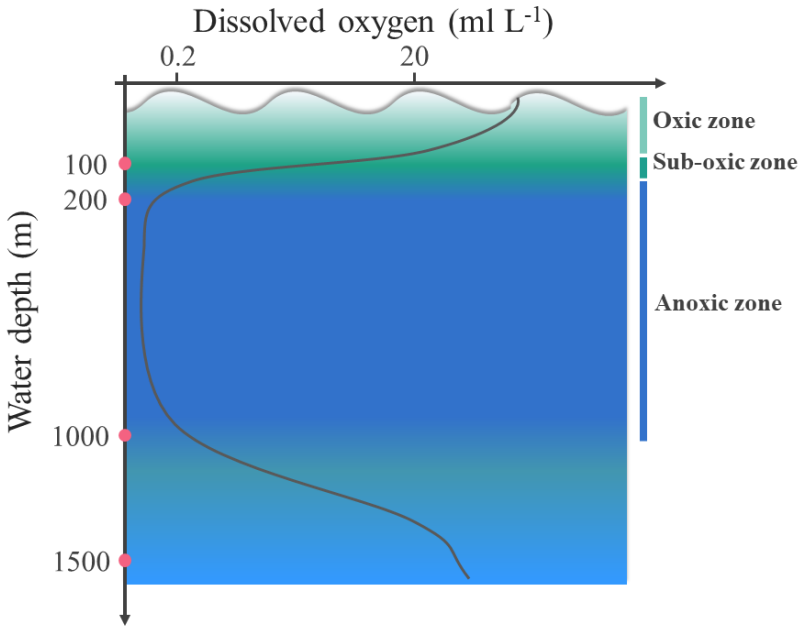

Figure 1.2. Depth profile of the oxygen minimum zone.

OMZs are involved in the cycling of important climate-relevant gases such as N_2O (Bange *et al*., 1996), H_2S (Dugdale *et al*., 1977) and sometimes CH_4 (methane) when OMZ is in contact with sediments (Cicerone and Oremland, 1988). Besides they limit atmospheric CO_2 by the ocean through remineralisation (Paulmeir *et al*., 2006).

Also, they are intriguing regions of biological and ecosystem studies. Transitions from highly oxygenated environments to low O_2 could provide useful insights about primitive life which must have been originated in the anoxic ocean. Rogers, 2000 believe that this could stimulate biodiversity on a paleoclimatic scale. Spreading of low O_2 areas across the basin can lead to massive fish mortality as seen in the past during Mid-Cretaceous. However, current events of episodic anoxia associated with eutrophicated waters are also inducing massive abnormal fish mortality (Chan *et al*., 2008).

1.1.4. *Microbial plankton ecology of oxygen minimum zones*

The nitrogen transformations in the marine system are controlled by the interaction of organisms of different size classes, including microplankton and bacteria. According to Campbell *et al*., 1988, the distribution of phytoplankton is influenced by a variety of factors including temperature, light, nutrient availability, water column stability, and grazing pressure. The Arabian Sea provides a range of nutrient conditions to examine the relative importance of the classical and microbial food webs. Small producers at the base of the microbial food web become more prominent in terms of biomass when physical conditions cannot sustain large standing stocks of larger producers (Landry *et al*., 1997). Phytoplankton, divided into three major groups based on the cell size; microphytoplankton (20 to 200 µm), nanophytoplankton (3 to 20 µm) and picophytoplankton (0.2 to 3 µm), are the key players for fixation of nitrogen in the upper layers of the water column. Large amounts of organic matter generated by these organisms' acts as a fuel for the occurrence of heterotrophic remineralisation in the system.

Bacteria predominantly control the nitrogen cycle, and their activities determine the distribution of nitrogen compounds. In turn, environmental conditions that regulate the activity of bacteria determine where each process occurs, the degree of exchange among various nitrogen pools, and the physical, chemical, and possible biological interactions. Although detailed biochemical information about bacterially mediated reactions is required, such knowledge alone is not sufficient to understand control of or to predict rates of bacterial processes in the environment (Kuenen and Robertson, 1988). Diverse assemblages of bacteria utilising one or more N compounds to carry out various

functions of the nitrogen cycle, control the microbial ecology of the OMZs. In the Arabian Sea, the standard nitrite maxima (SNM, between 150-400 m depth) is assumed to be the site of most intense denitrification and coincides with an oxygen concentration of <0.1 mL L^{-1} (Naqvi, 1994). A wide variety of microorganisms, including over 40 genera of bacteria, halophilic archaea, fungi, and foraminifera (Shoun *et al*., 1992; Zumft, 1997; Cabello *et al*.. 2004, Pina-Ochoa *et al*., 2010), have the capability to denitrify but rarely are they strict anaerobes (Zumft, 1997). Denitrifiers comprise of a phylogenetically differing group of heterotrophic bacteria having an ability to reduce nitrate and must have acquired this trait via evolutionary mechanisms (Jones *et al*., 2008). Hence, this ability to denitrify can be found in distantly related organisms. At the same time, However, all closely related species belonging to the same genus do not share this ability. Phillipot *et al*. (2007) have listed denitrifying ability among micro-organisms belonging to more than 60 genera. The most abundant phyla in the OMZs include Proteobacteria, Bacteroidetes, marine group A (a candidate phylum), Actinobacteria and Planctomycetes (Wright *et al*., 2012). Although most denitrifiers belong to the *proteobacteria*, denitrification is common among the *Firmicutes, Actinomycetes, Bacteroidetes*, as well as among the archaea. Pina-Ochoa *et al*. (2010) also showed denitrification to be widespread between Foraminifera and *Gromiida*. Microbes involved in the denitrification pathway are studied by their nitrate (NO_3) or nitrite (NO_2) utilisation capacity in the absence of oxygen (Manohar *et al*., 2014) and by an analysis of the enzymes involved in the denitrifying pathway (Shailaja *et al*., 2006). Newer methods include the functional gene analysis using marker sequences for denitrification utilising real-time quantitative studies (Braker *et al*., 2000; Henry *et al*., 2004). Besides studying the natural oxygen-depleted environments, conventional microbiological techniques involving studies using pure cultures of denitrifiers also is of substantial importance and has widened our understanding of the capability of these microbes in nitrogen cycling processes.

1.2. Review of literature

The oxygen minimum zone in the AS was first discovered in the 18th century during the Murray expedition, following which Wrytki (1962, 1966), described anoxia of the World Ocean. After Cline and Richards coined the term Oxygen minimum zone in 1972, numerous studies have been carried out describing the biogeochemistry associated with this unique habitat. Consolidated information on global scale denitrification and distribution of hypoxia in the Pacific and the Indian Ocean regions can be found in Kamykowski and Zentara (1990). The IIOE (International Indian Ocean Expedition), conducted during 1959-1965 collected a large volume of data on the chemical characteristics of the Arabian Sea OMZ (Mc Gill, 1973). Qasim (1982) presented a comprehensive document describing the oceanography of the northern AS based on data collected during 1973-1974, 1976 and the cruises carried out during the IIOE expedition in 1973. He described the temperature and salinity characteristics of the euphotic zone and the formation of two oxygen minima (between 100-400 m and between 800-1500 m). Oxygen minimum zones (OMZs) are identified by very low oxygen saturation. Oxygen concentrations $\leq$20 µmol L^{-1} are commonly observed between 100 and 1000 m in the eastern north and the south Pacific Ocean and the northern Arabian Sea (Paulmier and Ruiz-Pino, 2009; Bianchi *et al*., 2012). Swallow (1984), however, postulated that the north Indian Ocean experiences much stronger mixing processes than other oceans and that the acute deficiency of dissolved oxygen might be due to a combination of excessive oxygen consumption and low oxygen concentrations of the waters responsible for renewal. The surface waters of the Arabian Sea are highly productive and generate vast amounts of organic matter in the subsurface layers (Naqvi *et al*., 1987). Surface primary production ranging from 20-232 mg C m^{-3} d^{-1} have been reported in the Arabian Sea by Krey and Babenerd, 1976 and Sumitra-Vijayaraghavan and Kumari in 1989. Decomposition of this organic matter with the apparent utilisation of oxygen leads to excessive consumption of the same and development of oxygen minima in these waters (Kamkowski and Zentara, 1990; Helly and Levin, 2004). Besides this, sluggish ventilation due to lack of circulation of deep waters with this intermediate water adds on to the oxygen deficiency (Sarmiento *et al*., 1988). The subsurface layers are supplemented with warm, high saline and oxygen-poor waters from the Persian Gulf and

the Gulf of Aden (Neyman, 1961). Rao and Jayaraman (1970) also believed that the oxygen minimum in top layers is because of near-stagnant conditions in the northern and central parts of the Arabian Sea.

Depletion of oxygen in the ocean is primarily associated with nitrate concentrations in the water column. Sengupta *et al*., 1995 showed the quantitative relationships between nutrients and oxygen in the Arabian Sea. From the works of Deuser (1978), a clear association of the standard nitrite maxima with the average salinity maximum was evident. However, calculation of nitrate concentrations or nitrate deficits using the nitrate-salinity relationship was supposedly underestimated and was shown to be restricted to Persian Gulf Waters. In the northeastern Arabian Sea, nitrate minimum and nitrite maximum were observed between 700-1200 m which was associated with high particulate organic matter resulting from seasonal changes in primary productivity (Naqvi, 1987). The central and Eastern Arabian Sea was studied extensively under the JGOFS programme by De Sousa *et al*., 1996, who suggested seasonal variations in concentration of oxygen and nutrients in the water column. Kumar *et al*., 2001 related the physical forcing mediated through nutrient availability with the biological productivity in this region. The coastal and the open ocean waters remain highly productive during monsoons owing to upwelling and convective mixing while the intermonsoon has been marked by warm and stratified waters with low productivity (Madhupratap *et al*., 1996; Banse, 1987). The coastal upwelling systems around the world are generally known to be more productive and contribute extensively to the export flux of primary production to the ocean interior (Wiggert *et al*., 2005). Owing to the complete reversal of subsurface coastal circulation, seasonal changes in the denitrification regime occur in the Arabian Sea suggesting higher deficits in inorganic combined N during northeast monsoon and dynamic renewal processes during the southwest monsoon (Naqvi *et al*., 1990). Naqvi *et al*., 2000 have reported >1µM of nitrite in the Indian coastal waters when denitrification is at its peak. In a recent work by Gomes *et al*., 2017, nitrite concentrations higher than 0.2 µM have also been reported during the summer monsoon in the mid-depth and near-bottom waters of eastern Arabian Sea.

The coastal OMZ seem to have intensified in comparison to the open ocean OMZs over the past few decades. Seasonal hypoxia, following the southwest monsoon, is

a common feature along the west coast of India (Naqvi *et al*., 2006). Enhanced nutrient load from land create eutrophied waters which often generate higher demands of oxygen by bacteria for decomposition. High rates of DO consumption lead to hypoxic or anoxic conditions in the water column. The oxygen here is <0.5 mL L^{-1}, and the upwelling intensity increases from north to south (Naqvi *et al*., 2000). This annual occurrence of hypoxia results in increased denitrification in the region. Concurrent occurrence of nitrification and denitrification in these coastal waters lead to a buildup of nitrous oxide levels of ~500 nM, the highest ever recorded in seawater in the world oceans (Naqvi *et al*., 2000). Stramma *et al*., 2008 and Diaz and Rosenberg, 2008 have observed similar extension of hypoxic waters in coastal regions and at intermediate depths in the North Pacific and tropical oceans which has expanded and shoaled significantly. In the open ocean suboxic zone, an accumulation of nitrite is accompanied by the depletion of nitrous oxide whereas coastal suboxic zone high nitrite and very high nitrous oxide concentration frequently co-occur indicating net consumption and net production of nitrous oxide by denitrifiers (Naqvi *et al*., 2006).

Recent studies based on observations suggest that the volume of OMZs' suboxic waters has increased over the past decades (Stramma *et al*., 2008) and could expand further in response to ocean warming and increased stratification associated with climate change (Sarmiento *et al*., 1998; Keeling *et al*., 2009). Additions of fixed nitrogen via nitrogen fixation are smaller compared to its loss via pelagic denitrification. However, the major portion of this denitrification occurs in the water column compared to sedimentary denitrification (Bange *et al*., 2005). An imbalance is thus created in the oceanic inventory of nitrogen (Codispoti, 2007). Low-oxygen pelagic environments are responsible for ~35% of the marine-fixed nitrogen loss through microbially catalysed reductive processes (Codispoti, 2007). While low oxygen has been thought to be beneficial for N_2 fixation because oxygen inhibits nitrogenase (the enzyme complex that mediates nitrogen fixation), high nitrate concentrations, such as those present in oxygen deficit zones, are thought to be inhibitory to N_2 fixation in these systems (Postgate, 1998). Nitrogen is introduced into the biosphere by biological and chemical fixation of dinitrogen (N_2) and removed from there again by denitrification (Zumft, 1997). Denitrification is the major

loss term for fixed-N in the global N cycle and is therefore crucial for controlling the oceanic inventory of nitrogen (Codispoti *et al*., 2001).

Recent concerns related to denitrification began to foster research in this area. Eutrophication due to excess nitrate from land runoffs poses dangers to coastal waters forming blooms of phytoplankton some of which are even harmful. Secondly, nitrous oxide (N_2O) is next to carbon dioxide (CO_2) and methane (CH_4) in its importance as a potent greenhouse gas. N_2O and nitric oxide (NO) together are of much concern in terms of ozone chemistry of the atmosphere (Crutzen, 1981; Dickinson and Cicerone, 1986). The production of N_2O by nitrifiers is oxygen sensitive, and its emissions increase very substantially under hypoxic conditions (Wyman *et al*., 2013). The Arabian Sea is considered a 'hot spot' for N_2O emissions to the atmosphere. This is particularly important because N_2O is an atmospheric trace gas, which directly and indirectly influences the earth's climate. Overall, the nitrogen cycle of the Arabian Sea will probably respond sensitively to climate change, which might have an impact on climate via its N_2O and denitrification components.

Denitrification through nitrate and nitrite reducing bacteria and archaea balances the nitrogen fixation by nitrifiers aiding back transformation of fixed nitrogen to the elementary state on a global scale, thus contributing to biosphere maintenance (Zumft, 2005). Genetic and biochemical investigation of nitrification and denitrification have ameliorated our understanding of these processes which were initially thought to be limited to very specific habitats and microbes, but are more widely distributed (Zehr, 2002). Resplandy *et al*., in 2012 examined the factors controlling the oxygen balance in the Arabian Sea using eddy-resolving biophysical model and noted that the biological consumption of oxygen is most intense below the region of highest productivity. From a biological point of view, denitrification is an important process in the deoxygenated waters (Jayakumar *et al*., 2004; Stevens and Ulloa, 2008). Denitrification by bacteria was discovered in the second half of the nineteenth century by Gayon and Dupetit in the year 1886. Elmerich and Newton 2007 as well as Thamdrup *et al*., 2006 postulated that oceanic zones with DO concentration below detection limit support heterotrophic nitrate reducers as well as ammonium-oxidising bacteria that reduce nitrate via denitrification and anammox. Studies carried out by Jayakumar *et al*., in 2009 also stated that

denitrifying assemblages differ spatially as well as temporally and exhibit prominent changes in diversity associated with the development of denitrification from initial anoxia through nitrate depletion. Initial denitrifying assemblages is highly diverse, but succession leads to a less diverse assemblage and dominance by one or a few phylotypes. Also, heterotrophic denitrification, involving respiration of organic matter was the only known nitrogen loss pathway in nature for decades. The recent discovery of anammox, however, sheds more light on this complicated pathway. Lam and Kuypers, 2011 and Kuypers *et al*., 2005 have suggested anammox to be predominant pathway for N_2 formation in both marine sediments and water columns. Majority of the past and present estimates of the oceanic nitrogen budgets were estimated from the stoichiometric or stable isotope effects about denitrification only (Deutsch *et al*., 2001, 2004, Ganeshram *et al*., 2000; Brandes and Devol, 2002). Naqvi *et al*., 1982 reported denitrification rates of 3.2×10^{12} g y^{-1}. Denitrification rate measurements using modelled recycling mechanism was also demonstrated by Anderson *et al*., 1982 and were based on distributions of nitrite and nitrate deficits (nitrate consumed during denitrification) in OMZ.

Regardless of the numerous studies carried out on microbial processes, especially N_2 cycling in the OMZ's during the latter half of the twentieth century (e.g., Wooster *et al*., 1965; Fiadeiro and Strickland, 1968; Cline and Richards 1972; Codispoti and Christensen, 1985; Naqvi 1987; Ward and Zafiriou, 1988; Lipschultz *et al*., 1990), research interest in this topic is still at its peak because of the discovery of new key microbial players including archaeal nitrifiers that appear to be abundant in mesopelagic oceans (Francis *et al*. 2005, Konneke *et al*. 2005). The distribution of denitrification among prokaryotes does not follow a distinct pattern. It is carried out by diverse assemblages of bacteria belonging to Proteobacteria, archaea (including halophilic and hyperthermophilic branches of the kingdom and may have evolutionary significance). Kobayashi, 1996 also found the existence of denitrification enzymes in the mitochondria of certain fungi. An active role of fungi as denitrifiers in the oxygen-deficient waters of the Arabian Sea was also confirmed by Manohar *et al*., in 2014. Studies involving the diversity of micro-organisms involved in specific N cycle transformations are being carried out for quite some time now (Stevens and Ulloa, 2008; Jayakumar *et al*., 2013; Bandekar *et al*., 2018). However, knowledge about the diversity of the key players is an

important step to assay gene expression and the activity of these enzymes. Traditional culture-based studies provide a suitable means in establishing a link between genetic and phenotypic variation. Bergaust *et al*, 2010; Liu *et al* 2010, carried out studies using robotic gas sampling apparatus, which allowed for monitoring of O_2, CO_2, and gaseous denitrification products which demonstrated that different denitrifying species regulate the expression of denitrification genes differentially in response to O_2, NO_2, NO_3, and NO concentrations as well as other parameters such as pH and temperature. Zumft 1997 provided the most comprehensive review about the organisms involved in denitrification and their structural properties. A bacterium is either denitrifying or ammonifying. Apparently, there is no option within the cell to proceed either way. The ammonifying pathway is mostly not electrogenic, detoxifies nitrite and serves as an electron sink. Joano Falcao *et al*., 2012 also related phylogenetic and functional diversity among denitrifiers.

The behaviour and physiology of cultivated isolates cannot be generalised and can be misleading since it appears that many micro-organisms in situ have yet to be obtained in culture (Zehr and Ward, 2002). Specific enzymes are known to catalyse many of the energy metabolism reactions involved in the nitrogen cycle (Cabello, 2009). These enzymes and genes provide useful tools for studying microbial processes. The genetic basis of denitrification has been explored through some reviews (Hochstein and Tomlinson, 1988; Knowles, 1996; Zumft, 1997). Knowledge of genes and molecular biology has increased our understanding about the ecological role of the different organisms involved such as a widespread capability of nitrate assimilation among heterotrophic bacteria and an absence of the same amongst some of the most abundant photosynthetic picoplankton, as previously thought. Gammaproteobacteria are found to harbour the largest number of denitrifying genes viz. *Pseudomonas stutzeri*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* with 33, 32 and 20 genes sequenced, respectively (Zumft 1997; Arai *et al*. 1995). *Pseudomonas aeruginosa* was the first bacteria whose denitrification genes were accessed by conjugational and transductional mapping (Jeter *et al*., 1984; Hartingsveldt and Stouthamer, 1973).

Previous studies on OMZ have concentrated primarily on the diversity of microorganisms in these waters/sediments. Denitrifying bacteria belong to diverse phylogenetic groups and studies have been directed mainly towards amplification of

functional genes involved in denitrification from environmental samples (Braker *et al*., 1998; Henry *et al*., 2006; Casciotti and Ward, 2005 etc.). The occurrence of denitrification among denitrifying as well as anammox bacteria is well established (Codispoti *et al*., 2001; Lam *et al*. 2009). Recent studies have also reported microbial sulphate reduction and sulphur oxidation in OMZ (Canfield *et al*. 2010). Majority of the studies in the Arabian Sea OMZ, however, were based on metagenomic approach (Bandekar *et al*., 2018). Marker genes involved in denitrification such as *nirS* and *nirK* as well as the *16S rRNA* gene have been investigated to analyse the composition of anammox and denitrifiers in the OMZ (Jayakumar, *et al*., 2009). Research on microbial diversity conducted on OMZ of the AS is based primarily on culture-independent methods (Castro-Gonzalez *et al*., 2005; Stewart *et al*., 2012, Jayakumar *et al*., 2004, 2009; Ward *et al*., 2009; Kuypers *et al*., 2005). Molecular surveys reveal that denitrifying ability exists between organisms belonging to multiple taxonomic groups (Braker *et al*., 2000; Jayakumar *et al*., 2004; Scala and Kerkhof, 1998). Many micro-organisms inhabiting the OMZ's are capable of numerous functions in the elemental cycles. However, their versatile metabolic potentials compared with actual activities are challenging with respect to ecophysiological and biogeochemical measurements (Lam and Kuypers, 2011). Ecological studies on denitrifiers started with their cultivation from diverse environments. Gamble *et al*., 1977 carried out comprehensive research exploring soil denitrifier communities wherein they isolated over 1500 bacteria, and 146 of those were capable of complete denitrification or reduction to gaseous nitrogen. Aerobic denitrifying species have also been isolated from terrestrial and aquatic ecosystems that are known to simultaneously utilise oxygen and NO_3^- as electron acceptors. These reports conclusively demonstrate that denitrification is not necessarily exclusively anaerobic. Molecular studies on the ecology of denitrifiers based on the functional genes which are exploited for developing probes or primers. All of these studies conducted to analyse the diversity of denitrifying community rely largely on an isolation procedure involving screening of denitrifying organisms based on the evaluation of the nitrate to gaseous nitrogen reducing ability (Philippot, 2002). Earlier investigations of total microbial abundance reported microbial cell number maxima coincident with the secondary nitrite maxima in the OMZ (Jayakumar *et al*., 2009). The nearly complete dissolved oxygen

depletion coupled with the high concentration of nitrate provides an ideal niche for microbes that can respire nitrate, but function in addition to the distribution of denitrifying bacterioplankton is essential to determine the mechanisms responsible for the nitrogen deficits in the marine nitrogen cycle.

A few attempts have been made to study bacterial abundance and picoplankton in the Arabian Sea. (Ducklow and Harris, 1993; Chepurnova, 1984; Ramaiah *et al*., 1996). However, these studies have been confined to the open waters of the Arabian Sea. Bacterial dynamics and community composition in the Eastern Arabian Sea remains poorly understood. Knowledge of the diversity, distribution, and relative abundance of the nitrifying and denitrifying bacterioplankton will help to determine the mechanisms responsible for the observed nitrogen discrepancy and thus contribute to our understanding of marine nitrogen cycle. Axenic microbial cultures from the study area help to describe not only the diversity but also to understand their physiological characteristics and metabolic activities in biogeochemical cycles. Culture-based studies also allow the description of new species. The seasonal anoxic system in the coastal regions of India have been previously investigated for bacterial indications of nitrification and denitrification using a culturable approach, but the studies have been mainly confined to sediments (Krishnan *et al*., 2008; Divya *et al*., 2010). Due to this, there is a scarcity of studies on the diversity of culturable bacteria from the water column of the Arabian Sea.

1.3. Significance of work

The ocean accounts for at least one-third of all natural N_2O emissions, a large fraction of which is derived from OMZs via microbial respiration of nitrate (NO_3) and nitrite (NO_2) (Naqvi, 2010, Wright, 2012). Since nitrogen cycling is closely intertwined with that of carbon and phosphorous, any small change in the nitrogen cycle is likely to have significant alterations for other biogeochemical processes. These perturbations are becoming more and more apparent with climate change. Increased stratification and reduced ventilation of the thermocline can decrease the oxygen content of the ocean's interior (Matear and Hirst, 2003; Shepherd *et al*., 2017) leading to expansion of OMZ as currently observed which would further result in enhanced denitrification and N_2O production (Gruber *et al*., 2008). The persistent infusion of nitrogenous fertiliser runoff has placed significant additional pressure on natural nitrogen removal mechanisms.

Although improved by physical processes such as tidal flushing, denitrification is one of only two mechanisms that mediate removal of excess environmental nitrogen. Human activities exacerbate the natural O_2 deficiency in shallow coastal and estuarine environments, where nutrient run-off from agricultural and wastewater sources results in eutrophication.

Moreover, changes in wind-driven circulation patterns can induce upwelling of oxygen deficient waters from coastal OMZs onto continental shelves, increasing mortality of shelf-dwelling organisms. Shelf intrusion has produced dead-zones in some coastal regions of the world including the Gulf of Mexico, off the coast of Chile, Africa, and India, resulting in decreased production from commercial fisheries. Regardless of the water body (estuary, basin, coastal waters or open ocean), oxygen deficiency shifts energy away from pelagic macrofauna towards microorganisms, decoupling predator-prey interactions and changing the trophic exchanges that occur through existing food webs. Owing to the vast potential of OMZ regions regarding nutrient cycling, fisheries production or discovery of novel organisms, various studies have been carried out both in pelagic and benthic realms of this region. OMZ of the Arabian Sea has been illustrated geochemically, but studies on the microbial ecology have been sporadic, and microbes that are responsible for the observed characteristic chemical distributions are still unknown (Jayakumar *et al*., 2009). Microbial diversity studies thus come handy while understanding the functions and ecological role of these organisms in biogeochemical processes.

1.4. Research aims and objectives

i. Estimation of the abundance and distribution of bacterioplankton.
ii. Identification of denitrifying bacteria using a molecular approach.
iii. Investigating the denitrifying potential of selected species of marine bacteria.
iv. Quantification of some functional genes (*nir*, *nos*, *nor* genes) involved in denitrification.

Chapter 2

Bacterioplankton in the Arabian Sea

2.1. Picophytoplankton and heterotrophic bacteria

Bacterioplankton, defined as the bacterial component of the plankton drifting in the water column. There are two main groups of bacterioplankton in water; (a) autotrophic picophytoplankton which includes photosynthetic prokaryotes and eukaryotes derives energy from photosynthesis or chemosynthesis and (b) heterotrophic bacteria which obtain energy by consuming organic matter produced by other micro-organisms. The autotrophic picoplankton community includes different species from genera *Synechococcus* and *Prochlorococcus* and picoeukaryotes, the small eukaryotic cells from diverse taxa. These picoplankton range from 0.2 to 3 µm in size and their significance arises from its ubiquity and abundance in aquatic environments. This smallest group of phytoplankton, mainly cyanobacteria, forms a major component in marine (and freshwater) including nutrient-rich to poor ecosystems (Shiomoto *et al*., 1997) carrying out about 25% of the total carbon fixation in the ocean. Thus, these size classes strongly impact the primary production as well as carbon cycle in the marine ecosystem (Worden *et al*., 2004; Richardson & Jackson, 2007) acting as primary producers as well as primary consumers and hence play critical roles in global biogeochemical cycles (Azam *et al*., 1983).

Among the picophytoplankton, *Synechococcus* was the first group to be studied in detail. These cells ranging from 0.8 to 1.5 µm in size are a rod or coccoid shaped organisms which reproduce by binary fission (Waterbury *et al*., 1979). These cyanobacteria predominantly contain phycobilisome and are found in marine as well as freshwater ecosystems, generally being more abundant in nutrient-rich than oligotrophic regions. Two major types of *Synechococcus* are known based on the phycobilisome composition one containing phycoerythrin (PE; *SYN*-PE) and the other phycocyanin (PC; *SYN*-PC) as a major accessory pigment. The former group is present in all kinds of aquatic systems whereas the latter is present only in freshwater and estuarine environments. Based on PE fluorescence intensity, different clades of *SYN*-PE have been observed (Partensky *et al*., 1999). *SYN*-PE group abundance is generally high in clear waters where the blue light of short wavelength can penetrate deeper in the water column, whereas, *SYN*-PC is higher in turbid waters (Stomp *et al*., 2007). Variation of light quality is one of the factors for altering the picophytoplankton composition in marine

waters and reflects the importance of the blue-green light on the PP accessory pigments (Scanlan, 2003).

Prochlorococcus is the smallest known photosynthetic organism having a size of 0.5-0.7 µm diameter. It was first discovered by Chisolm *et al*. (1988) using the advanced technique of flow cytometry as the cells were not visible through traditional microscopy. Although being smaller than coccoid cyanobacteria, these cells are numerically dominant primary producers throughout much of the world's oceans, their average concentration being greater than 10^5 cells ml^{-1} in the deep euphotic zone. It is widely found within the 40°S to 40°N latitudinal band of oceans and occurs at high density from the surface down to depths of 200 m. The population size declines beyond these latitudinal limits, and *Prochlorococcus* is thought to be absent at temperatures below 15°C (Johnson *et al*., 2006). This makes it presumably the most abundant photosynthetic organism on Earth. *Prochlorococcus* possesses a unique pigment composition, which includes divinyl derivatives of chlorophyll *a* and chlorophyll *b* that are unique to this genus (Partensky *et al*., 1999). They are well adapted to living in oligotrophic oceanic regions and contain two ecotypes which are genetically and physiologically distinct, adapted to either low- or high-light niches (Bibby *et al*., 2003). Their diversity has been linked to environmental factors, such as light, temperature and iron availability (Johnson *et al*., 2006; Rusch *et al*., 2010). Although *Prochlorococcus* is most abundant in oligotrophic waters relative to the other photosynthetic populations, it is by no means restricted to nutrient-depleted waters. Their abundance has been well established in coastal waters across basins (Campbell *et al*., 1998; Rebeiro *et al*., 2016).

Picoeukaryotes are the eukaryotic component of the picoplankton. They are a taxonomically diverse group that includes representatives from all of the major algal groups (e.g., green algae, haptophytes, stramenopiles, and dinoflagellates). Although not present as individual cultures, their existence as extraordinary contributors to global biogeochemical cycles has been demonstrated using DNA sequencing and PCR techniques. The nuclear genome of *Ostreococcus,* an autotrophic picoeukaryote belonging to the class Prasinophyceae, has been recently sequenced and is shown to be one of the smallest known for a free-living eukaryote. For instance, the abundance and distribution of picoeukaryotic algae in world oceans are quite uniform exist with

concentrations ranging between 10^2 and 10^4 cells mL^{-1} in the photic zone (Fogg, 1995). Marine picoeukaryotes belong to different phylogenetic groups, and three novel algal classes have been recently described for picoeukaryotic isolates (Guillou, 2004). Studies by Caron *et al*., 2012; Simon *et al*., 1994 have demonstrated that open ocean picoeukaryotes are usually coccoid or flagellated forms with chloroplasts (phototrophic) or without chloroplasts (heterotrophic). Picoeukaryotes are usually considered to be a cosmopolitan group capable of mixotrophy. Their characterisation to decipher ecological roles is thus complicated (Zubkov and Tarran, 2008; Hartmann *et al*., 2013). Picoeukaryotes, however, can contribute significantly to biomass and productivity in a wide variety of aquatic environments, even at much lower abundances than cyanobacteria. This is due to their bigger size and higher intracellular chlorophyll *a* and carbon content than found in cyanobacteria. Picoeukaryotes are preyed upon by grazers, thus forming a link to higher trophic levels, which has a variety of implications for the fate of their fixed carbon (Partensky *et al*., 1996).

The heterotrophic bacteria contribute a large portion of total plankton biomass, acting as important producers of new biomass along with their proven role of decomposers of organic matter. In comparison with data from other open-ocean observations (Ducklow *et al*., 1992; Kirchman *et al*., 1995), the heterotrophic bacterial stocks observed in the Arabian Sea are quite substantial. Heterotrophic bacteria are present at the base of the microbial food web and are involved in transformation and respiration of autotrophically synthesised organic compounds. In the marine environment, the aggregation and sinking flux of organic matter following phytoplankton bloom, transport the materials out of the euphotic zone (Takahashi, 1986). An association of heterotrophic bacteria with the sinking organic matter forms the bulk of carbon biomass in the pelagic ecosystems that support life below the euphotic zone. Besides this, heterotrophic bacteria are essential components of important processes such as utilisation of labile dissolved organic matter, the operation of microbial loop and cycling of bio-essential elements. Often, these are the key players governing important biogeochemical processes in the oceans. High levels of primary production facilitate their growth and abundance (Sai Elangovan, *et al*., 2019). The biomass of heterotrophic bacteria becomes available at higher trophic levels through grazing, by flagellates and ciliates. Hence,

heterotrophic bacteria, as a part of the bacterioplankton community, undoubtedly have an important role in carbon flow through the marine system.

Biological processes occurring in the Arabian Sea are highly variable due to strong seasonal changes that exist within a year. Resultant seasonal variations in nutrient supply are reported to have a profound effect on the bacterioplankton activity. The ubiquitous and self-regulating bacterioplankton communities provide the foundation to our understanding of the processes in marine food-web (Landry *et al*., 2001). The present study was carried out to describe the spatial and temporal distribution of bacterioplankton in relation to the physicochemical processes in cross-shelf transects of the northeastern and central Arabian Sea. This will help us assess their community structure and their involvement in carbon stocks of different water masses.

2.2. Methodology

2.2.1. *Study area: Coastal and central Arabian Sea*

The study area covers the coastal and open ocean region of northeastern and central Arabian Sea, located in the north Indian Ocean region. The abundance and community structure of bacterioplankton were studied from seawater samples collected during four cruises; SSK 56, SSK 69, SSK 79 and SSD 26 onboard research vessels Sindhu Sankalp (SSK) and Sindhu Sadhana (SSD). Sampling was carried out under the SIBER (Sustained Indian Ocean Biogeochemistry and Ecosystem Research) program during late southwest monsoon season (October 2013, October 2014 and September 2016) and during late northeast monsoon season (February 2015). A total of 9 stations in the coastal and seven stations in open ocean region of the Arabian Sea were covered within two transects. The coastal transect was covered during two cruises; SSK 56 and SSK 69 along with a few stations in the offshore region while cruise SSK 79 and SSD 26 covered an open ocean transect in the central Arabian Sea. The coastal stations G4, G5, G6, G10, G11, G12, G13, G14, and G15 comprised of a transect perpendicular to the Goa coast (15°N, 73°E) located on the west coast of India. The offshore transect included seven stations along 68°E (II14, 21°N; II12, 19°N; ASTS, 17°N; II8. 15°N; II6, 13°N; II4, 11°N; II2, 9°N). Details of the cruises and the stations sampled during each cruise are given in Table 2.1 and Figure 2.1.

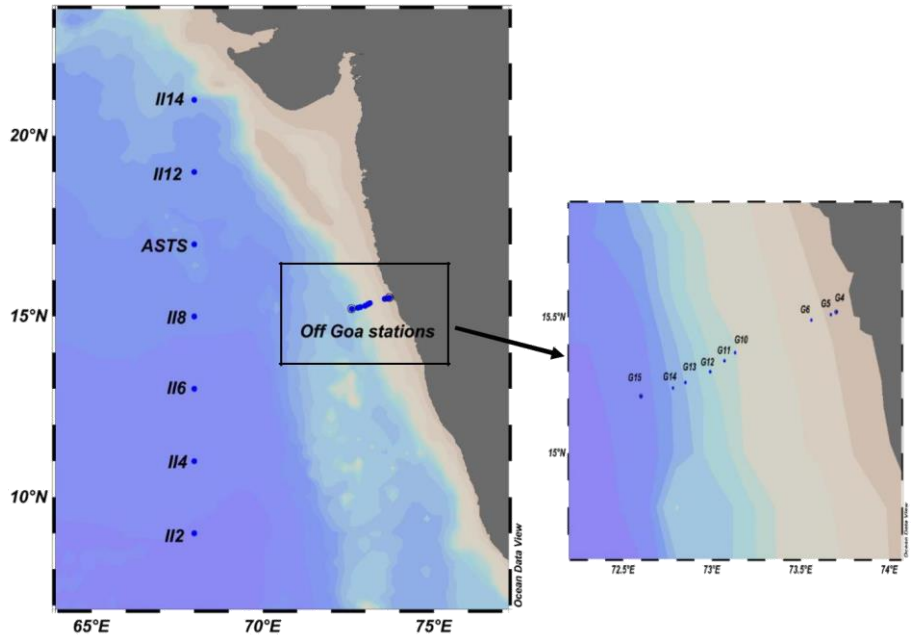

Figure 2.1. Sampling locations in the Arabian Sea. Stations II2, II4, II6, II8, ASTS, II12 and II14 are the open ocean stations along a longitudinal transect at 68°E. Coastal stations along a transect perpendicular to the Goa coast in the eastern Arabian Sea are denoted as G4, G5, G6, G10, G11, G12, G13, G14, G15

Table 2.1. Details of the cruises and stations sampled in the Arabian Sea during the study period

Cruise	**Sampling (Month/Year)**	**Stations sampled (Maximum depth of station in meters)**
SSK 056	October (2013)	G4 (20), G5 (30), G6 (40), G10 (100), G12 (400), G14 (1160), II2 (4500), II4 (4300), II8 (4000), ASTS (3800)
SSK 069	October (2014)	G4 (20), G5 (30), G6 (40), G11 (200), G13 (600), G15 (1200), II2 (4500), II4 (4300), ASTS (3800), II12 (3300), II14 (3000)
SSK 079	February (2015)	II2 (4500), II4 (4300), II6 (4000), II8 (4000), ASTS (3800), II12 (3300), II14 (3000)
SSD 026	September (2016)	II2 (4500), II4 (4300), II6 (4000), II8 (4000), ASTS (3800), II12 (3300), II14 (3000)

2.2.2. *Hydrography*

The physicochemical properties of the water column were measured using sensors fitted onto a CTD (Sea Bird Electronics, Inc., USA). The portable CTD was attached to a rosette containing 24 bottles of Niskin water samplers having a capacity of 10 L each. Temperature, salinity, Fluorescence, and depth profiles were used to select the sampling depths within the water column during each cruise.

Dissolved Oxygen (DO) - The concentration of DO in the water column was determined using oxygen sensors fitted onto the CTD. Dissolved oxygen was also determined onboard from water samples collected in glass bottles. Bottles were filled with seawater from Niskin samplers, avoiding any contamination of atmospheric oxygen. One ml each of manganese chloride and sodium iodide-sodium hydroxide reagents was added to fix the sample. Winkler titration based on standard idiometry was carried out using a digital Dosimat, following the protocol given by Carpenter (1965).

Nutrients - The nutrients (nitrate and nitrite) were measured from frozen samples carried to the on-shore laboratory using segmented flow Autoanalyser (Skalar Analytical, The Netherlands) following standard methods (Grasshoff *et al*., 1983).

2.2.3. *Microscopic determination of total bacterial counts (TBC)*

Bacterial abundance was determined microscopically, following JGOFS Protocols (Knap *et al*., 1996). 100 mL seawater samples from each depth were preserved with particle-free (0.2 µm filtered) 25% glutaraldehyde (2% final conc.) and stored in a refrigerator until slide preparation. From this, 2 ml aliquots were incubated with DAPI (4′, 6-diamidino-2-phenylindole; 20 µL of 1 mg mL^{-1}) for 10 min and subsequently filtered onto 0.22 µm black polycarbonate membrane filters (Whatman) in an all-glass filtration assembly. Backing filters of 0.45 µm (mixed cellulose ester membrane, Whatman) were used under the polycarbonate filters to ensure even distribution of cells. The filters were mounted on microscope slides using non-fluorescent immersion oil and stored at -20°C in slide boxes. From each sample slide, 20 random microscopic fields were counted using an epifluorescence microscope (Olympus, BX51) and total bacterial abundance was calculated.

2.2.4. *Flow cytometric analysis of picoplankton*

Seawater (1.5 mL) was preserved with glutaraldehyde (0.2% final concentration) in cryovials, frozen in liquid nitrogen. The samples were kept at -80°C until lab analysis. Samples were thawed before analysis and analysed in a BD FACS Calibur flow cytometer equipped with blue and red lasers following Ahmed, *et al*., 2016. It facilitated the quantification of smaller autotrophic plankton groups including cyanobacteria and picoeukaryotes based on the forward angle light scatter (FALS; proxy for cell size), Right angle light scatter (RALS-proxy for cell granularity) and cell fluorescence from photosynthetic pigments such as chlorophyll a, >650 nm, phycocyanin-630 nm and phycoerythrin-564/606 nm. Beads of 1 µm (Fluoresbrite Polysciences) were added for internal calibration. An initial analysis (of 3 min at a rate of 70 µL min^{-1}) was carried out to enumerate picoplankton. The acquisition was set on FL3-H channel (chlorophyll fluorescence) using a threshold of 200, for excluding any heterotrophic cell. Subsequently, the second analysis included an enumeration of heterotrophic prokaryotes. A DNA staining dye SYBR Green (Molecular probes™, Invitrogen, U.S.A) was added to the samples (final concentration of 1/10,000). The samples were then incubated in the dark for 15 minutes at room temperature. Flow cytometry acquisition was set on the FL1 channel (threshold value-500), and analysis was carried out for 2 minutes (flow rate-60 µL min^{-1}). Data were analysed with the BD CellQuest™ Pro software. For autotrophic picoplankton analysis, chlorophyll fluorescence, phycoerythrin fluorescence, forward scatter and side scatter were employed to distinguish between three major groups, i.e., *Synechococcus, Prochlorococcus* and picoeukaryotes. For SYBR Green-stained samples, only heterotrophic bacteria were included in the analysis (Olson, *et al*., 1990, Zubkov, *et al*., 1998).

2.2.5. *Carbon conversions*

Cell-to-carbon conversion factors of 20 fg C $cell^{-1}$ for heterotrophic bacteria (Lee and Fuhrman, 1987), 53 fg C $cell^{-1}$ for *Prochlorococcus*, 175 fg C $cell^{-1}$ for *Synechococcus*, and 2,100 fg C $cell^{-1}$ for picoeukaryotes (Campbell *et al*., 1998) were applied to flow cytometry abundance data for estimating the total carbon contribution of each group.

2.3. Results and discussions

2.3.1. *Hydrography of the Arabian Sea during late summer and winter monsoon*

SSK 56 (October 2013)

The hydrographic features of the water column (temperature, salinity, dissolved oxygen concentration) during late summer (October) cruise SSK 56 are given in Figure 2.2. The stations are divided into two sections, i.e. coastal section (including continental shelf region) and the offshore section. The sea surface temperature (SST) varied from 25.7 to 29.6°C in the coastal section and between 28.9 and 29.1°C in the offshore section showing a gradual increase in surface water temperature from the coast towards the open ocean. Similarly, the sea surface salinity (SSS) in the coastal region ranged from 33.8-35.3 PSU and increased to 36.4 PSU in the offshore region. In general, the horizontal gradient between coastal and offshore waters at the surface was about 1.5 units for both temperature and salinity.

The vertical distribution of temperature indicated a weakly stratified region in the nearshore stations (G4, G5, G6) of the coastal section. However, the thermocline deepened as the distance from the shore increased towards the open ocean and water with higher temperatures (>26°C) were observed at these stations (Station G10, G12, G14). The offshore waters depicted a well-stratified water column across latitudes (17°N; ASTS to 9°N; II2). Higher temperatures (>27.5°C) were recorded in the upper 50m of the entire section and gradually decreased with depth. The offshore section showed a deeper mixed layer depth (MLD) starting at about 60m as compared to the coastal section (MLD; approximately 30 m). The salinity profiles of the coastal and offshore section depicted lower values (33.8 - 35.7) in the coastal section than in the offshore section (>36 PSU).
The nearshore stations in the coastal section had water with high salinity (>35 PSU) below 20m, whereas stations G10, G12 and G14 of the coastal section had water with lower salinities (34-35 PSU). In the offshore transect, salinity values varied narrowly between stations with high saline waters (>36 PSU) up to 80m. Depth-wise stratification at stations ASTS (17°N) and II8 (15°N) with respect to salinity was quite evident with low salinity waters at intermediate depth; 0-80 m (salinity >36.5 PSU), 80-130 m (35.6-36.2 PSU), below 130 m (<35.5 PSU). The dissolved oxygen concentrations at the

surface were lower (2.7-4.3 mL L^{-1}) in the coastal section than the offshore section (4.3-4.5 mL L^{-1}). The offshore waters remain well oxygenated (>3.8 mL L^{-1}) upto a depth of ~60 m, below which there is a decrease in oxygen concentration with depth, whereas, the coastal section had shallower oxyclines (~20-40 m deep).

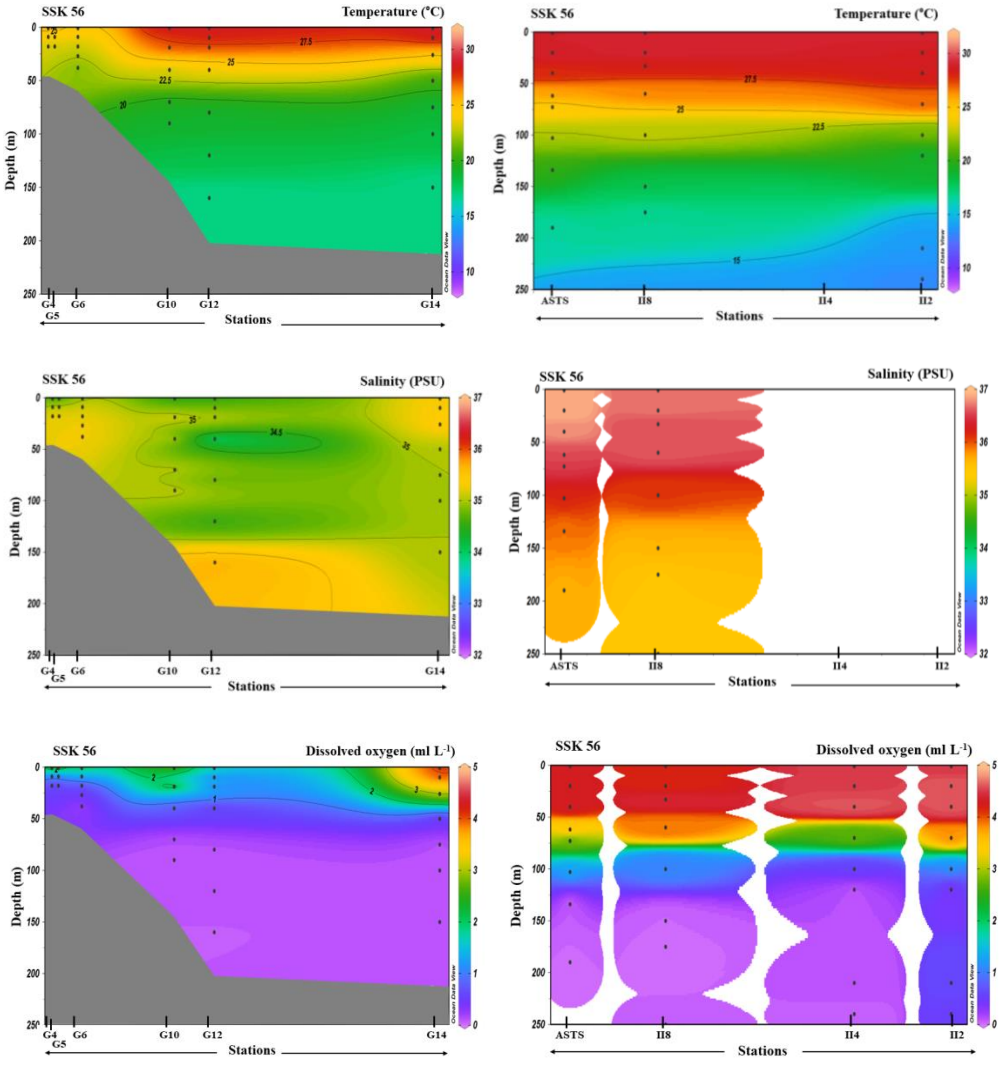

Figure 2.2. Vertical distributions of temperature (T°C), salinity (PSU) and dissolved oxygen concentration (mL L^{-1}) in the coastal and offshore stations during cruise SSK 56 (October 2013) in the Arabian Sea.

SSK 69 (October 2014)

The temperature and DO profiles of the water column (upto 200 m) during SSK 69 cruise are shown in figure 2.3. The profiles for salinity have not been shown due to technical errors in the salinity data from the CTD sensor. Surface temperatures at the coastal section varied between 26.4-28.7°C and between 28.6-29.4°C in the offshore section. The temperature of the water column at the coastal section varied between 13.4-28.7°C with higher temperatures at the G11, G13 and G15 stations than the nearshore stations. The offshore section depicted stratified waters with thermocline being at depths between 40-60 m and reduced temperatures (<20°C) below 100 m depth. The coastal section showed high levels of dissolved oxygen (>4 mL L^{-1}) at the surface and a decrease in oxygen concentration with depth. The nearshore stations depicted the presence of low oxygen waters (DO; 0.05-3 mL L^{-1}). Oxycline deepening was seen with distance away from the coast. All the stations in the offshore section were well oxygenated until depths of approximately 100m, below which zone of low oxygen began with DO concentrations of <2 mL L^{-1}.

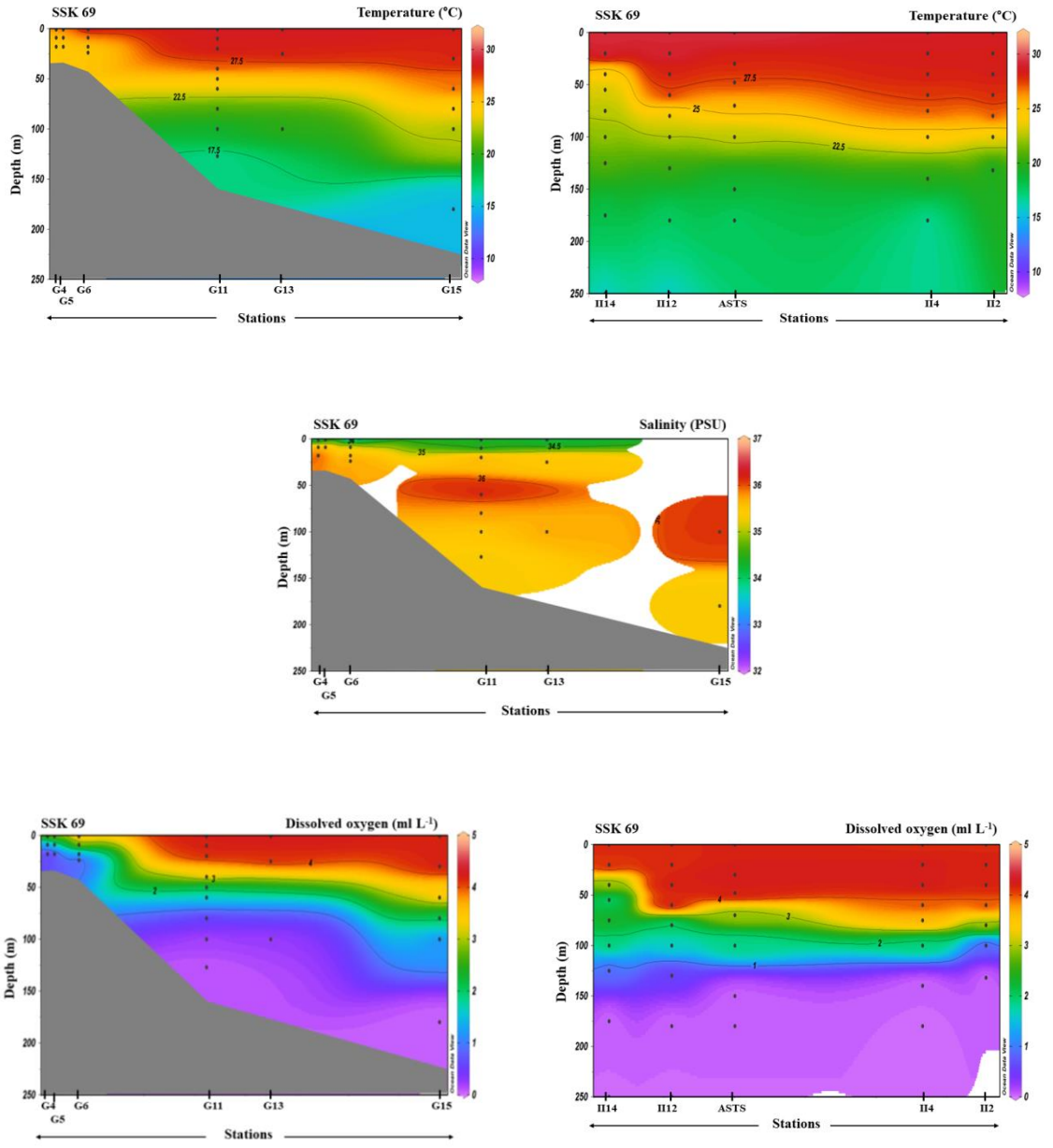

Figure 2.3. Vertical distributions of temperature (T°C) and dissolved oxygen concentration (mL L^{-1}) in the coastal and offshore stations during cruise SSK 69 (October, 2014) in the Arabian Sea.

SSK 79 (February 2015)

The SSK 79 cruise occupied a transect in the open ocean with seven stations located between 21°N and 9°N. For convenience, stations to the north of 15° were assigned northeastern Arabian Sea stations (NEAS) while those below it were called southeastern Arabian Sea stations (SEAS). The temperature, salinity and dissolved oxygen profiles are given in figure 2.4. Water column upto a depth of 1000 m was studied which gave a picture of latitudinal variation in the Arabian Sea. Higher temperature was recorded in stations towards the south (II2; 28°C) as compared to the northern stations (II14, 25°C). The 27°C isotherm showed a distinct downward slope from NEAS to SEAS. This confirms mixing within the upper ~120 m of water column in NEAS (prevalence of convective mixing) and deepening of the thermocline in the SEAS. Station II14 showed a wider mixed layer depth (200 m) as compared to station II2 (120 m). The salinity profile represented the presence of high saline water at station II14 (>36.3 PSU) and lower salinity values close to 35 PSU at stations II8 and II6. The dissolved oxygen profile clearly showed the existence of suboxic/hypoxic zone between 150-1000 m in all the stations, although the starting depth of the oxygen minimum zone varied between stations.

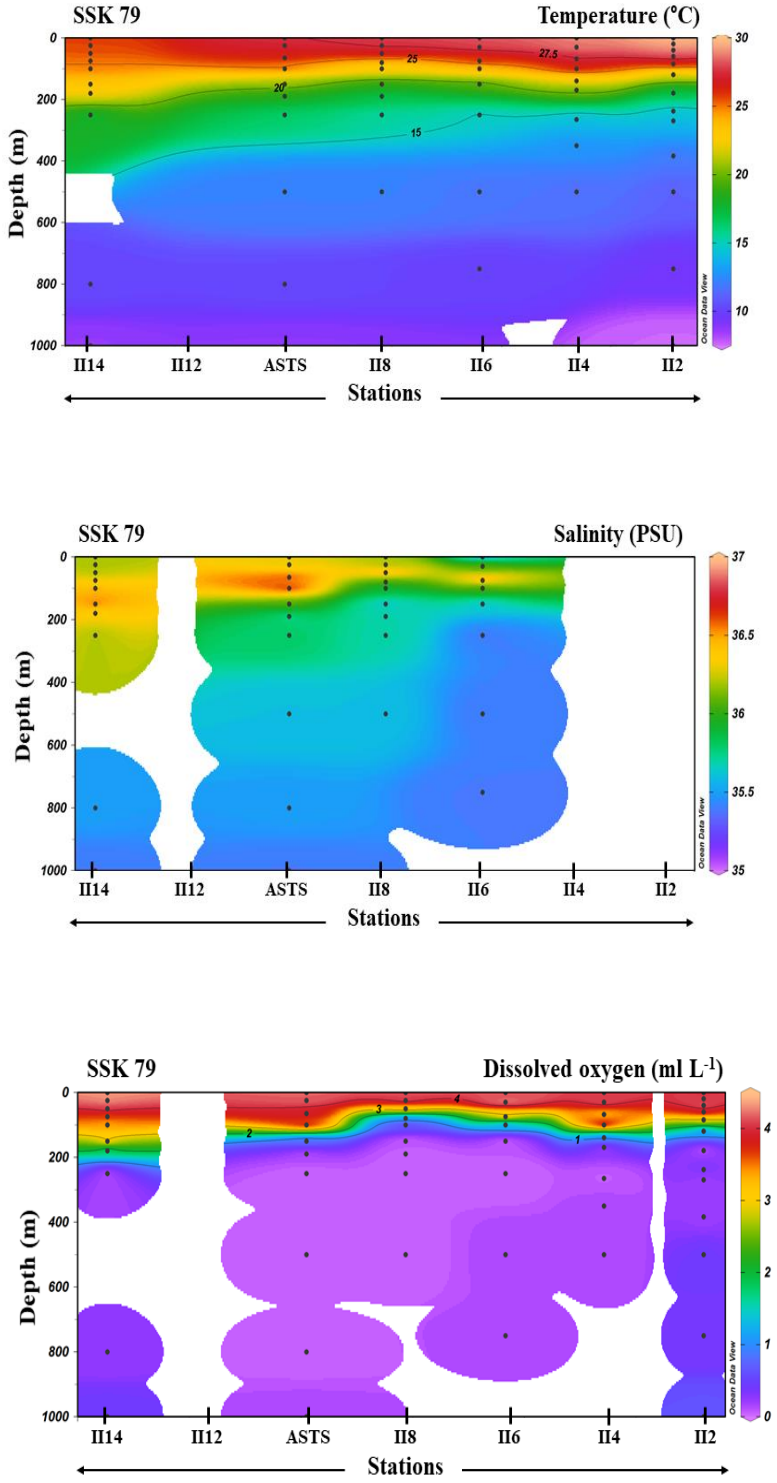

Figure 2.4. Vertical distributions of temperature (T°C), salinity (PSU) and dissolved oxygen concentration (mL L^{-1}) in the offshore stations during cruise SSK 79 (February, 2015) in the Arabian Sea.

SSD 26 (September 2015)

Hydrography of the Arabian Sea during SSD 26 cruise in the Arabian Sea is depicted in figure 2.5, giving latitudinal variations are shown in temperature, salinity, and dissolved oxygen at seven stations in the open ocean region. The surface water temperature was nearly constant across north-south stations. For instance, stations northernmost station, II14 and southernmost station II2 had a surface temperature of 28.5°C, and it was slightly lower at stations II8 and II6 (27.9°C). The surface salinities were however consistently high, ranging between 36.5-36.8 PSU. Vertically, at each station the water column temperature reduced with depth, decreasing gradually upto 50-80 m followed by a rapid fall upto a depth of 100 m. Salinity variation across stations revealed the existence of high saline water (>36 PSU) at 21°N which reduced (~35 PSU) towards 9°N. Vertically, high saline water (>36 PSU) existed from 0-200 m, below which salinity ranged from 35.4-35.8 PSU. In terms of dissolved oxygen, water column at all the stations was well oxygenated till 100 m. Suboxic conditions began to appear below this depth and continued until 1000 m water column.

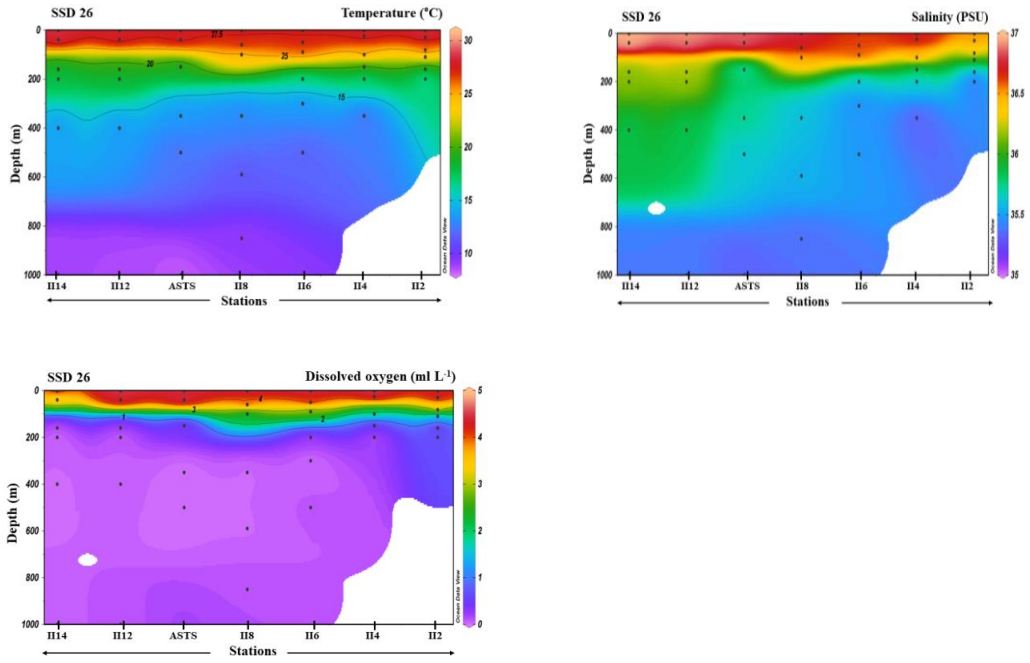

Figure 2.5. Vertical distributions of temperature (T°C), salinity (PSU) and dissolved oxygen concentration (mL L^{-1}) in the offshore stations during cruise SSD 26 (September 2016) in the Arabian Sea.

2.3.2. *Hydrography of the coastal Arabian Sea (summer monsoon)*

The west coast of India experiences upwelling which is driven by the annual reversal of monsoonal winds. The process of upwelling is characterised by the renewal of surface waters with nutrient-rich water from deeper depths. A decline in temperature and DO associated with an increase in salinity and surface nutrients is a signature of upwelling (Rochford, 1975). During the two cruises SSK 56 and SSK 69, hydrographic properties changed mostly in the upper 200 m. Horizontal and vertical sections of temperature, salinity and dissolved oxygen suggested upwelling signature along the coastal transect. Stations off the shelf edge essentially displayed the same vertical structure as found in the central Arabian Sea.

An upsloping of isotherms in the coastal region was observed due to the entry of deep cold waters from below the thermocline brought by the southwest monsoon. The 20°C isotherm generally lies in the middle of the upper thermocline in the north Indian Ocean (Wyrtki, 1971). In our study, it rose from a depth of more than 100 m to a depth of less than 75 m across the section. The mixed layer depth also decreased from 65 m to 20 m from the station farthest (G15, G14) to the one nearest to the coast (G5). Shetye *et al*. (1990), have regarded this upsloping of isotherms along with the equatorward movement of the winds to be affirmative for wind-induced eastern boundary upwelling phenomenon. Below 50 m (SSK 56) the upward lift of isotherms indicated the equatorward undercurrents along the shelf waters of the study area. Likewise, studies by Kumar *et al*., 2008 have shown the upsloping isolines from 40-50 m to the surface layer on the shelf to be a prominent feature during the late summer monsoon, causing a considerable fall in surface temperature indicative of coastal upwelling.

Water mass structure in the Arabian Sea is complex. Low-salinity waters of southern origin are advected into the region by the Somali and North Equatorial Currents, and during the southwest monsoon, the waters that upwell along the Arabian coast have relatively low salinities as a consequence of poleward advection associated with the Somali current (Morrison *et al*., 1998). Advection of high saline subsurface waters from offshore to the coast was observed during both cruises, although the intensity was higher in 2015 than 2013. The surface waters along the coastal side of the 13°N transect was comparatively less saline (34.9 PSU) than the open ocean surface waters (>35.8 PSU).

An intrusion of high saline waters (>36 PSU) from the open ocean was evident in this transect between 25-100 m depth. A well-stratified salinity structure was observed below 50 m, except a patch of high saline (36 PSU) waters between 60-75 m depth indicating the presence of Arabian Sea High Salinity Water (ASHSW) in the transect. Sources of high salinity waters include local evaporation and contributions from the adjoining Red Sea and Persian Gulf evaporation basins. Excessive evaporation over precipitation leads to the formation of higher surface salinities in the Arabian Sea which sink and renew deep waters (Morrison *et al*., 1998). The occurrence of an undercurrent was also suggested by the presence of low salinity water hugging the continental slope with its core at a depth of about 150 m. This water with salinities less than 35 PSU was well below the depth of the upper thermocline and hence below the region of direct surface mixing (Shetye *et al*., 1990)

The offshore waters remain well oxygenated (> 3.8 mL L^{-1}) upto a depth of ~60 m, below which a decrease in oxygen concentration prevailed with depth, whereas, the coastal sections showed shallower oxyclines (~20-40 m deep). The coastward movement of low oxygen water from the offshore region was apparent with the existence of low oxygenated waters having values less than 2 mL L^{-1} in the coastal stations. The oxygen concentration at the surface shows a gradual decrease in concentration towards the coast and the values vary from 4.2 mL L^{-1} to 3.4 mL L^{-1}.

Using hydrographic and sea level data, Banse (1968) stated that cool upwelled water was present on the entire shelf of the west coast of India during the southwest monsoon. Studies along 13°N and 15°N during the late southwest monsoon season have also described the withdrawal phase of upwelling with relatively high sea surface temperature (>29°C) and low salinity (33.8 to 35.4) (Kumar *et al*., 2008). Although upwelling off the southwest coast of India starts with the onset of the southwest monsoon, the factors controlling the coastal processes were attributed to seasonal changes of mass distribution and currents in the Arabian Sea at large (Darbyshire *et al*., 1967; Shetye *et al*., 1990).

2.3.3. *Hydrography of the open ocean Arabian Sea (summer and winter monsoon)*

The Arabian Sea is a unique geographic area known for its high seasonally oscillating biological primary productivity resulting from monsoon-driven circulation. The present study also focused on a transect in the central Arabian Sea along 68°E, comprising of seven stations between 21°N and 9°N. These stations could be grouped into northeast Arabian Sea stations (NEAS, North of 15°N) and south-east Arabian Sea stations (SEAS; south of 15°N) based on the hydrographic features of the water column. The temperature, salinity and dissolved oxygen profiles depicted well-known characteristics within this region varying with seasons (summer monsoon and winter monsoon). During the summer monsoon, upwelling was an important process which sustained high biological productivity in the central Arabian Sea whereas, during winter, productivity was enhanced by convective mixing and winter cooling processes. Studies carried out in the eastern Arabian Sea by Madhupratap (1996), Banse (1987) and Kumar *et al*., 2001, have established the influence of seasons over the hydrographic and biological properties in this region.

The summer monsoon was associated with warmer sea surface (SST; > 26°C). Shoaling of MLD was observed from 100 m in the SEAS (9°N) to 50m in the NEAS (21°N). These results are consistent with the reports by Kumar *et al*., 2000 who have suggested that shallowing of MLD north of 15°N was an indication of termination of the summer monsoon. Increased SST, as a result of increased insolation along with weak winds during this season, leads to the formation of a shallow and stratified upper layer.

Recent measurements in the open waters of the Arabian Sea ascribe the high productivity in the northern region to a combination of processes such as open ocean upwelling induced by the cyclonic wind stress curl and advection of nutrient-rich waters from the Arabian upwelling zone. In the southern central waters, higher productivity occurs due to advection of nutrient-rich, upwelled waters from Somalia. Winter cooling due to reduced incoming insolation and elevated evaporation leads to the formation of ambient density gradient which in turn initiates convection. Transport of nutrients from thermocline to euphotic zone increases the biological productivity in this region. The NEAS is shown to experience convective mixing during winter, influencing phytoplankton community. This was also supported by satellite data from the region

which showed relatively low temperatures in the north (<26°C) associated with the well-mixed water column down to ~120 m due to convection (Chndrasekharrao *et al*., 2018).

Various hypotheses have been proposed for the maintenance of sub-oxic conditions prevailing at intermediate depths in the Arabian Sea. In the Arabian Sea, other sources of oxygenated water are Persian-gulf water (PGW) and Red-Sea water (RSW), with PGW entering the Arabian Sea just beneath the thermocline (Bower *et al*., 2000; Prasad *et al*., 2001) and RSW at intermediate depths (300-1000 m) (Beal *et al*., 2000). The excess evaporation over precipitation and the presence of several high-density waters provide AS a unique property of sustaining high saline water mass. Morrison (1998), described local evaporation and advent of water from the adjoining Red Sea and Persian Gulf basins to be sources of high salinity waters, which sink and renew deep waters. The Arabian Sea High Salinity Water Mass is also formed during the winter season which on sinking deepens the mixed layer. The evaporative heat loss and sinking of the surface waters initiate convective mixing (Madhupratap *et al*., 1996).

2.3.4. *Community structure of autotrophic picoplankton in the Arabian Sea*

The community structure of autotrophic picoplankton during the late southwest monsoon was studied during two cruises in the Arabian Sea i.e. SSK 56 and SSK 69. A comparison was made in the community composition between coastal and open ocean sections of both cruises. Figure 2.6, represents the vertical and horizontal variations in total abundance at each station. Three different groups of autotrophic picoplankton, distinguished by flow cytometry analysis displayed spatial variations in terms of maximum abundances. *Synechococcus* abundances were generally higher at the coast than in the offshore region. The maximum abundance of *Synechococcus* cells was seen at station G6 during SSK 56 ($98x10^3$ cells mL^{-1}), and station G4 during SSK 69 ($67x10^3$ cells mL^{-1}). The offshore abundances varied between $5.9x10^3$ cells mL^{-1} (SSK 56) and $20x10^3$ cells mL^{-1} (SSK 69). *Prochlorococcus* dominates the oligotrophic oceanic waters. Their presence has also been reported in coastal Mediterranean Sea, Japan and China (Vaulot *et al*., 1990; Shimada, 1995). In our study, *Prochlorococcus* was observed at most of the sampling stations, their percentage contribution being highest in the offshore regions compared to that in the coastal regions. Maximum abundances of *Prochlorococcus* in the coastal transect ranged between $0\text{-}20x10^3$ cells mL^{-1} while that in

the offshore stations were above $50x10^3$ cells mL^{-1}. *Prochlorococcus* typically show an opposite pattern compared to the distribution of *Synechococcus* along the trophic gradient. They become less significant in picoplankton community from oligo- to eutrophic conditions (Calvo-Diaz and Moran, 2006). Also, owing to high affinity towards inorganic PO_4 and higher uptake rates, *Synechococcus* hold the advantage over the genus *Prochlorococcus* and thrive in phosphorous depleted environments, as reported (Martiny *et al*., 2009). For picoeukaryotes, the abundances were reasonably constant within the coastal stations of both cruises, ranging between $0\text{-}7x10^3$ cells mL^{-1}. Their total numbers at the offshore region were similar except when population maxima ($13.8x10^3$ cells mL^{-1}) occurred at ASTS station during SSK 69 cruise. Abundance maxima for all the picoplankton groups were seen at the base of the thermocline and usually coincided with the chlorophyll maxima depth at each station. A similar observation in picoplankton abundances has been previously reported in the Arabian Sea (Campbell *et al*., 1998) and the Atlantic basins (Ribeiro *et al*., 2016).

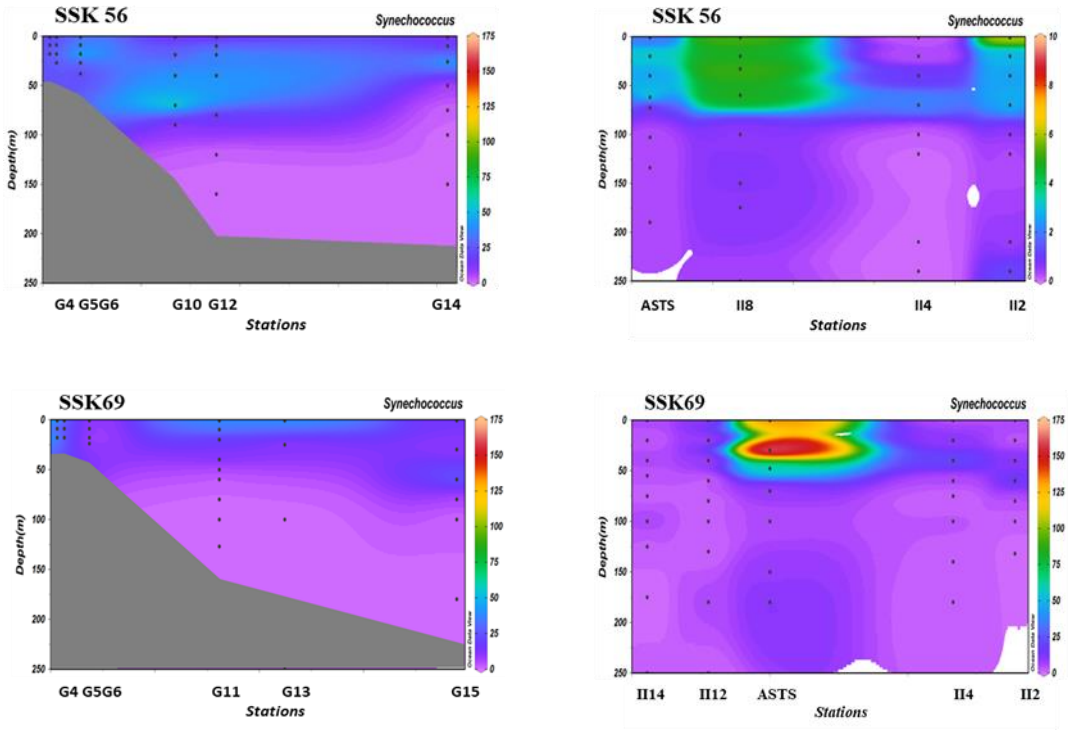

Figure 2.6A. Vertical distribution of *Synechococcus* abundance (10^3 cells mL^{-1}) for the coastal and open ocean transects of SSK 56 and SSK 69 cruises in the Arabian Sea.

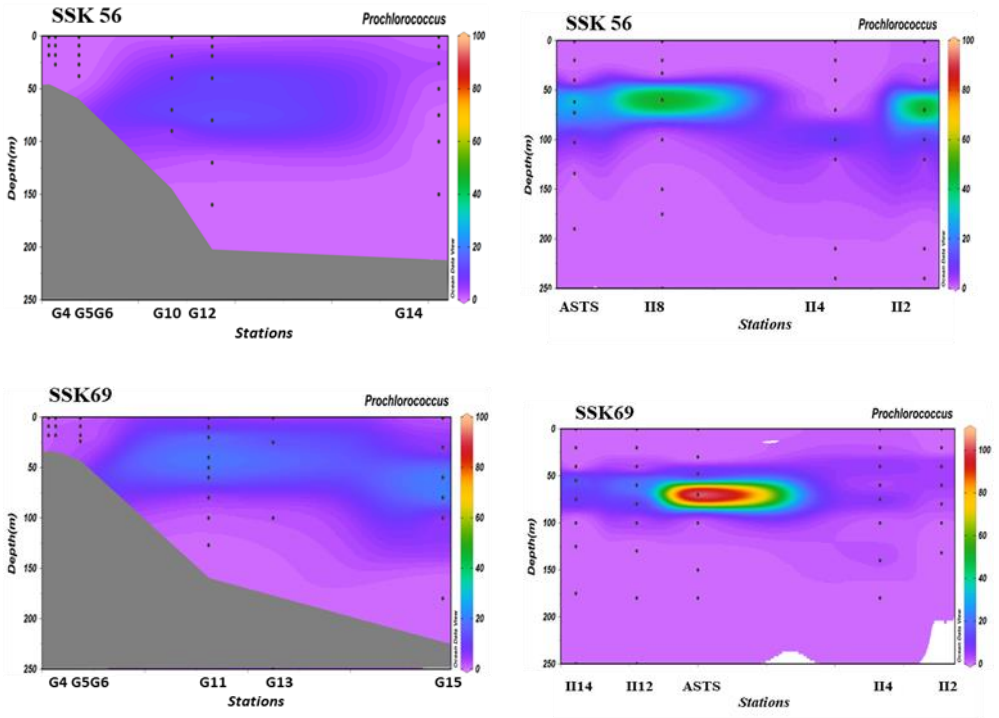

Figure 2.6B. Vertical distribution of *Prochlorococcus* abundance (10^3 cells mL^{-1}) for the coastal and open ocean transects of SSK 56 and SSK 69 cruises in the Arabian Sea.

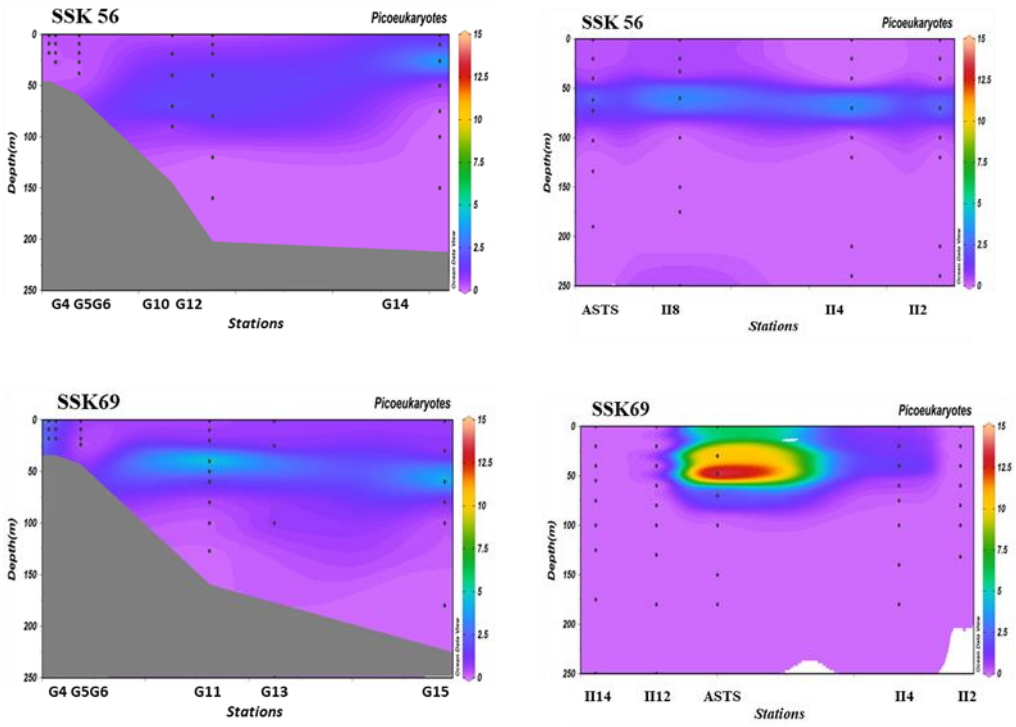

Figure 2.6C. Vertical distribution of picoeukaryotes abundance (10^3 cells mL^{-1}) for the coastal and open ocean transects of SSK 56 and SSK 69 cruises in the Arabian Sea.

2.3.5. *Dynamics of bacterioplankton in the open ocean Arabian Sea*

Seasonal and spatial variations in picoplankton and bacterial abundances were determined in the water column across seven stations between 21°N and 9°N in the central Arabian Sea. Figure 2.8 represents the depthwise and latitudinal variation of the autotrophic picoplankton and heterotrophic bacteria within stations. High picoplankton densities ranging up to $45x10^6$ cells L^{-1} have been previously reported during February, particularly in the northern Arabian Sea (Ramaiah *et al*., 1996). The northwestern region of the Arabian Sea remains productive owing to the wind-driven upwelling during the southwest monsoon. Even after the southwest monsoon, high primary production is sustained owing to cold and dry winds which result in convective mixing (Madhupratap *et al*., 1996). The central AS displays vertical structure typically known for oligotrophic oceanic provinces with a pronounced subsurface chlorophyll maximum at the nitricline and maximum primary productivity in the upper half of the subsurface maximum (Jochem, 1995).

Vertical distribution of phototrophic picoplankton followed the general pattern of a subsurface maximum. Highest abundance of *Synechococcus* was $54.6x10^3$ cells mL^{-1} during SSD 26 and $202x10^3$ cells mL^{-1} during SSK 79. Total abundance was higher in the northern stations (II14; 44-$202x10^3$ cells mL^{-1}) and reduced below ASTS station (Maximum abundance, 14-$92x10^3$ cells mL^{-1}). Similarly, during SSD 26, II14 station (21°N) displayed a maximum abundance of $54.6x10^3$ cells mL^{-1} while that at II2 (9°N) were $14.2x10^3$ cells mL^{-1}. The population of *Prochlorococcus* generally ranged from 2-$53x10^3$ cells mL^{-1} in the northern stations during SSK 79 cruise, while the total numbers varied from 1.5-$73x10^3$ cells mL^{-1} during SSD 26 cruise. Higher abundance of about 60-$63x10^3$ cells mL^{-1} was observed at stations II6 and II2 indicating their preference towards lower latitudes. Picoeukaryotes distribution was restricted to the base of the mixed layer and not surface. During SSK 79, picoeukaryotes were spread uniformly throughout the transect. However, abundance maxima were seen between 65-90 m depth at the II6 and II4 stations (47, $101x10^3$ cells mL^{-1}) during SSK 79 and at II12 station ($23.9x10^3$ cells mL^{-1}) during SSD 26 cruise. The depth distribution of Picoeukaryotes abundance at oligotrophic, offshore stations is usually associated with a subsurface maximum near the

base of the mixed layer often coincident with the depth of the insitu chlorophyll *a* fluorescence maximum. This feature was most prominent during the Spring Intermonsoon as noted by Campbell (1988).

Heterotrophic bacterial densities varied between 0.18-0.58x10^9 cells L^{-1} during the late summer monsoon and between 0.21-0.78x10^9 cells L^{-1} during the winter monsoon season. A vertical variation observed during the two cruises (SSK 79 and SSD 26) indicated higher abundances in the upper water column (~300 m) which decreased with depth. Also, horizontally, heterotrophic bacteria values showed an increase from northern (21°N) to southern (9°N) the Arabian Sea. Maximum bacterial abundance was observed to the south of 17°N during both cruises (SSK 79, II4; 0.78 x10^9 cells L^{-1} and SSD 26, II8; 0.58x10^9 cells L^{-1}). Higher primary production during the southwest and the northeast monsoon generates enormous amounts of particulate and dissolved organic carbon (DOC and POC) which becomes available for consumption by heterotrophic bacteria. The dissolved organic matter also tends to increase from the north to the south owing to decay of phytoplankton in that region as reported by Bhattathiri *et al*., 1996.

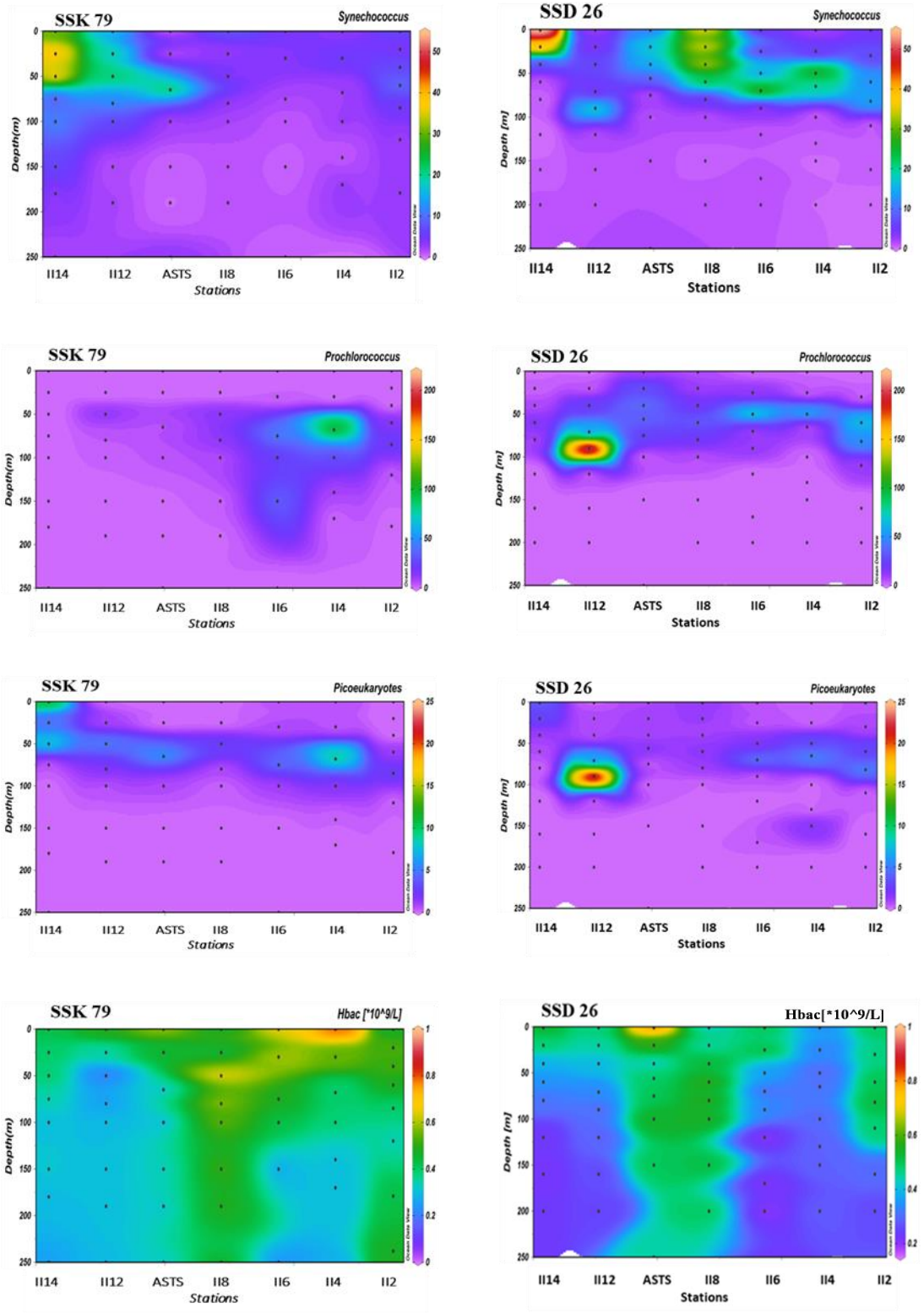

Figure 2.7. Vertical distributions of *Prochlorococcus*, *Synechococcus*, picoeukaryotes abundance (10^3 cells mL^{-1}) total heterotrophic bacteria, (Hbac; in 10^9 cells L^{-1}) for the open ocean transect (21°N to 9°N) of SSK 79 and SSD 26 cruise.

2.3.6. *Bacterioplankton abundance and distribution in the Arabian Sea*

In the Arabian Sea, strong upwelling during summer monsoon and convective mixing during winter facilitate the growth of phytoplankton (Banse, 1987). The dramatic seasonal changes can provide a range of nutrient conditions influencing primary production and emphasising the role of small producers in the microbial food web. Picoplankton forms important components in oligotrophic regions of the ocean as well as in stratified waters. Our reports are in accordance with the total abundance within each planktonic group described for coastal shelf systems and oligotrophic oceanic waters (Ducklow *et al*., 2001; Campbell *et al*., 1998). Accumulation of dissolved and particulate organic matter influences the abundance of total heterotrophic bacteria as seen in their higher numbers at the thermocline in our study. Their increased abundances coincided with secondary nitrite maximum indicating their probable role in denitrification. Higher bacterial biomass usually occurred where *Synechococcus* and picoeukaryotes were more abundant (Zhao *et al*., 2013) Our study suggests that *Prochlorococcus* tends to be a more important component of picoplankton community in the offshore stations while *Synechococcus* contributed substantially to total abundance in the coastal stations. Picoeukaryotes had a fairly uniform distribution across locations. Vertical distribution of autotrophic picoplankton was linked in most cases to the depth of the surface mixed layer.

Prochlorococcus are numerically dominant and form a major fraction of the total chlorophyll *a* biomass (Partensky *et al*., 1996; Veldhuis *et al*., 1997) in oligotrophic regions. Their role in primary productivity is well documented with maximum abundances being in highly stratified upper layers (Johnson, 2006). The coexistence of differently adapted ecotypes of *Prochlorococcus* explains their wide vertical distribution between subsurface depths (Farrant *et al*., 2016). Their local maxima correlated with the upper edge of the thermocline upto 100 m of the water column. In addition, their presence in the inshore waters could be attributed either due to the advection from offshore waters or their in situ growth capabilities (Mitbavkar *et al*., 2011). While *Synechococcus* and picoeukaryotes were present at all the stations and presented higher concentration in the inner shelf as compared to offshore, *Prochlorococcus* was 10-fold lower or not detectable from the near-shore stations. They encompass a considerable

fraction of the total bacterial biomass in the euphotic zone of oligotrophic waters whereas *Synechococcus* and the Picoeukaryotes prefer eutrophic conditions (Jiao, *et al*., 2005).

High cell abundances of *Synechococcus* are associated with mesotrophic waters and influenced by upwelling processes (Zubkov *et al*., 1998). In the present study, higher abundances of *Synechococcus* were found mostly in shelf waters, along with the upward movement of the thermocline. Higher abundance in northern latitudes has been previously reported (Jiao *et al*., 2002). *Synechococcus* niche partitioning is influenced by individual clade preferences for temperature and nutrients, which is reflected in the latitudinal shift of their abundance (Sohm, *et al*., 2016). Campbell *et al*., 1998 have also pointed out a higher abundance of 'bright' *Synechococcus* subpopulation in the offshore regions while 'dim' subpopulation is occupying the coastal stations. Observed lack of a consistent pattern in the distributions of *Synechococcus* abundance between the two monsoons could be a result of multiple *Synechococcus* populations defined by their phycoerythrin chromophore composition, can be distinguished based on their fluorescence signatures (Wood *et al*., 1985; Olson *et al*., 1990).

Photosynthetic picoeukaryotes are generally less abundant than *Synechococcus* and *Prochlorococcus* (Cai *et al*., 2007), but they may contribute a large proportion of primary production in the picophytoplankton fraction. In our study, picoeukaryote abundances peaked between 50 m and 100 m. In tropical and subtropical oligotrophic waters, picoeukaryotes are known to form maximum in deeper layers as well as in upper layers when upwelling/eddy systems bring nutrient-rich waters onto the euphotic zone (Jiao *et al*., 2002; Zubkov *et al*., 1998). The average picoeukaryotic abundance obtained in this study ($1.3x10^3$ cells mL^{-1}) is in accordance with averages observed in oligotrophic waters (Rebeiro *et al*., 2016). A close relationship between deep chlorophyll maximum layers and picoeukaryote abundance have been previously reported in Atlantic waters (Rebeiro *et al*., 2016). This dominance also suggests their better adaptation to low light levels compared to larger phytoplankton and *Synechococcus* (Worden *et al*., 2004).

During late southwest monsoon and late northeast monsoon, depth profiles displayed a peak at the base of the mixed layer. Latasa and Bridigare, 1998 explained the development of subsurface chlorophyll a fluorescence peaks in the central Arabian Sea analogous to the ones observed in our study. Advection of denser water from the north

and Ekman pumping deepens the thermocline during late southwest monsoon (Morrison, 1998). *Prochlorococcus* and the picoeukaryotes population extended deeper (~80 m) in the offshore region, whereas vertical distributions of *Synechococcus* also were limited principally to the surface mixed layer and extended deepest during the SW and NE monsoons and at offshore relative to coastal stations. The higher *Synechococcus* abundance owing to entrainment of nutrients in the mixed layer was observed during peak winter monsoon, while the concomitant changes in nitrate concentration, light and oxygen environment restricted *Prochlorococcus* growth resulting in lower abundance during the same period (Bemal *et al*., 2018). Due to the high affinity for inorganic phosphorous and higher phosphate uptake rates, *Synechococcus* hold the advantage over the genus *Prochlorococcus* and thrive in phosphorous depleted environments, as reported. (Moutin *et al*., 2002)

The depth distributions of picoplankton are expected to be more strongly structured during the Spring Intermonsoon period than during the SW Monsoon (Jochem *et al*., 1993). The spatial variation observed within picoplankton community structure indicated that *Synechococcus* abundance was higher in northeastern AS, a region influenced by convective mixing, while *Prochlorococcus* dominated southeastern AS owing to the presence of a warm core eddy (Chndrasekharrao *et al*., 2018). These physical processes maintain essential nutrient concentrations in these regions influencing the abundance of a particular group of picoplankton. It is well known that *Synechococcus* can respond quickly to nitrate input (Glover *et al*., 1988).

On the other hand, *Prochlorococcus* abundance and growth rates were found to be negatively correlated with both nitrate and phosphate concentrations (Partensky *et al*., 1999). Most *Prochlorococcus* strains are known to lack the genes involved in NO_3 uptake as well as reduction (Moore *et al*., 2002), a negative correlation between *Prochlorococcus* and nitrates is expected. Recent studies have shown that cultures of *Prochlorococcus* cannot utilise nitrate for growth (Rippka *et al*., 2000), but they can take up organic nitrogen compounds (Zubkov *et al*., 2003).

2.3.7. *Bacterioplankton distribution associated with the oxygen and nitrite in OMZ of the Arabian Sea*

The station ASTS (17 °N, 68 °E) located in the central Arabian Sea was used as a representative station to display the distribution of bacterioplankton in relation with the chemistry of the oxygen minimum zone. The dissolved oxygen and nitrite concentration profiles depicted in figure 2.8 are characteristic of oxygen minimum in the central Arabian Sea. Water remains well oxygenated upto a depth of ~200 m, below which the oxygen concentration falls below 0.2 mL L^{-1}. This perennial zone of oxygen minima lies within 200-1000 m of the water column due to more productivity in surface waters and a limited supply of oxygen to the intermediate waters (Naqvi, *et al*., 2006). Even though the renewal of intermediate waters is quite rapid (Swallow, 1984, Naqvi, 1987), this high oxygen demand leads to the development of intense oxygen minimum at intermediate depths. When oxygen is present at trace levels, bacteria utilise nitrate as an oxidant for decomposition of organic matter. Vigorous denitrification occurs in this zone where oxidised nitrogen species such as nitrate and nitrite are reduced to molecular nitrogen (Naqvi, 1991).

The presence of nitrite below the thermocline indicates the occurrence and the intensity of the denitrification process (De Sousa, *et al*., 1996). As seen from our data, high nitrite concentrations occurred at two depths, one just below the thermocline having NO_2 concentration of 0.5 µM (primary nitrite peak). The secondary nitrite maximum, however, occurred at ~200 m depth which coincided with the upper interface or the start of the oxygen minimum zone. The NO_2 concentration at this depth was 4 µM. Gupta (1976) have suggested that nitrite present in the thermocline region originates from the nitrification process while that in the suboxic waters indicates the occurrence of denitrification. The heterotrophic bacteria also displayed a similar profile wherein abundance maxima coincided with the depth of the secondary nitrite maxima. Hence, we speculate that the role of heterotrophic bacteria in denitrification in this zone is important as compared to the autotrophic picoplankton which may be involved in nitrate consumption in the upper layers.

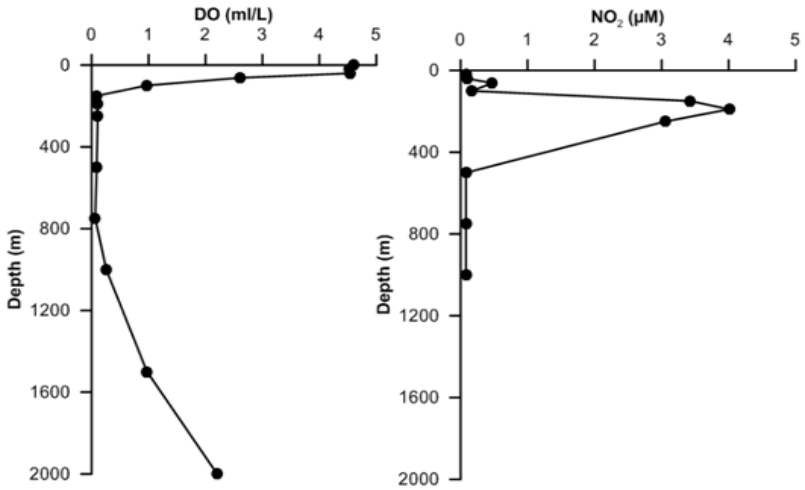

Figure 2.8A. Depth profiles of dissolved oxygen (mL L^{-1}) and nitrite concentration (µmol L^{-1}) at the ASTS station of SSK 69 cruise.

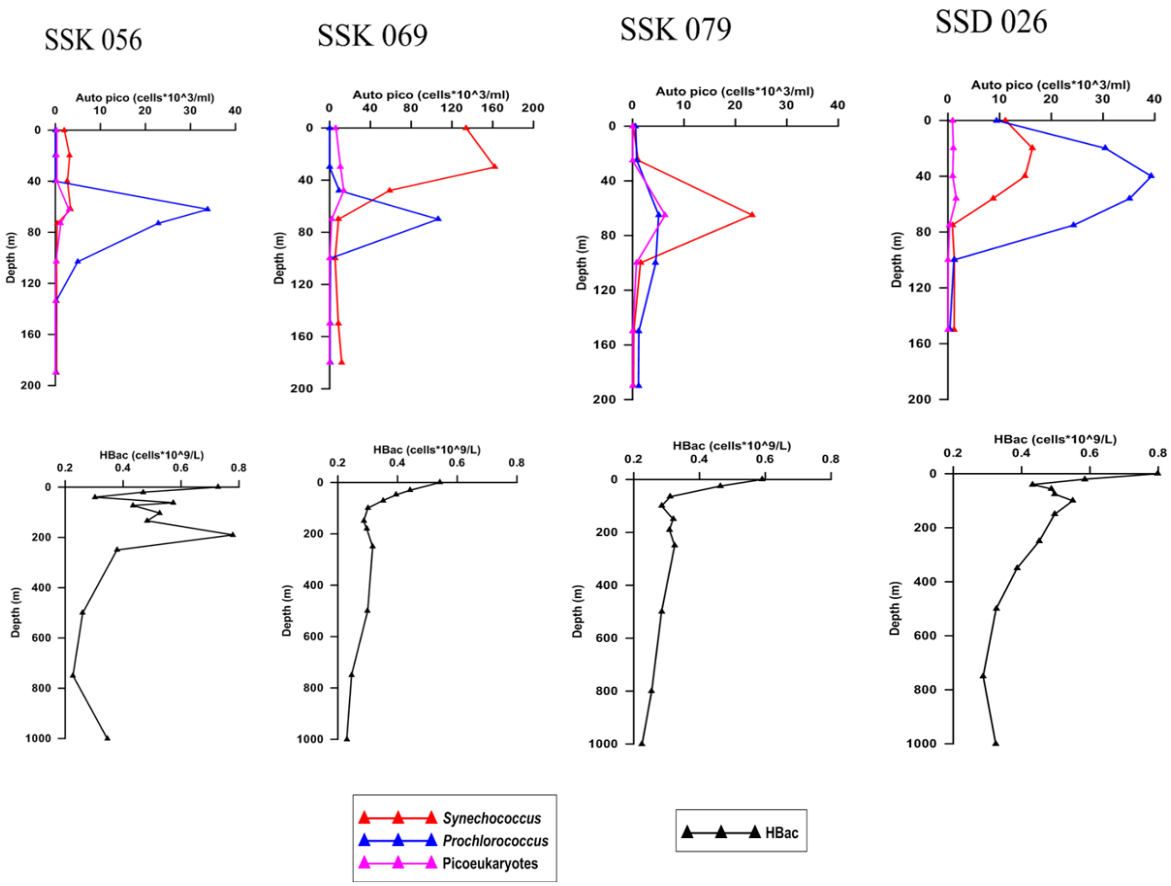

Figure 2.8B. Depth profiles of the abundance of autotrophic picoplankton (Auto pico; 10^3 cells mL^{-1}) and heterotrophic bacteria (Hbac; 10^9 L^{-1}) at the ASTS station of cruises, SSK 56, SSK 69, SSK 79 and SSD 26 in the Arabian Sea.

2.3.8. *Standing carbon stock of bacterioplankton in the central Arabian Sea*

Carbon biomass estimates at offshore locations during two cruises; SSK 79 and SSD 26 were used to assess the relative importance of each population within the bacterioplankton community. Table 2.2 gives the range of biomass values for each group of bacterioplankton. Depth-wise carbon biomass values for each station are represented in Figure 2.9. Heterotrophic bacteria had the highest percentage contribution to the total carbon biomass and ranged between 23 and 97% as compared to the autotrophic component (5-75%) in the euphotic zone of the water column (upper 250 m). Heterotrophic bacteria are known to constitute a major component of total microbial carbon and nitrogen, and their biomass accounting 20% to 50% of primary production in the sea (Cho and Azam, 1990; Jones *et al*., 1996). *Synechococcus* biomass was higher in the northern stations (II14) while *Prochlorococcus* dominated the total autotrophic biomass in the southern region (II4). Photosynthetic picoeukaryotes are usually scarce as compared to *Synechococcus* and *Prochlorococcus* (Cai *et al*., 2007). However, their contribution to the total autotrophic biomass was as high as 40%. Overall, these tiny primary producers are known to contribute substantially to both total phytoplankton biomass and production in marine ecosystems. Their importance in oligotrophic waters is well-established accounting upto 90% of the total photosynthetic biomass and carbon production (Campbell *et al*., 1994; Li *et al*., 1992).

Table 2.2. Carbon biomass estimates (µg C L^{-1}) for each population of bacterioplankton during SSK 79 and SSD 26 cruises in the central Arabian Sea.

Bacterioplankton groups	**Carbon biomass (µg C L^{-1})**	
	SSK 79	**SSD 26**
Synechococcus	0.04-6.48	0.02-9.57
Prochlorococcus	0-5.3	0.01-11.46
Picoeukaryotes	0-21.3	0-47.8
Heterotrophic bacteria	3.5-15.5	3.5-15.9

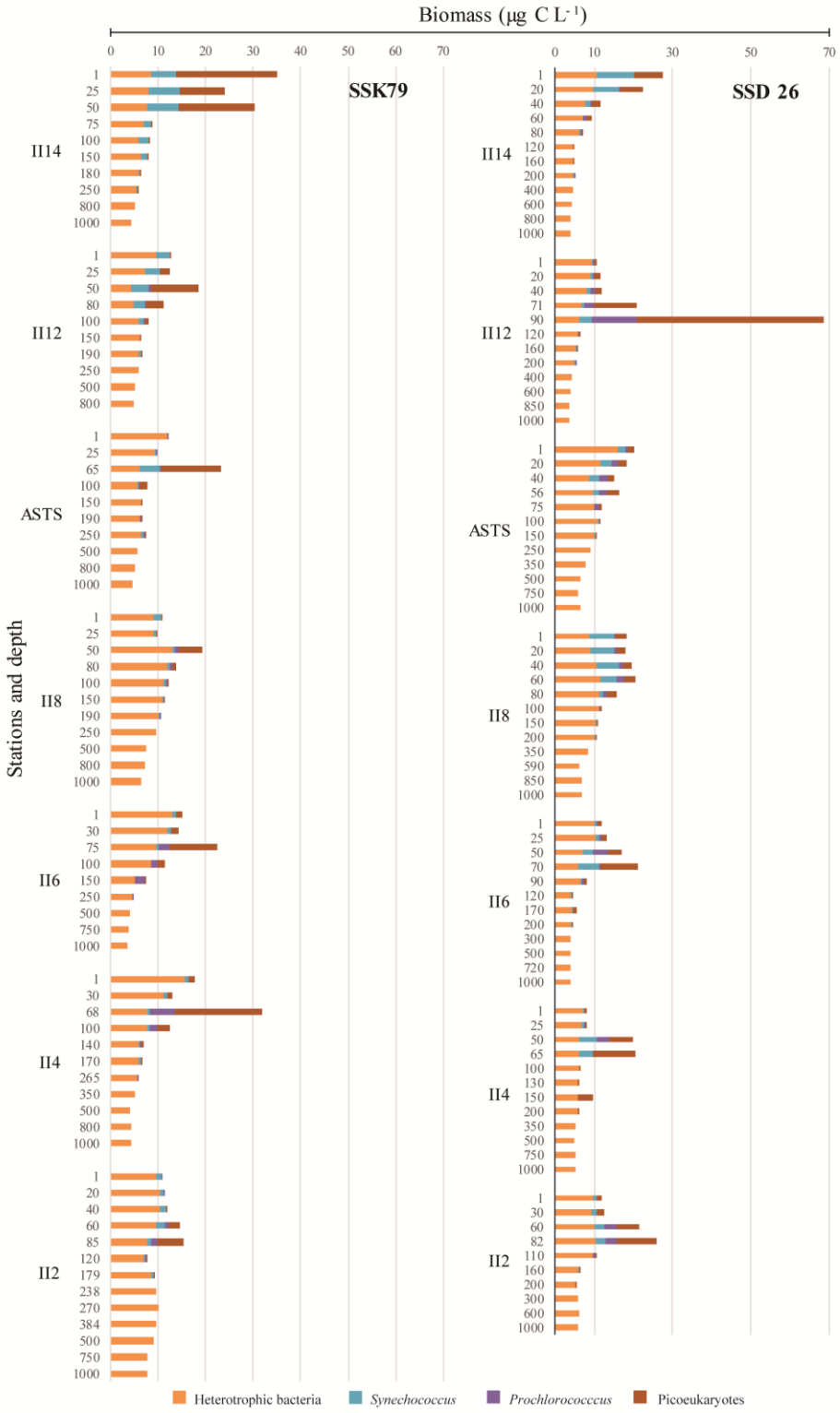

Figure 2.9. Biomass (µg C L^{-1}) estimated for total heterotrophic bacteria, *Synechococcus*, *Prochlorococcus*, and picoeukaryotes for SSK 79 and SSD 26 cruises in the Arabian Sea.

Chapter 3

Culturable diversity of nitrate-reducing bacteria from OMZ

3.1. Denitrification and associated bacteria

The role of nitrogen as a limiting factor for governing various life processes on the planet is well established (Kuypers *et al*., 2018). Atmospheric dinitrogen is without a doubt the largest inventory of freely available nitrogen. Ammonium and nitrate are examples of two such substrates which are indispensable and can be easily absorbed as well as assimilated by an organism. The availability of these forms of nitrogen depends on the diverse nitrogen transforming reactions mediated by various metabolically versatile microorganisms. Nitrogen transforming microorganisms are generally classified according to one of the six processes they are involved in such as nitrogen fixation, nitrification, denitrification, anammox, assimilation and ammonification. Nitrifiers carry out nitrification; denitrifiers, denitrification; nitrogen-fixers, nitrogen fixation; and so on. The discovery of nitrogen cycle around 100 years back, fostered the idea that two groups of chemolithoautotrophic bacteria were responsible for carrying out nitrification while denitrification was carried out by denitrifying bacteria under anaerobic conditions (Zumft, 1997). However, genomic data collected during the past decade challenge this classification, as they have revealed tremendous metabolic versatility within nitrogen transforming microorganisms. We now know that several microorganisms can fix dinitrogen gas and denitrify simultaneously (Stein and Klotz, 2016; Yan *et al*., 2008). The ability to denitrify is restricted not alone to bacteria but also to eukarya and archaea which form essential components of natural denitrifying assemblages.

Denitrification, termed as a canonical reduction of nitrate (NO_3^-) into nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), and lastly to dinitrogen gas (N_2) is carried out by diverse groups of bacteria (Zumft, 1997; Yu *et al*., 2014). Most of these bacteria are facultative and can switch between aerobic and anaerobic modes of respiration. Nitrate reduction is a significant source of nitrite for other nitrogen cycling processes, including aerobic nitrite oxidation and anammox (Bristow *et al*., 2017). Nitrate reduction to nitrite, known as dissimilatory nitrate reduction is used for respiration and nitrogen assimilation into biomass. Dissimilatory nitrate reduction to nitrite is not merely the first step in denitrification and can be linked to the oxidation of electron donors like organic matter (Zumft, 1997), methane (Haroon *et al*., 2013), sulfur compounds (Cardoso *et al*., 2006), hydrogen or iron (Weber, 2006).

Denitrifying organisms are ubiquitous, and thus denitrification occurs widely throughout terrestrial, freshwater, and marine systems where the combined conditions of nitrate or nitrite availability, low oxygen concentrations, and sufficient organic matter occur. The waters of the northern AS display a distinct oxygen minimum layer associated with high nitrite maxima which provide suitable conditions for the survival and activity of organisms that can respire nitrate. The oxygen concentrations of 0.2 mg L^{-1} support denitrification and complete anoxic conditions are not a prerequisite for the occurrence of this process (Seitzinger *et al*., 2006).

Identification of microbes involved in these transformations is quite a challenge primarily because natural denitrifying assemblages contain many different phylotypes. Despite a huge diversity of marine microbes, there is a lack of laboratory cultures that are most abundant in the environment or the ones that perform major functions in nutrient cycles. Cultivation of some of the bacterial representatives of natural communities is laborious because of their specific nutrient requirements. However, isolation and culture techniques provide a strong base to associate essential cellular functions with diversity studies. This is of paramount importance to understand the implications of microbial diversity in contributing to the rate and distribution of biogeochemical transformations (Jayakumar *et al*., 2009).

This chapter deals with the phylogenetic diversity of nitrate-reducing bacterial communities from the Arabian Sea OMZ following a culturable approach.

3.2. Methodology

3.2.1. *Sampling*

The water samples were collected onboard four cruises in the Arabian Sea, SSK 56 (October 2013), SSK 69 (October 2014) SSK 79 (February 2015) and SSD 26 (October 2016) to study the bacterial diversity within OMZ. Samples were collected from the central Arabian Sea at seven different locations along 68°E. Stations chosen were II14 (21°N, 68°E), II12 (19°N, 68°E), ASTS (17°N, 68°E), II8 (15°N, 68°E), II6 (12°N, 68°E), II4 (11°N, 68°E) and II2 (8°N, 68°E). Water samples were derived within the oxygen minimum zone at each station, and sampling depths were decided based on the

dissolved oxygen profile of the water column. The dissolved oxygen concentration of <2 mL L^{-1} was considered as the limit for sampling.

3.2.2. *Hydrography*

The methodology followed to determine the physicochemical parameters of the water column have been discussed in detail in chapter 2. In brief, sensors fitted on a portable CTD were used to measure the temperature, salinity and concentration of dissolved oxygen (DO) in the water column.

3.2.3 *Nutrients*

Nutrients (nitrate and nitrite) were measured from frozen samples carried to the on-shore laboratory using Skalar Autoanalyser (Skalar Analytical, The Netherlands) following standard methods (Grasshoff *et al*., 1983).

3.2.4. *Isolation of Nitrate-reducing bacteria- Culturable approach*

Seawater (100 mL) was filtered through a 0.22 µm nitrocellulose membrane and placed on Zobell's marine agar (ZMA) medium. Water sample (200 µL) was also spread plated on ZMA in order to obtain a higher number of isolates. All the agar plates were incubated at 28°C for 5-7 days to allow even the slow-growing organisms to appear. After incubation, plates were examined, and colonies of different morphology were selected and transferred to new agar plates several times for purification by quadrant streaking technique. Purified bacterial isolates were then transferred to nitrate agar medium (peptone, beef extract, potassium nitrate, and agar) for selectively isolating bacteria capable of utilising nitrate for their growth.

3.2.5. *Molecular characterisation by 16S rRNA sequencing*

3.2.5.1 *Genomic DNA extraction*

Molecular characterisation of cultured isolates was done following DNA extraction for which each bacterial culture was grown for 2-4 days in 5 mL nitrate broth at 28°C. The total genomic DNA was extracted using the ZR Fungal/Bacterial DNA Kit

(Zymo Research). Agarose gel (0.8%) was prepared by adding electrophoresis grade agarose (HiMedia) to 1X TAE (Tris Acetate EDTA) buffer and heating in a microwave for 30 seconds. Molten agarose was poured into a gel casting tray with combs and set to solidify for 30 minutes. The comb was then removed, and the gel was immersed in an electrophoresis tank with sufficient 1X TAE buffer. DNA samples were mixed with 6X loading dye in the ratio 1:4 and loaded into the gel wells. A DNA ladder was loaded for reference. The samples were run for 20 minutes at 200 mV. The gel was then viewed under UV transilluminator for the presence of bands.

3.2.5.2 *Spectrophotometric estimation of DNA*

NanoDrop 1000 spectrophotometer (Thermo Scientific) was used to quantify DNA by measuring absorbance at 260 nm. The purity of DNA was checked by measuring the ratio of absorbance at 260 nm and 280 nm. If the ratio A260/A280 is approximately equal to 1.8, it indicates pure DNA. If the ratio is lower, this may indicate the presence of co-purified contaminants.

3.2.5.3. *16S rRNA gene amplification and purification*

The amplification of the 16S *rDNA* gene was carried out using the primers 27F (5´-AGAGTTTGATCMTGGCTCAG-3´) and 1492R (5´-TACGGYTACCTTGTTACGACTT-3´). PCR amplification was performed in a reaction volume of 50µL containing 5µL of 10X PCR buffer (750mM Tris-HCl (pH-9), 500 mM KCl, 200 mM (NH_4) SO_4 and 20 mM $MgCl_2$), 2.5 mM of each dNTP, 0.6 µL of Taq polymerase (Genaxy scientific pvt.ltd.), 1 µL each of forward and reverse oligonucleotide primers (Eurofins Genomics India Pvt.Ltd.) and 3 µL of template DNA. The final volume of PCR was adjusted to 50 µL with sterile distilled water. PCR amplification was carried out as - initial denaturation at 95°C for 1 min, 35 cycles of primer annealing at 54°C for 45 seconds, elongation at 72°C for 1 minute. The last step was the final extension at 72°C for 10 min.

DNA in the PCR product was quantified using spectrophotometer as described above, and the reaction product was visualised on an agarose gel (1%) under UV light after ethidium bromide staining. The amplified PCR products of the bacterial gene

fragments were purified using Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, US) and stored at -20°C for further use.

3.2.5.4. *Sequencing of PCR products and BLAST analysis*

The purified amplicons were sequenced using an in-house sequencing facility (3130xl Genetic Analyzer, Applied Biosystems, California, USA). The sequences obtained were edited using DNA Baser and compared with sequences in the NCBI database through BLAST searches (https://blast.ncbi.nlm.nih.gov). The phylotypes were defined based on sequence similarity higher than 90%. Identities of the sequences were also verified on EzBioCloud software (Chun *et al*., 2007). The *16S rDNA* gene sequences obtained in this study have been deposited in the NCBI GenBank under accession numbers KY679583-KY679590, KY678488-KY678496, KY678470-KY678485, KY678487, KY926902, KY926903, MH660280-MH660375.

3.2.6. *Construction of phylogenetic tree*

Through BLAST search, sequences most closely related to the sequences of isolates were searched. All the sequences with a similarity index of >97% were included in the tree. These sequences were aligned in ClustalW, and a phylogenetic tree was constructed to elucidate the evolutionary history of the isolates using the maximum likelihood method in MEGA X (Kumar *et al*., 2018). The evolutionary distances denoted by the scale bar were computed using the Tamura-Nei method (1993).

3.2.7. *Diversity and statistical analyses*

The species abundance and range of diversity indices at each station was calculated using the statistical software primer 6, version 6.1.9 (PRIMER-E). Effect of environmental parameters on bacterial community structure was carried out using cluster analysis programme or Bray Curtis similarity in PRIMER. Correlation analysis was also carried out using a statistical data analysis tool in Microsoft Excel.

3.3. Results and discussion

3.3.1. *Hydrography*

The hydrological parameters for the entire water column have been described in chapter 2. Table 3.1. shows the temperature, salinity, dissolved oxygen concentration and the nutrient values within the oxygen minimum zone for cruises SSK 56 (October 2013), SSK 69 (October 2014), SSK 79 (February 2015) and SSD 26 (September 2016) respectively.

3.3.1.1. *Temperature*

Water column temperature profile followed the usual pattern wherein the temperature reduced with depth. The average water column temperature of the OMZ region during SSK 56 was 15.71°C, SSK 69 was 14.8°C, SSK 79 was 13.01°C and SSD 26 was 14.9°C.

3.3.1.2. *Salinity*

Average values for salinity ranged between 35.93 to 35.62 PSU. A spatial variation in salinity values was evident from measurements during SSK 79, and SSD 26 wherein the salinity values in the upper layers of the OMZ were ~36.2 and dropped down to ~35.5 in the core of the OMZ or deeper depths. This variation in salinity is due to the existence of different water masses in the Arabian Sea (Joseph and Freeland, 2005) and notably did not have any influence on the total diversity of culturable NO_3 reducing bacteria in our study.

3.3.1.3. *Dissolved oxygen (DO)*

During the study period the DO values were in the range of 0.09-1.6 mL L^{-1} (SSK 56), 0.04 mL L^{-1} (SSK 69), 0.04-0.79 mL L^{-1} (SSK 79) and 0.01-2 mL L^{-1} (SSD 26). These measured values of dissolved oxygen indicate that the sampling depths were indeed within the low oxygen threshold assigned in our study, i.e. below 2 mL L^{-1}.

3.3.1.4. *Nutrients*

An accumulation of NO_2^- (secondary nitrite maxima) and partial depletion of NO_3^- is a distinguishing factor for the presence of denitrifying waters (Castro-Gonsalez *et al*., 2005). The intensity of which is assumed to correlate with denitrification rates. In our

study, higher nitrite concentrations were particularly found at a depth of 200-350 m, which also coincided with deep chlorophyll maxima depths of the water column. The nitrite concentration in the water column varied between 0.01-3.8 µmol L^{-1} (SSK 56), 0.03-3.7 µmol L^{-1} (SSK 69), 0.01-3.2 µmol L^{-1} (SSK 79) and 0-2.9 µmol L^{-1} (SSD 26). Nitrate concentrations ranged between 24.49-48.61 µmol L^{-1} (SSK 56), 17.17-29.68 µmol L^{-1} (SSK 69), 9.38-27.12 µmol L^{-1} (SSK 79) and 15.06-36.95 µmol L^{-1} (SSD 26).

Table 3.1. Measurements of temperature, salinity, dissolved oxygen concentration, nitrate and nitrite values for four cruises (SSK 56, SSK 69, SSK 79 and SSD 26) in the Arabian Sea.(ND: Not determined)

Cruise	Station	Depth (m)	Temp (°C)	Salinity (PSU)	DO (mL L^{-1})	NO_3^- (µmol L^{-1})	NO_2^- (µmol L^{-1})
SSK 56	ASTS	150	19.1	35.8	0.09	24.49	3.43
	II8	150	18.3	35.7	0.04	24.52	3.85
	II2	120	18.8	ND	0.35	33.60	-0.03
		210	13.9	ND	0.63	37.35	-0.04
		240	13.3	ND	0.53	38.62	-0.04
		500	10.9	ND	0.62	44.05	-0.05
	II14	1500	ND	ND	1.62	48.61	-0.01

Cruise	Station	Depth (m)	Temp (°C)	Salinity (PSU)	DO (mL L^{-1})	NO_3^- (µmol L^{-1})	NO_2^- (µmol L^{-1})
SSK 69	ASTS	180	17.2	35.9	0.04	17.17	3.76
		250	15.4	36.0	0.04	18.19	2.69
		500	12.0	35.9	0.05	29.68	0.03

Cruise	Station	Depth (m)	Temp (°C)	Salinity (PSU)	DO (mL L^{-1})	NO_3^- (µmol L^{-1})	NO_2^- (µmol L^{-1})
SSK 79	II14	250	17.5	36.2	0.11	21.80	0.02
		500	12.9	35.8	0.16	27.03	0.29
		800	10.3	35.5	0.25	27.12	0.01
		1000	8.7	35.4	0.43	20.37	0.01
	ASTS	150	21.6	36.1	0.79	ND	ND
		250	16.2	35.8	0.04	22.76	3.23
		500	12.2	35.6	0.05	32.06	0.01
		750	ND	ND	ND	ND	ND
		800	10.3	35.5	0.06	36.85	0
		1000	8.9	35.4	0.14	39.385	0.005
	II6	150	19.1	35.6	0.04	9.38	0.73
		250	14.5	35.4	0.05	10.75	0.00
		500	11.8	35.4	0.15	8.02	0.01
		750	10.0	35.4	0.14	20.35	0.01
	II2	175	17.2	ND	0.12	23.84	0.01
		270	13.8	ND	0.16	16.58	0.02
		384	12.0	ND	0.20	29.19	0.01
		750	9.5	ND	0.37	19.23	0.01
		1000	7.7	ND	0.62	24.28	0.01

Cruise	Station	Depth (m)	Temp (°C)	Salinity (PSU)	DO (mL L^{-1})	NO_3^- (µmol L^{-1})	NO_2^- (µmol L^{-1})
SSD 26	II14	160m	19.8	36.17	0.12	21.6	0.17
		200m	17.3	36.14	0.00	19.96	0.9
		400m	13.6	35.85	0.04	21.95	2.13
		1000m	8.9	35.40	0.07	35.42	0
	II12	160m	19.9	36.23	0.19	23.81	0
		200m	17.8	36.15	0.01	20.94	0
		400m	13.5	35.83	0.06	23.02	1.61
		1000m	8.8	35.39	0.11	36.7	0
	ASTS	150m	18.5	35.66	0.20	23.79	0
		350m	13.6	35.74	0.00	22.34	1.44
		500m	12.2	35.61	0.03	26.78	0
		1000m	8.5	35.33	0.19	36.95	0
	II8	100m	24.4	36.47	1.80	15.06	0.05
		590m	11.4	35.56	0.04	30.00	0
		850m	9.3	35.37	0.13	33.46	0
	II6	200m	16.3	35.57	0.06	19.37	2.75
		300m	13.1	35.42	0.00	27.64	0.19
		500m	11.7	35.48	0.11	24.46	0.04
	II4	200m	15.9	35.56	0.05	24.62	0.26
		350m	12.0	35.29	ND	32.21	0
	II2	110m	23.7	35.95	0.55	20.13	0.06
		160m	17.5	35.46	0.07	26.46	0.03
		201m	15.0	35.33	0.77	21.74	0.08

3.3.2. *Nitrate-reducing bacteria from the OMZ*

Initial isolation using the spread plate technique yielded approximately 230 bacterial cultures on Zobell agar medium. Few of which (~50 numbers) were lost during subsequent subcultures on nitrate agar medium. The remaining 180 isolates were carried forward for DNA extraction and PCR amplification.

3.3.3. *Identification of bacterial isolates by PCR and 16S rRNA sequencing*

The bacterial *16S rDNA* gene fragments were successfully amplified from all extracts of DNA samples. The PCR amplification of the DNA samples extracted from pure cultures of nitrate-reducing bacteria using universal bacterial primers yielded amplicons of ~1500 bp. The PCR products were further purified and used for sequencing.

Sequencing of PCR amplified *16SrDNA* gene samples was carried out by Sanger's dideoxy chain termination method using universal primers, 27F and 1492R. Out of 180 samples, 152 were successfully sequenced. BLAST analysis of the test sequences revealed partial identities with sequences of 152 bacteria deposited in the NCBI GenBank. The closest match with type strain was considered for identification up to genus level.

3.3.4. *Bacterial diversity from OMZ*

Bacterial denitrification is induced by the presence of either NO_3^- or NO_2^- under a limited O_2 supply and suppressed by excess O_2 (Zhou *et al*., 2001). Our results suggest that the denitrifier community structure in the OMZ could be associated with the prevalent biogeochemical properties within the study area. The highest diversity of culturable nitrite reducers was found at depths with high nitrite levels (ASTS, 1.4-3.4 µM) considered to be the zones of high denitrification activity, similar to results reported by Jayakumar *et al*., 2004. High levels of NO_3^- and very low levels of NO_2^- could determine the establishment of a characteristic community located at the limit of the open ocean denitrifying area (Codispoti and Packard, 1980). The interaction and competition for resources with other micro-organisms could be an additional factor influencing the diversity and composition of denitrifiers in OMZs (Castro-Gonzalez *et al*., 2005).

A total of 152 nitrate-reducing bacterial isolates were obtained from water samples collected during four cruises in the Arabian Sea oxygen minimum zone, between

depths of 100-1500 m (Table 3.2, 3.3). The isolated bacteria were identified belonging to three phylogenetic groups; Proteobacteria, Firmicutes and Actinobacteria by partial sequencing of the *16S rDNA* gene. One hundred thirty-four isolates belonged to Proteobacteria, which was the most abundant group at all the stations studied, while nine belonged to Firmicutes and nine to Actinobacteria (Figure 3.2). Diversity analysis at the genera level revealed a total of 16 genera namely, *Alcanivorax, Alteromonas, Bacillus, Brevibacterium, Glutamicibacter, Halomonas, Idiomarina, Marinobacter, Micrococcus, Nesterenkonia, Nitratireductor, Pseudoalteromonas, Pseudomonas, Staphylococcus, Sulfitobacter,* and *Vibrio* (Figure 3.3). Out of these, isolates belonging to *Halomonas* were the most abundant (38 organisms) followed by *Marinobacter* (37 organisms) and *Pseudoalteromonas* (21 organisms). In cultivation-based studies of this functional community, the genera *Pseudomonas, Ralstonia, Alcaligenes, Paracoccus, Rhodobacter, Rubrivivax, Thauera, Burkholderia, Bacillus,* and *Streptomyces* have been pointed out as the dominant denitrifiers, along with *Thiobacillus denitrificans, Micrococcus denitrificans,* and some species of *Serratia, Pseudomonas,* and *Achromobacter* present in various environments (Fuka *et al*., 2007). *Pseudomonas aeruginosa*, a ubiquitous and metabolically flexible member of the Gammaproteobacteria, can perform (complete) denitrification under anaerobic conditions and the presence of nitrate (Arat *et al*., 2015).

Our study reveals that culturable bacterial community in the AS-OMZ was dominated by Proteobacteria. Retrieval of bacteria belonging to the class gammaproteobacteria is apparent enough because proteobacteria are readily cultivable microorganisms from the marine environment (Furman and Hagstrom, 2008). The dominance of this organism testifies the endurance and adaptability to environmental stressors. Rappe *et al*., 2000 reported the presence of alpha-proteobacteria and gamma proteobacteria from a variety of pelagic environment. Also, proteobacteria are the most diverse group of the microbial assemblage and hence is of biological significance (Gupta, 2000). The dominance of gamma proteobacteria has also been reported earlier from the OMZ of the Arabian Sea (Jain, *et al*., 2014; Jayakumar *et al*., 2004; Fuchs, *et al*., 2005) and the ETSP (Castro-Gonsalez, *et al*., 2005; Ganesh, *et al*., 2014; Stevens and Ulloa, 2008; Stewart, *et al*., 2012), although some of the phylotypes identified belonged to not yet cultivated species. Studies on sediments of Arabian Sea revealed the dominance of

gamma proteobacteria from clone libraries as well as retrieval of *Micrococcus* sp., *Halomonas* sp. and *Alteromonas* sp. from the culturable fraction of bacteria from coastal AS (Divya *et al*., 2010, 2011). Other studies by Walsh *et al*., 1991 also displayed the bacterial diversity and community composition in the sediments as well as water column which mainly comprised of gamma proteobacteria, particularly *Pseudoalteromonas* sp. Analyses of *16S rRNA* and *nas*A genes from bacterial communities in the Indian Ocean revealed 63 OTUs which clustered into gammaproteobacteria (Jiang and Jiao, 2016). Sun *et al*., 2017, studied the community composition of nitrous oxide consuming bacteria in the ETSP sediments and found *Marinobacter* sp. to be abundant. As suggested by Orsi *et al*., 2017, the genera *Marinobacter, Alcanivorax* and *Pseudoalteromonas* being relatively abundant serve as indicator taxa for strong OMZ periods.

However, the OMZ in AS exhibits differential water column properties which confer wide variation in the bacterial community structure in this region. The seasonal dynamics and associated bacterial community studies by Bandekar *et al*., 2018 suggest significant variation in the abundance of major bacterial groups between surface layers and OMZ depths. Similarly, Bryant *et al*., 2012 and Stewart *et al*., 2012 demonstrated microbial communities from different depths within the OMZ cluster together. However, the population from surface layers was entirely different. Although the bacterial community changes seasonally between surface and OMZ, it is more or less identical within the OMZ.

Figure 3.4 represents the phylogenetic analysis of all the isolates obtained in this study. Actinobacteria and Firmicutes have been represented in Figure 3.4 (a) while proteobacteria are represented in Figure 3.4 (b). A total of 9 isolates clustered with type strains of organisms belonging to actinobacteria group, while 9 isolates grouped in the firmicutes class. Among the gamma proteobacteria, 134 strains clustered into 9 different genera. The selection and prevalence of a few phylotypes of bacteria in the OMZ are governed by the lower redox potentials and less readily available organic matter (Jain, *et al*., 2014). Also, DO, organic matter and pH are considered key factors driving the changes in microbial community composition in aquatic ecosystems (Lindstrom and Bergstrom, 2005; Haukka, *et al*., 2006). Studies on the effect of environmental factors on

bacterial community suggest that DO and TOC (total organic carbon) are responsible for this vertical separation (Jain, *et al*., 2014).

Results from this study ascertain the existence of a culturable heterotrophic bacterial community in the low oxygen waters. These facultative anaerobes were capable of growing on low strength Zobell's medium aerobically indicating their versatility. Here we assume that there is no much difference in the bacterial community within the OMZ region on the basis of studies carried out by Jain *et al*., 2014 wherein they suggested least or minimal variation in the OMZ bacterial community within OMZ implying temporal homogeneity and to some extent seasonal predictability in the Arabian Sea OMZ.

Table 3.2. Sampling details and the total number of bacterial isolates obtained in each cruise

Cruise No.	**SSK 56**	**SSK 69**	**SSK 79**	**SSD 26**	**Total**
No. of stations sampled	4	1	4	7	16
Cumulative depths sampled	6	3	17	37	63
No. of isolates obtained	8	9	39	96	**152**

Table 3.3. Nitrate reducing bacterial isolates obtained during cruises in Arabian Sea and their closest representative in NCBI database

Sr. no.	Total no. of isolates	Isolates	Closest match in NCBI (% similarity > 90)
1	1	(NIOSSK056#77)	*Alcanivorax dieselolei*
2	11	(NIOSSK056#78, NIOSSK056#79, NIOSSD026A#1, NIOSSD026A#2, NIOSSD026A#3, NIOSSD026A#4, NIOSSD026A#5, NIOSSD026A#6, NIOSSD026A#7, NIOSSD026A#8, NIOSSD026A#9)	*Alteromonas macleodii*
3	1	(NIOSSK056#95)	*Bacillus firmus*
4	3	(NIOSSK056#37, NIOSSK056#36, NIOSSK056#39)	*Bacillus flexus*
5	1	(NIOSSK056#34)	*Bacillus infantis*
6	1	(NIOSSK056#41)	*Bacillus megaterium*
7	1	(NIOSSK056#40)	*Brevibacterium casei*
8	1	(NIOSSD026A#10)	*Brevibacterium epidermidis*
9	2	(NIOSSD026A#11, NIOSSD026A#12)	*Brevibacterium sanguinis*
10	1	(NIOSSD026A#13)	*Glutamicibacter nicotianae*

11	10	(NIOSSK056#35, NIOSSK079#30, NIOSSK056#81, NIOSSK079#5, NIOSSK079#48, NIOSSD026A#14, NIOSSD026A#15, NIOSSD026A#16, NIOSSD026A#17, NIOSSD026A#18)	*Halomonas aquamarina*
12	8	(NIOSSK079#11, NIOSSD026A#19, NIOSSD026A#20, NIOSSD026A#21, NIOSSD026A#22, NIOSSD026A#23, NIOSSD026A#24, NIOSSD026A#25)	*Halomonas axialensis*
13	1	(NIOSSK056#83)	*Halomonas denitrificans*
14	1	(NIOSSK056#82)	*Halomonas halodenitrificans*
15	17	(NIOSSK056#38, NIOSSD026A#26, NIOSSD026A#27, NIOSSD026A#28, NIOSSD026A#29, NIOSSD026A#30, NIOSSD026A#31, NIOSSD026A#32, NIOSSD026A#33, NIOSSD026A#34, NIOSSD026A#35, NIOSSD026A#36, NIOSSD026A#37, NIOSSD026A#38, NIOSSD026A#39, NIOSSD026A#40, NIOSSD026A#41)	*Halomonas meridiana*
16	1	NIOSSD026A#42	*Halomonas venusta*
17	7	(NIOSSK069#1, NIOSSK069#2, NIOSSK056#84, NIOSSK056#80, NIOSSK056#85, NIOSSD026A#43, NIOSSD026A#44)	*Idiomarina zobelli*
18	2	(NIOSSK056#86, NIOSSD026A#66)	*Marinobacter algicola*
19	3	NIOSSD026A#63, NIOSSD026A#64, NIOSSD026A#65)	*Marinobacter alkaliphilus*

20	25	(NIOSSK069#3, NIOSSK069#4, NIOSSK069#5, NIOSSK056#87, NIOSSK056#88, NIOSSK056#89, NIOSSK056#90, NIOSSD026A#45, NIOSSD026A#46, NIOSSD026A#47, NIOSSD026A#48, NIOSSD026A#49, NIOSSD026A#50, NIOSSD026A#51, NIOSSD026A#52, NIOSSD026A#53, NIOSSD026A#54, NIOSSD026A#55, NIOSSD026A#56, NIOSSD026A#57, NIOSSD026A#58, NIOSSD026A#59, NIOSSD026A#60, NIOSSD026A#61, NIOSSD026A#62)	*Marinobacter hydrocarbonoclasticus*
21	2	NIOSSD026A#67, NIOSSD026A#68)	*Marinobacter flavimaris*
22	5	(NIOSSK079#53, NIOSSK079#75, NIOSSK079#66, NIOSSK056#91, NIOSSK056#96)	*Marinobacter litoralis*
23	3	(NIOSSK069#6, NIOSSK069#7, NIOSSD026A#69)	*Micrococcus luteus*
24	1	(NIOSSK069#8)	*Nesterenkonia lacusekhoensis*
25	1	(NIOSSD026A#70)	*Nitratireductor aquibiodomus*
26	3	(NIOSSD026A#71, NIOSSD026A#72, NIOSSD026A#73)	*Nitratireductor kimnyeongensis*
27	8	(NIOSSK056#92, NIOSSK079#29, NIOSSK079#20, NIOSSK079#22, NIOSSK079#42, NIOSSK079#60, NIOSSD026A#74, NIOSSD026A#75)	*Pseudoalteromonas arabiensis*
28	4	(NIOSSK079#27, NIOSSD026A#76, NIOSSD026A#77, NIOSSD026A#78)	*Pseudoalteromonas lipolytica*

29	5	(NIOSSK079#74, NIOSSK079#15, NIOSSK079#47, NIOSSD026A#79, NIOSSD026A#80)	*Pseudoalteromonas nigrifaciens*
30	3	(NIOSSK079#52, NIOSSK079#4, NIOSSK079#61)	*Pseudoalteromonas prydzensis*
31	1	(NIOSSD026A#81)	*Pseudoalteromonas ruthenica*
32	2	(NIOSSD026A#82, NIOSSD026A#83)	*Pseudomonas stutzeri*
33	1	(NIOSSD026A#84)	*Staphylococcus arlettae*
34	1	(NIOSSD026A#85)	*Staphylococcus cohnii*
35	1	(NIOSSK069#9)	*Staphylococcus saprophyticus*
36	2	(NIOSSD026A#86, NIOSSD026A#87)	*Sulfitobacter dubius*
37	2	(NIOSSD026A#88, NIOSSD026A#89)	*Sulfitobacter faviae*
38	2	(NIOSSD026A#90, NIOSSD026A#91)	*Vibrio campbellii*
39	4	(NIOSSD026A#92, NIOSSD026A#93, NIOSSD026A#94, NIOSSD026A#95)	*Vibrio harveyi*
40	1	(NIOSSK079#62)	*Vibrio splendidus*
41	2	(NIOSSK056#94, NIOSSD026A#96)	*Vibrio parahaemolyticus*

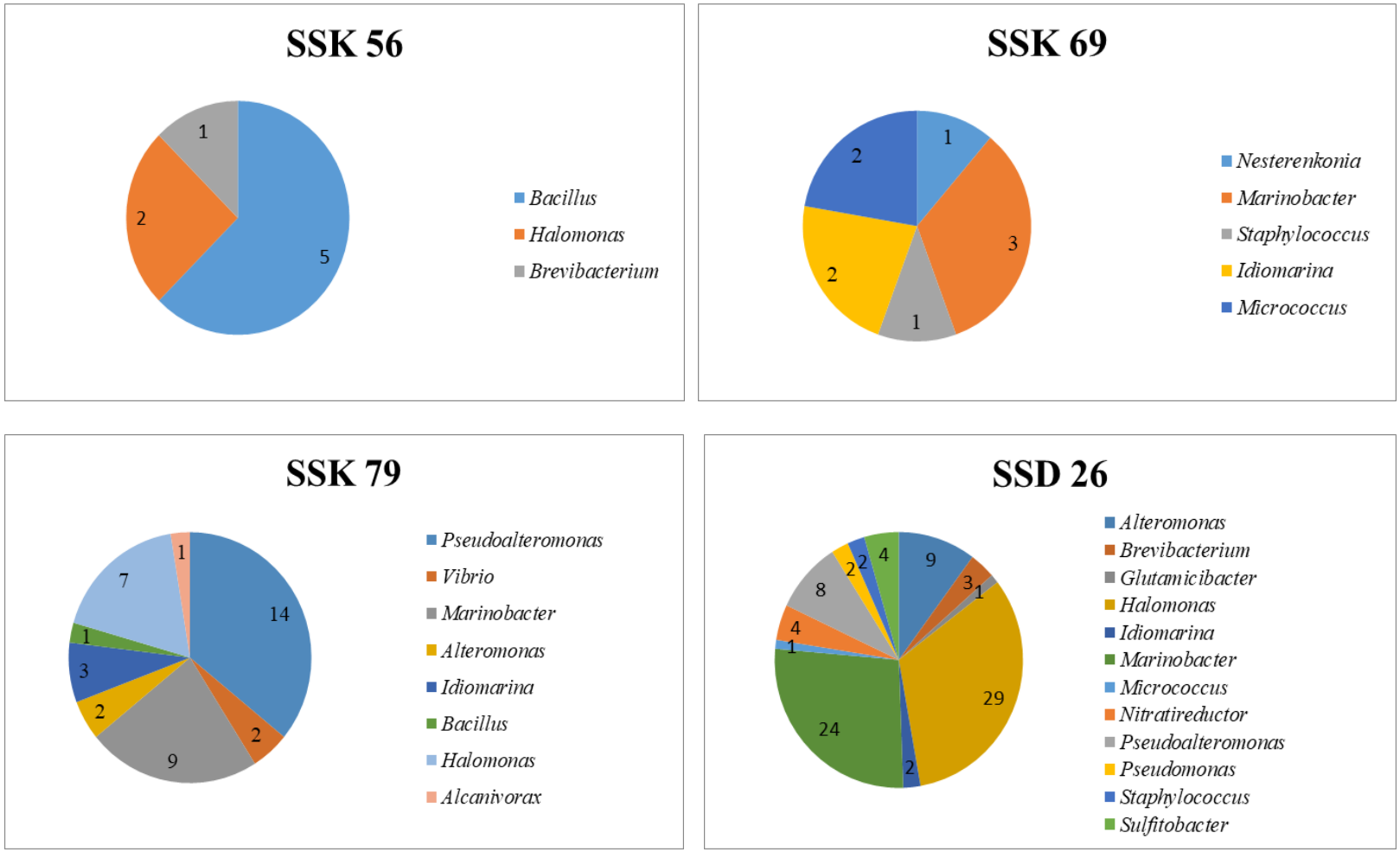

Figure 3.1. Culturable bacterial diversity obtained during four cruises (SSK 56, SSK 69, SSK 79 and SSD 26) in the Arabian Sea

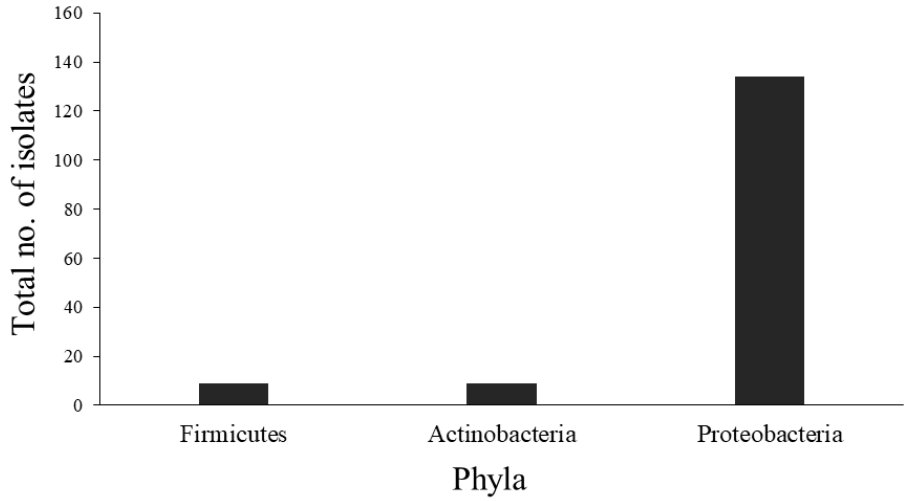

Figure 3.2. Culturable diversity of nitrate reducing bacteria from the AS-OMZ at the phylum level

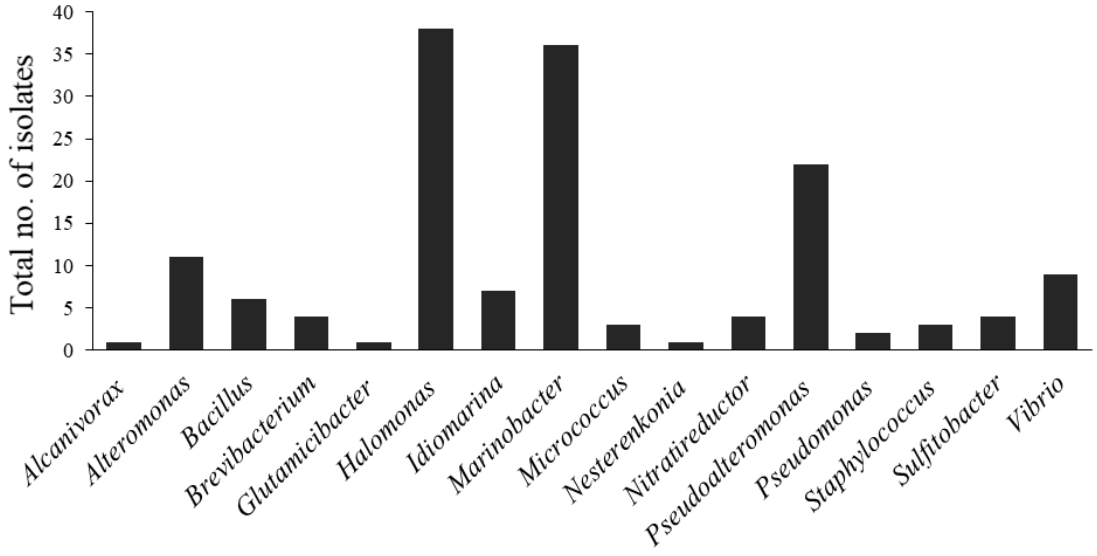

Figure 3.3. Culturable diversity of nitrate-reducing bacteria from the AS-OMZ at the genus level

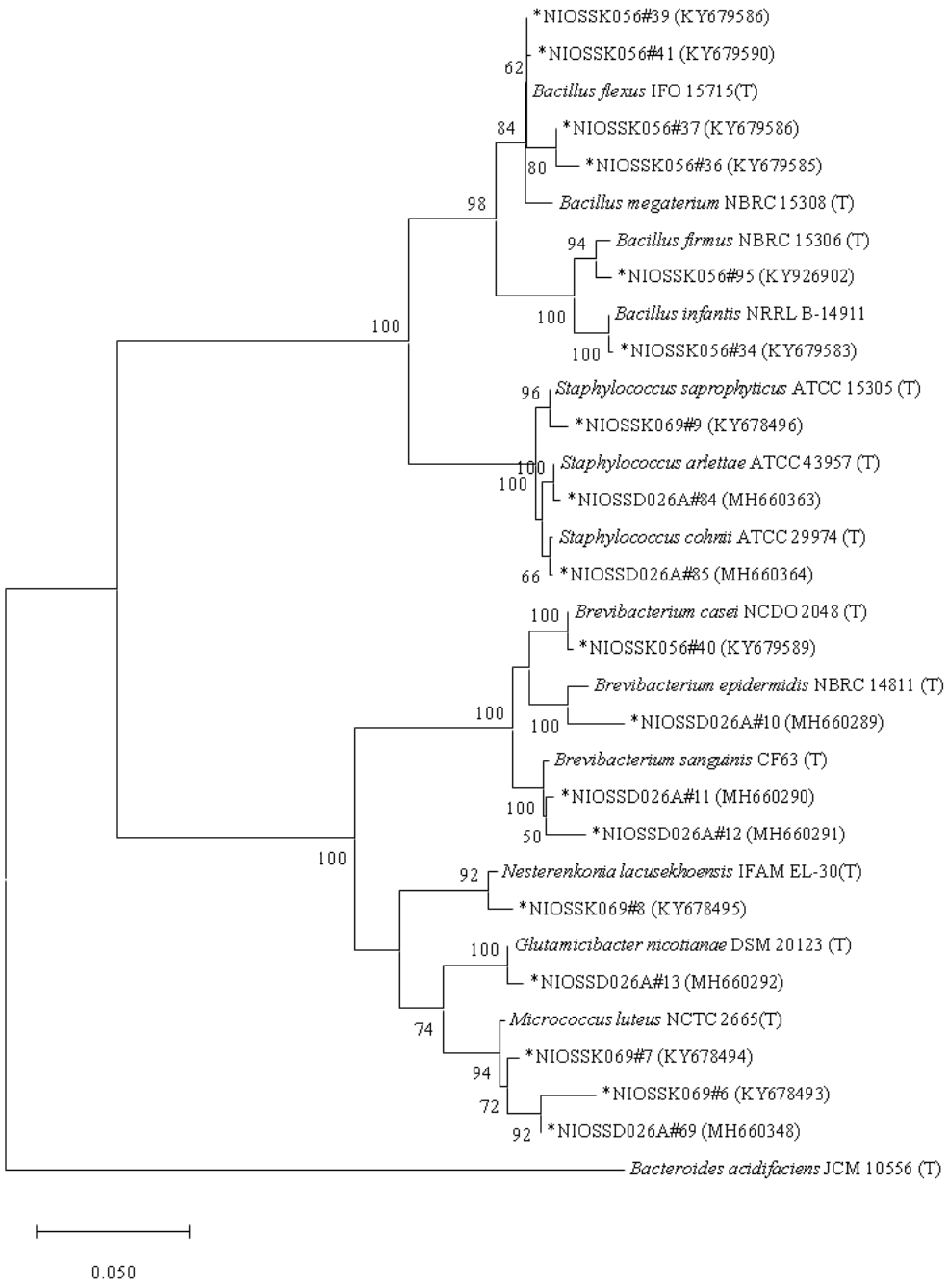

Figure 3.4A. Phylogenetic tree (maximum likelihood) of Actinobacteria and Firmicutes inferred from 16S rDNA gene sequences. Bootstrap values are shown at the nodes (values <50% are not shown). Scale bar represents 0.5% nucleotide substitution. *Bacteroides acidifaciens* as used as outgroup. Sequences generated in this study are represented by *.

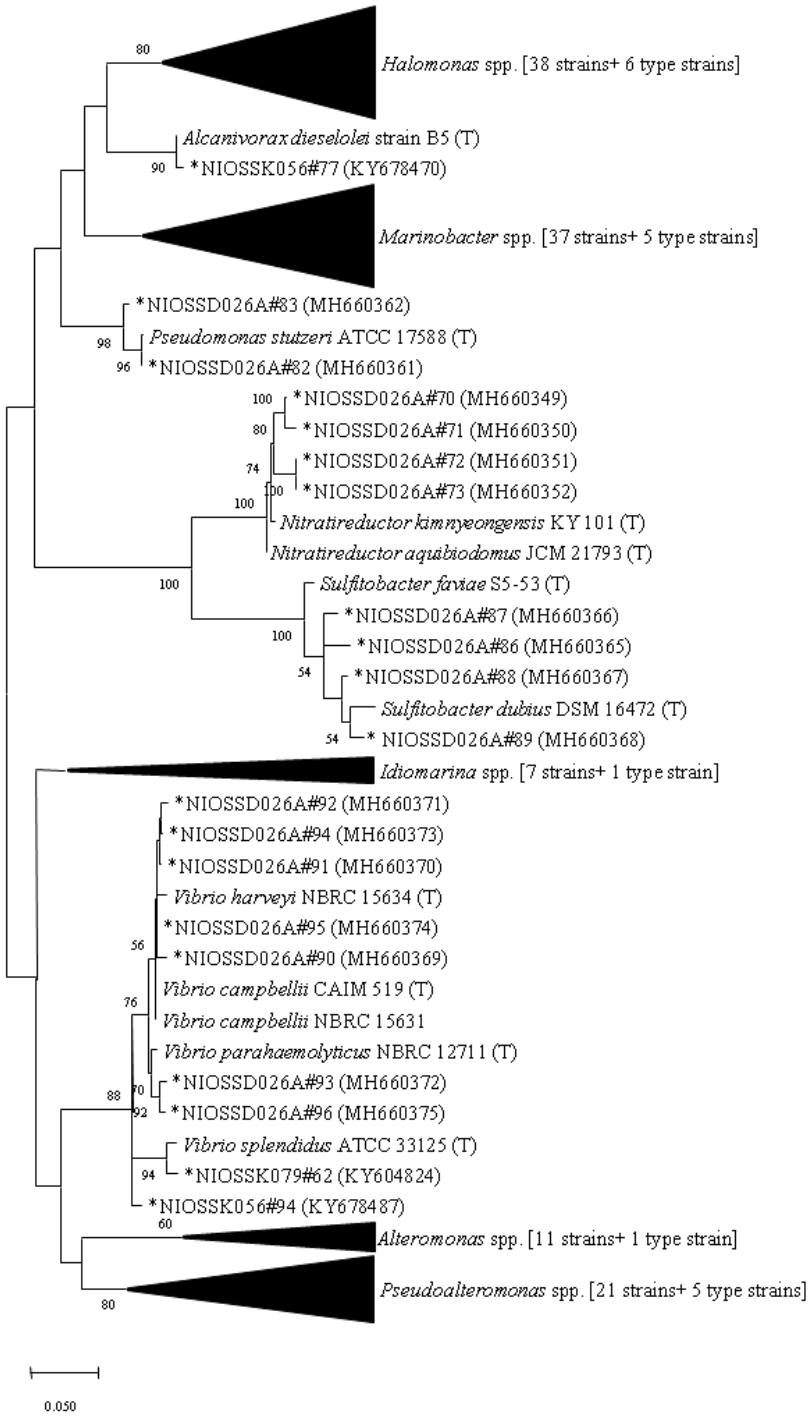

Figure 3.4B. Phylogenetic tree (maximum likelihood) of gammaproteobacteria inferred from 16S rDNA gene sequences. Bootstrap values are shown at the nodes (values <50% are not shown). Scale bar represents 0.5% nucleotide substitution. Sequences generated in this study are represented by *.

3.3.5. *Statistical analyses*

3.3.5.1. *Diversity indices*

Diversity indices describe general properties of communities which allow a comparison between different regions, taxa or trophic levels, besides providing vital information about rarity and commonness of species in a community. Measuring species diversity is an elementary task in ecology and an important tool for biologists trying to understand community structure. Species diversity within a landscape or region can be partitioned into components representing the average diversity within a sample (alpha-diversity) and the diversity among all the samples (beta-diversity). These measurements take into consideration not only species richness but also the evenness between species, namely the distribution throughout the stations of the number of individuals belonging to the species. Richness quantifies how many different species of a particular community are present, while evenness represents the degree to which the species are separated. Low values indicating that one or a few species dominate, and high values indicate that a relatively equal number of individuals belonging to each species.

The total number of isolates obtained per cruise was dependent on the number of stations and depths sampled during each cruise. The maximum number of bacterial cultures was obtained during the SSD 26 cruise (96 isolates from seven stations). The species abundance and range of diversity indices at each station (Table 3.4) were calculated using the statistical software primer 6, version 6.1.9 (PRIMER-E). The species diversity was calculated based on the number of isolates obtained at each station. The highest number of species was recorded at ASTS (20) and least from II4 (7). Also, high diversity value at ASTS (H'=4.23) was associated with high species richness (d=5.7).

Table 3.4. Diversity indiex (Shannon-Weiner) at all the stations during four cruises in the Arabian Sea

Station	*S* (No. of species)	*d* (Richness)	*J'* (Evenness)	*H'* (Diversity)
II14	12	4.22	0.99	3.56
II12	13	4.44	0.99	3.66
ASTS	20	5.70	0.98	4.23
II8	10	3.60	0.98	3.27
II6	12	4.08	0.97	3.50
II4	7	2.99	1.00	2.80
II2	14	4.41	0.99	3.75

3.3.5.2. *Effect of environmental parameters on bacterial community structure*

Alpha diversity between all the nitrate reducers obtained in this study was deduced using Cluster analysis in PRIMER 6. For this, the isolates were divided into 3 zones; an upper interface (UI), Core OMZ (C) and lower interface (LI) based on the DO concentration and the depth from which the bacteria were isolated. All samples having a DO concentration of <0.1 mL L^{-1} were considered in the core zone while those with DO>0.1 mL L^{-1} were included in the UI/LI zones. This distinction based on DO was made based on the CTD profile at each station. Water column profile is shown in chapter 2. Bray-Curtis similarity which is the most commonly used similarity coefficient for the biological community was used to determine species similarity between different zones (Figure 3.5). Isolates belonging to the Upper interface and the lower interface clustered together and showed around 50% similarity while the bacteria isolated from the core of the OMZ were 70% different from the rest of the isolates.

Bacterial community in relation to the environmental variables was analysed using Canonical correspondence analysis (CCA). First two axes explain the variation of the species-environment relation (Figure 3.6). It can be inferred that DO, NO_3^- and NO_2^- influence the variation in bacterial community. The sum of Eigen values indicated a variance of 1.84 in the dataset. The first two ordination axes of CCA accounted for

57.95% of the total variance. The observed variance denotes an influence caused by DO (R=0.56). CCA indicated that DO and nitrate may have an influence on the culturable bacterial community in the OMZ.

Correlation between various environmental parameters and a total number of bacteria isolated was assessed by using Spearman's rank correlation analysis. Since the highest diversity and the maximum number of isolates were obtained from the ASTS station, the correlation was tested only at this station. A positive correlation between the isolated bacteria and DO, NO_3^-, NO_2^- concentrations was thus observed, and temperature/ salinity may not have any specific influence on the bacterial community in this region (Table 3.5)

Castro-Gonzalez *et al*., 2005 carried out a canonical correspondence analysis of the *nirS* genes and biochemical parameters indicating that oxygen, nitrate and nitrite gradients and depth within the water column are critical environmental factors influencing the denitrifier community composition. Other studies by Liu *et al*., 2003 and Taroncher-Oldenburg *et al*., 2003 have also reported oxygen, nitrate, dissolved organic carbon, inorganic nitrogen and salinity gradients to be critical factors governing the composition of denitrifier communities.

Table 3.5. Spearman's rank correlation coefficients (r) between various environmental parameters and the total number of bacteria at ASTS station in the AS-OMZ.

	Temperature	**Salinity**	**DO**	**NO_3^-**	**NO_2^-**	**Bacteria**
Temperature	1					
Salinity	0.912971	1				
DO	0.284063	0.294422	1			
NO_3^-	-0.81508	-0.80547	-0.58428	1		
NO_2^-	0.342888	0.287553	-0.29445	-0.35083	1	
Bacteria	-0.48886	-0.495	0.22006	0.05062	0.0423	1

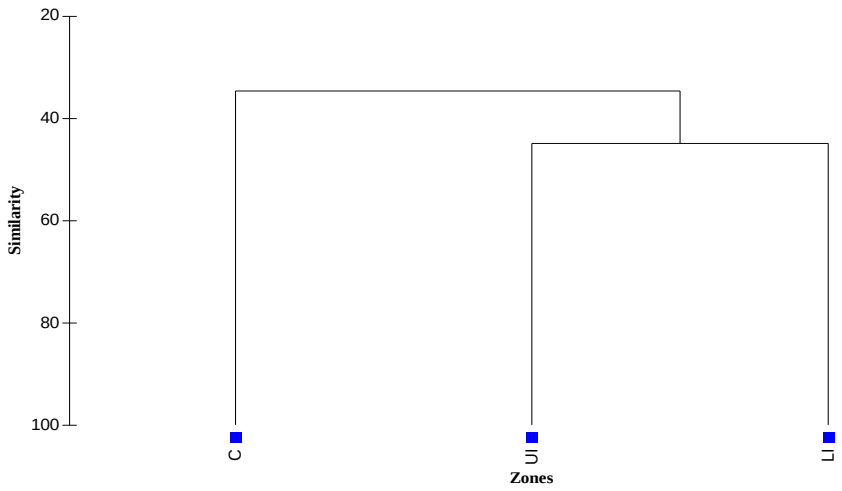

Figure 3.5. Hierarchial cluster analyses of the bacterial community profiles during cruise SSD 26 from the AS-OMZ (UI-upper interface, C- Core, LI- the Lower interface of OMZ).

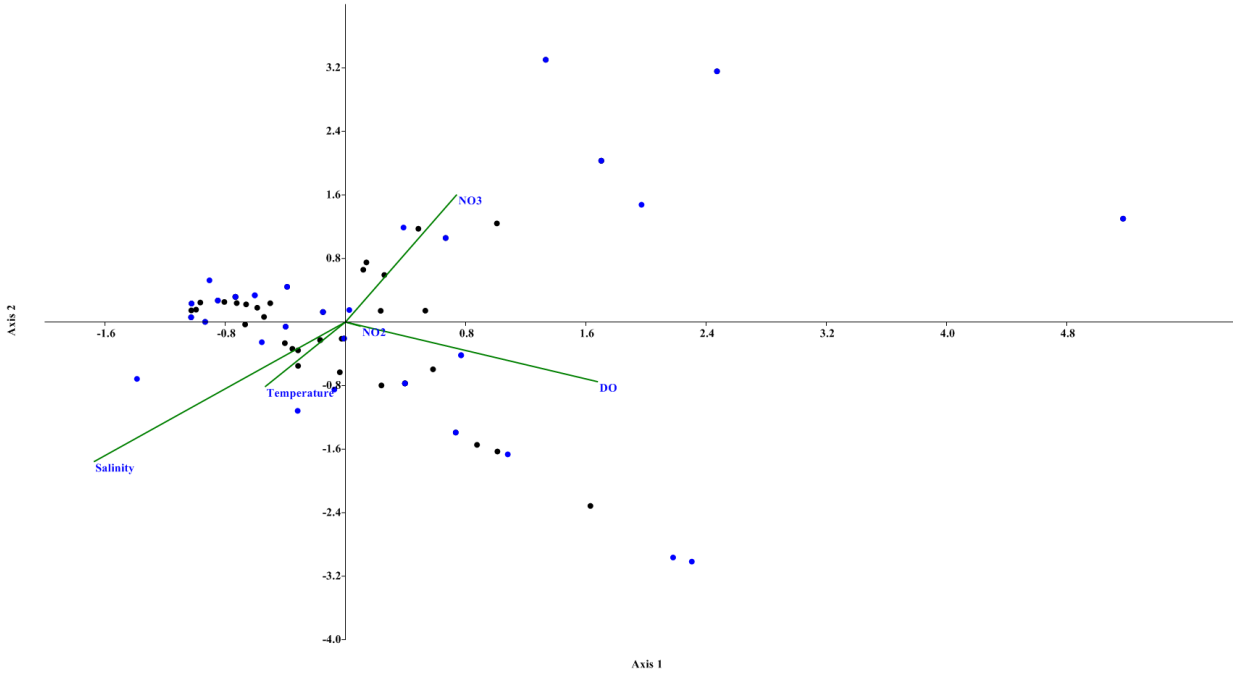

Figure 3.6. Canonical correspondence analysis (CCA) ordination diagram of bacterial communities associated with environmental variables. Green lines are the environmental parameters, black dots station and depth and blue dots are the bacterial isolates.

Chapter 4

Nitrate reducing potential and functional gene analysis of nitrate reducing bacteria

4.1. Genes involved in denitrification

Bacterial denitrification is mostly facultative. A taxonomically differing diverse group of bacteria can reduce nitrate into nitrite, with low oxygen concentrations and availability of nitrogen oxide governing its expression. Traditional methods such as microscopy do not provide an adequate distinction between denitrifying bacteria. Hence, molecular methods targeting functional genes offer a precise characterisation of these organisms (Philippot, 2002). Biochemical and genetic analyses have enhanced our understanding of an array of enzymes catalysing the four steps of the denitrification pathway. Besides the genes encoding denitrification reductases, genes encoding electron donors, electron carriers, chaperonins, regulatory proteins and proteins involved in metal processing have been successfully characterised (Zumft, 1997; Berks *et al*., 1995; Richardson *et al*., 2001).

In canonical denitrification pathway, nitrate reduces to dinitrogen in a series of four reactions. Enzymes encoded by a distinct gene are responsible for each of these steps (Figure 4.1). The first step in denitrification is catalysed by nitrate reductase enzyme coded by the nitrate reductase gene (*nar*). Several nitrate reductases exist in bacteria, i.e., dissimilatory, periplasmic, and assimilatory enzymes (Zumft, 1997). This step, in contrast to the others, is less suitable for assessing the occurrence of denitrification in bacteria. Nitrite reductase is the first enzyme of denitrification involved in catalysing the first committed step that leads to a gaseous intermediate and is encoded by the *nir* gene, which can be catalysed by two entirely different dissimilatory nitrite reductases, either cytochrome cd_1 or Cu-containing enzymes (Zumft, 1997). The following conversion of nitric oxide (NO) to nitrous oxide (N_2O) is done by nitric oxide reductase (encoded by the *nor* gene). Finally, N_2O is reduced to dinitrogen (N_2), catalysed by the Cu-containing nitrous oxide reductase encoded by the *nos* gene (Kloos *et al*., 2001).

Diverse combinations of genes involved in the denitrification pathway are observed within denitrifying organisms (Jones *et al*., 2008). The genes for denitrification encoding functions for nitrate, nitrite, nitric oxide and nitrous oxide reduction are assembled in various prokaryotes such as *Pseudomonas stutzeri* (Braun & Zumft, 1992), *Pseudomonas aeruginosa* (Arai *et al*., 1995), *Paracoccus denitrificans* (Berks *et al*., 1995, de Boer *et al*., 1994), *Sinorhizobium meliloti* (Holloway *et al*., 1996), and

Achromobacter cycloclastes (Zumft, 1997). Environmental studies have shown that denitrification genes are widespread in environments that receive some exposure to oxygen. Most model denitrifiers support growth by carrying out denitrification when oxygen is limiting. Nonetheless, this may not be the case in all bacteria that contain genes encoding nitrogen oxide reductases associated with denitrification. Some microorganisms harbour all denitrification enzymes and can potentially perform complete pathway, while others have a condensed mechanism such as lack of the nitrous oxide reductase gene (*nosZ*) and produce only nitrous oxide (N_2O) as the end product (Philippot *et al*., 2011). Bacteria with limited chains consisting of a single enzyme may use that enzyme for alternative functions as seen in case of *Staphylococcus aureus* that only contains nitric oxide reductase, involved in the detoxification of nitric oxide. Similarly, some bacteria only contain nitrite reductase.

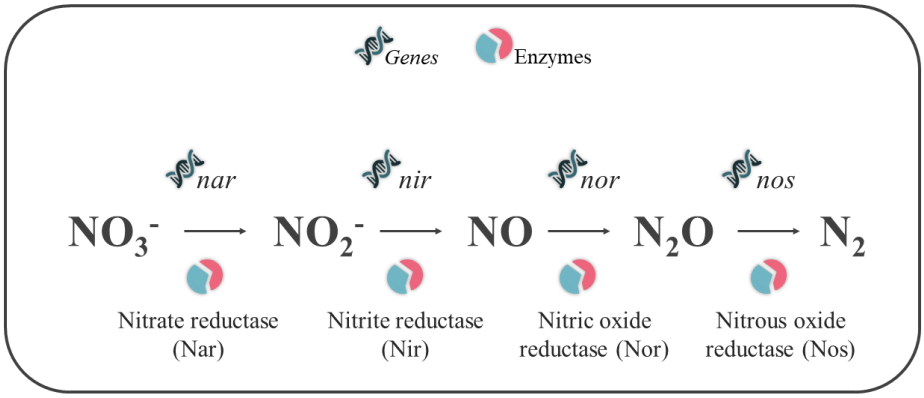

Figure 4.1. Schematic representation of the genes and the enzymes involved in the denitrification process.

However, the function of this enzyme is uncertain in these organisms since it will lead to toxicity due to the production of nitric oxide. Recent reports have also shown non-denitrifying nitrous oxide reducers with atypical *nos*Z, which are potential contributors to N_2O reduction in soils (Jones *et al*., 2013). The *nir-nor* gene clusters harbour the structural information for both reductases and the functions for metal processing, cofactor synthesis, electron donation, protein maturation, assembly processes, and regulation (Zumft, 1997). Studies on denitrifying genes have been carried out in only

a few species despite the tremendous diversity of bacteria capable of denitrification. Besides their role in the nitrogen cycle, the denitrifying genes also provide a good model for studying the evolutionary relationship of functional genes.

4.1.1. *Nitrate reduction genes (nar)*

NO_3^- reduction to NO_2^- is the first step in denitrification and is carried out by two pathways namely, assimilatory and dissimilatory nitrate reduction. Three types of NO_3^- reductases catalyse these reactions; a soluble assimilatory type NO_3^- reductase (*nas*) and two dissimilatory reductases (*nar/nap*). Some eukaryotes, bacteria, and archaea contain assimilatory nitrate reductases, and nitrate is a significant source of nitrogen in these organisms. *Nar* or *nap* catalyses dissimilatory nitrate reduction to nitrite (Moreno-Vivian *et al*., 1999). These enzymes are used for respiration and also for nitrogen assimilation. *Nar* and *nap* nitrate reductases can be present in a single organism but differ in the mechanism of action, i.e., *nar* reduces NO_3^- in the cytoplasm and releases protons into the periplasm whereas *nap* reduces NO_3^- in the periplasm (Kuypers *et al*., 2018).

Many bacteria have more than one of the three types of NO_3^- reductases. Gene sequencing and mutational studies on *Ralstonia eutropha* and *Paracoccus denitrificans* have provided enough evidence for the coexistence of all the NO_3^- reductases. Nitrite-oxidising bacteria contain the *nxr* (NO_2 oxidoreductases) which aids in nitrate reduction. Bacterial and archaeal *nas*, together with *nap*, *nar*, and *nxr*, belong to the dimethyl sulfoxide reductase family, while eukaryotic assimilatory NO_3^- reductases belong to the sulfite oxidase family (Stolz and Basu, 2002). This suggests multiple origins of NO_3^- reductases.

4.1.2. *Nitrite reductase genes (nir)*

The definitive step in the denitrification process is the reduction of nitrite to nitric oxide by nitrite reductase enzyme (Zumft, 1997). The possession of the *nir* gene distinguishes denitrifying bacteria from all other bacteria capable of nitrate reduction and exists in two physiologically equivalent but structurally different forms: cytochrome cd_1-Nir, a heme protein encoded by the *nirS* gene, and the Cu-Nir encoded by the *nirK* gene. Cd_1 enzymes are periplasmic soluble proteins and involved in respiratory NO_2 reduction. Cytochrome cd_1 are homodimeric proteins of two identical subunits, each containing one

heme C and one unique heme $d_{1.}$ whereas the Cu-Nir are trimeric enzymes. Purification and characterisation of *nir* from several bacterial sources have shown the presence of these two distinct classes of dissimilatory nitrite reductases that yield NO as the main reaction product.

The community structure of denitrifiers with *nirS* can differ from that of *nirK* communities along environmental gradients, indicating niche differentiation between the two denitrifying types (Enwall *et al*., 2010). The copper nitrite reductase was purified from several gram-negative bacteria, such as *Achromobacter cycloclastes* (Iwasaki and Matsubara, 1972), *Nitrosomonas europaea* (Dispirito *et al*., 1985) and *Ralstonia sphaeroides* (Sawada *et al*., 1978), gram-positive bacteria (Denariaz *et al*., 1989), and even from the archaea *Haloferax denitrificans* (Inatomi and Hochstein, 1996) and *Haloarcula marismortui* (Ichiki *et al*., 2001). The enzymes containing Cu and heme never coexist within the same bacterial species. These genes are examples of convergent evolution. Nonetheless, functional complementation of a cd_1NIR-deficient strain of *Pseudomonas stutzeri* with the Cu-*nir* from *Pseudomonas aureofaciens* indicates functional similarity. In contrast to the above theory, Murugapiran *et al*., 2013 found the co-occurrence of *nirK* and *nirS* genes contained within a circular megaplasmid of a hot spring bacterial strain *Thermus oshimai* JL-2, and in the chromosome of *Thermus scotoductus* SA-01.

The *nirS* gene exhibits less sequence homology over a narrow phylogenetic range as opposed to *nirK* which is conserved over a broad phylogenetic range. It is thought to be more widely distributed. Environmental *nirK* type denitrifiers are currently underestimated in PCR dependent surveys due to primer coverage limitations which can be attributed to their broad taxonomic diversity and large *nirK* sequence divergence. As both *nirK* and *nirS* are single-copy genes and are mutually exclusive in the genomes of denitrifying bacteria (Beckman and Kranz, 1993), they can be individually targeted as proxies for determining the genetic potential of a denitrifying bacterial community to remove excess bioavailable nitrogen from the environment.

4.1.3. *Nitric oxide reductase genes (nor)*

Nitric oxide in prokaryotes, is a true intermediate of denitrification and is produced from nitrite by the nitric oxide reductase gene (*nor*). The reduction of nitric oxide (NO) to nitrous oxide is catalysed by the *nor* enzyme which is a membrane-bound protein connected to an electron transport chain. Nitric oxide reductases purified from denitrifying bacteria are known to occur in two subtypes referred to as cNor and qNor. The cNor enzyme receives electrons from cytochrome *c*. These heterodimers consist of a catalytic subunit, *norB*, and a small subunit, *norC* (cb-Nor enzyme) (Busch *et al*., 2002). The qNOR possesses a homologous N-terminal extension and is thought to play a role in quinol binding besides its oxidation (Shapleigh, 2013). The resemblance of the catalytic sites of cNOR and qNOR suggests that the mechanism of NO reduction is very similar in these enzymes. The cytochrome oxidases of the heme/copper family cannot efficiently reduce NO under anaerobic conditions, although some convert it very slowly (Giuffre *et al*., 1999). The replacement of copper in the active site of the oxidases with iron in NOR is presumed to be an essential factor for the efficient reduction of NO. All bacteria that contain cNOR are capable of denitrification (Hendriks *et al*., 2000).

NO is reduced by and evolved from denitrifying bacterial communities, pure cultures, and extracts of induced cells (Zumft, 1997). Sequence analysis of the *nor* genes has revealed that *norB* has sufficient sequence similarity to terminal oxidases to include nitric oxide reductase within the family of heme-copper oxidases. Regulatory factors involved in the enzymatic production and reduction of NO are known to be in close association at the gene expression level. This regulation is thought to be important for preventing the accumulation of toxic NO during denitrification (Hendricks *et al*., 2000). The first evidence for a membrane-bound NO reductase activity was obtained with *Alcaligenes faecalis* IAM 1015 (Matsubara & Iwasaki, 1971). Nitric oxide reductases were studied in *Pseudomonas stutzeri* (Zumft *et al*., 1994) and *Pseudomonas aeruginosa* (Arai *et al*., 1995) wherein the cytochrome cb-NOR was found to be encoded by the *norCBQ* operon. In *Paracoccus denitrificans*, the *nor* operon consists of six genes, *norCBQDEF* (de Boer *et al*., 1996), and is formed by five genes in *Rhodobacter sphaeroides* (Bartnikas *et al*., 1997). The quinol-oxidising NOR has been biochemically

characterised in *Ralstonia eutropha* (Cramm *et al*., 1999) and *Synechosystis* (Busch *et al*., 2002).

4.1.4. *Nitrous oxide reductase genes (nos)*

The functional gene *nosZ* encodes for nitrous oxide reductase, an enzyme catalysing the final step of denitrification (Scala & Kerkhof, 1999). Many denitrifying bacteria grow at the expense of N_2O as the sole electron acceptor for the oxidation of organic compounds. Nitrous oxide reductases are homodimers containing only copper as the metal constituent. However, there are two types of copper centres; Cu_A and Cu_Z which are involved in electron transfer and catalysis respectively. Much of the early biochemical and spectroscopic knowledge about nitrous oxide reductase and its several forms arose from the studies by Zumft and co-workers on the enzyme from *Pseudomonas stutzeri*. Each monomer within this homodimeric enzyme is composed of a C-terminal cupredoxin domain that carries the mixed valent Cu_A binuclear centre and an N-terminal seven-bladed β-propeller domain which hosts the active Cu_Z site. The crystal structures of the enzyme from several bacterial species have been determined and show the same basic structure. Ji *et al*., 2015 carried out a detailed study of the biochemistry of this enzyme.

The N_2O reductase has been purified from various gram-negative denitrifiers but not yet from a gram-positive bacterium (Henry *et al*., 2006). Several authors have reported the production of N_2O by denitrifying isolates as an end product of denitrification (Cheneby *et al*., 2004; Greenberg & Becker, 1977). In a quantitative study, Graf *et al*., 2014, have found that bacteria with only *nosZ* genes were most common in the genomes of marine bacteria compared to other denitrification genes. The *nirS* gene was noted to be however preferentially associated with bacteria exhibiting a complete denitrification pathway. Just as nitrate respiration is not coupled obligatorily to denitrification, bacteria that respire N_2O without being denitrifiers are known. For example, *Wolinella succinogens* was the first strain that was shown to grow on N_2O. However, NO is not utilised by whole cells, and N_2 is not formed from nitrate reduction (Yoshinari, 1980). Nitrous oxide consumption is the final step of denitrification and is the least oxygen tolerant step (Bonin *et al*., 1989; Korner and Zumft, 1989). This certainly reflects the role of N_2O as an obligatory free intermediate during denitrification. N_2O

consumption rates have been measured in oxygen deficient zones at depths having very low oxygen concentration (Wyman *et al*., 2013; Ji *et al*., 2015). Studies in oxygenated waters by Farias *et al*., 2009 and Wyman *et al*., 2013 have also detected genes involved in N_2O reduction and N_2O consumption. Environmental regulation of N_2O consumption and emission is necessary for absolute quantification of the N_2O budget, thus improving our ability to envisage oceanic emissions of N_2O under global climate change. The distribution of *nosZ* genes in a recent study by Castro-Gonzalez *et al*., 2015, was related to oxygen concentration suggesting that the quantity, composition of *nosZ* genes along with the diversity of denitrifying bacteria might influence the microbial potential for N_2O consumption.

4.2. Methodology

4.2.1 Screening of bacterial isolates for their nitrate reduction potential

4.2.1.1 *Bacteria and growth conditions*

All the strains were grown aerobically at 27°C. A representative isolate from each species identified tentatively based on partial *16S rDNA* sequencing was used to study the nitrate reduction ability. Starter cultures of these bacteria were grown in nitrate broth medium having a concentration of 10 mM nitrate. The cultures were maintained in aerobic conditions in glass test tubes plugged with cotton containing 5 ml media. These were used further for the nitrate reduction assay.

4.2.1.2 *Nitrate reduction assay*

The nitrate reducing potential of the bacterial culture isolates was determined qualitatively by nitrate reduction test (Griess test). Determination of nitrate reduction to nitrite is a two-step process. In the first step, the reduction of nitrate to nitrite is tested with addition of nitrate reagents A and B, then if necessary, the reduction of nitrate beyond nitrite is determined by the addition of nitrate reagent C (zinc dust).

An inoculum of the organism to be tested was incubated in nitrate broth. After 24 hrs of incubation at 28°C and 120 rpm, the sample was tested for the reduction of nitrate (NO_3) to nitrite (NO_2) by adding five drops each of nitrate reagent A (sulfanilic acid) and nitrate reagent B (α-naphthylamine). If the organism had reduced nitrate to nitrite, the

nitrites in the medium would form nitrous acid. When sulfanilic acid was added, it would react with the nitrous acid to produce diazotised sulfanilic acid which in turn reacts with the naphthylamine to form a red-coloured compound. Hence, a positive test for nitrite reduction is denoted by the appearance of a deep red colour change after the addition of nitrate reagents A and B. However, if the medium did not turn red on the addition of the reagents, it meant that the organism was incapable of reducing the nitrate. It could also mean that the organism converted the nitrate or nitrite and produced ammonia or molecular nitrogen. To test this, a small amount of powdered zinc was added. Development of a red colour after the addition of nitrate reagent C (zinc dust), confirmed a negative test obtained earlier. No colour development after the addition of zinc dust indicated that nitrate was reduced beyond nitrite to nitrogen gas. Powdered zinc was then added to check for the presence of residual nitrate. The isolates that showed no nitrate reduction and tested positive for residual nitrate were categorised as non-reducers (--). The isolates that were positive for nitrite production but still had some residual nitrate were marked as incomplete nitrate reducers (+-), while the isolates that were positive for nitrite accumulation and (or) negative for residual nitrate are grouped as nitrate reducers (++) (Manohar *et al*., 2014).

4.2.2 Functional gene analysis

4.2.2.1. *Bacterial culture conditions and DNA extraction*

Representative isolates of each bacterial species were grown in 5 ml of Zobell marine broth at 28°C. A pure strain of *Pseudomonas stutzeri* (MTCC No. 101) obtained from Microbial Type Culture Collection, CSIR-IMTECH, India, was also inoculated along with the isolates obtained in this study. The total genomic DNA from all the cultures was extracted using ZR Fungal/Bacterial DNA Kit (Zymo Research) as specified by the manufacturer. The quality of the extracted DNA was checked spectrophotometrically using NanoDrop 1000 spectrophotometer (Thermo Scientific). The purity of DNA was determined by measuring the ratio of absorbance at 260 nm and 280 nm. This DNA was stored at -20°C for further use.

4.2.2.2. *Primer-specific amplification of denitrifying genes*

PCR primers targeting genes specific for nitrite reductase (*nirK, nirS*), nitric oxide reductase (*norB*) and nitrous oxide reductase (*nosZ*) were used to amplify genes involved in denitrification process. Nucleotide sequence and other details of each primer used are given in Table 4.1. Genomic DNA extracted from *Pseudomonas stutzeri* (MTCC 101) along with DNA from *Marinobacter hydrocarbonoclasticus strains* isolated from this study were used to standardise primers for specific gene amplification. The negative control mixture (sterile distilled water + PCR mix) and a positive containing (DNA from *Pseudomonas stutzeri* + PCR mix) were prepared and run along with the samples.

Standardisation of primers involved several trials with varying PCR conditions for specific amplification. Initially, all PCR reactions were performed in a total volume of 50 µL containing 5 µL of 10X PCR buffer, 25 mM $MgCl_2$, 2.5 mM of each deoxynucleotide triphosphate, 0.6 µL of Taq Polymerase, 1 µL each of forward and reverse oligonucleotide primers and 3 µL of DNA. The final volume of PCR mixture was adjusted to 50 µL with sterile distilled water. PCR conditions followed included initial denaturation at (95°C, 1 min.), 30-35 cycles of primer annealing for 45 seconds followed by an elongation at 72°C for 1 min, and a final extension (72°C, 10 min.) The annealing temperature was adjusted between 48°C-53°C according to the different primer pairs used. These conditions yielded nonspecific binding and multiple amplicons regardless of the gene targeted. The PCR conditions were hence modified, and the genes were amplified as per procedure stated below.

Table 4.1. Primer sequences and positions used to amplify fragments from *nir*, *nos* and *nor* genes

Primer	**Position**[a]	**Primer sequence (5´-3´)**	**Reference**
nirS1F	763-780	CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T	Braker, *et al* 1998; Throback, *et al* 2004
nirS3F	1002-1019	TTCCT(T/C/G)CA(C/T)GACGGCGGC	Braker, *et al* 1998; Throback, *et al* 2004
nirS4R	1317-1336	TTCGG(G/A)TG(C/G)GTCTTGA(T/C)GAA	Braker, *et al* 1998; Throback, *et al* 2004
nirS6R	1638-1653	CGTTGAACTT(A/G)CCGGT	Braker, *et al* 1998; Throback, *et al* 2004
Cunir3F	504-521	CGTCTA(C/T)CA(C/T)TGCGC(A/C/G)CC	Casciotti & Ward 2001; Throback, *et al* 2004
nirK5R	1023-1040	GCCTCGATCAG(A/G)TT(A/G)TGG	Braker, *et al* 1998; Throback, *et al* 2004
nirS4F	1317-1336	TTC(A/G)TCAAGAC(C/G)CA(C/T)CCGAA	Braker, *et al* 1998
nos661 F	303-320	CGGCTGGGGGCTGACCAA	Scala & Kerkhof, 1998; Throback, *et al* 2004
nos1773 R	1396-1415	AACGA(A/C/G)CAG(T/C)TGATCGA(T/C)AT	Scala & Kerkhof, 1998; Throback, *et al* 2004
nos1527R	1171-1188	CTGTTC(A/C/T)TCGACAG(T/C)CAG	Scala & Kerkhof, 1998; Throback, *et al* 2004
nosZF	1169-1188	CG(C/T)TGTTC(A/C)TCGACAGCCAG	Kloos, *et al* 2001
nosZR	1849-1869	CATGTCCAG(A/C/G/T)GC(A/G)TGGCAGAA	Kloos, *et al* 2001
norB2F	592-610	GACAARHWVTAYTGGTGGT	Casciotti & Ward, 2005
norB6R	967-986	TGCAKSARRCCCCABACBCC	Casciotti & Ward, 2005
norB7R	1000-1019	CCRTGGSTRWARWARTTSAC	Casciotti & Ward, 2005

[a]Positions in the *nir* S gene of *Pseudomonas stutzeri* ZoBell ATCC 14405 (X56813), *nir* K gene of *Alcaligenes faecalis* S-6 (D13155), *nos* Z gene of *Pseudomonas aeruginosa* DSM 50071 (X65277) and in *nor* B gene of *Pseudomonas stutzeri* (Z28384)

4.2.2.3 *Amplification of nitrite reductase gene (nir)*

Fragments of *nirS* gene were amplified using primer pairs (a) nirS1F-nirS4R, (b) nirS3F-nirS6R and (c) nirS4F-nirS6R developed by Braker *et al*., 1998. A touchdown PCR with primer combinations a, b and c was carried out, following modified methods given by Braker *et al*., 1998. The details of the PCR conditions are given in Table 4.3.

4.2.2.4 *Amplification of nitric oxide reductase gene (nor)*

A set of two primer combinations were tested for *norB* gene amplification; (a) norB2F-norB6R and (b) norB2F-norB7R (Cassiotti and Ward, 2005). The PCR conditions have been presented in Table 4.4.

4.2.2.5 *Amplification of nitrous oxide reductase gene (nos)*

Amplification of the *nosZ* gene was carried out with the primer combination; nosZF-nosZR (Kloos *et al*., 2001). A touchdown PCR protocol was followed with these primers following modified methods of Throback *et al*., 2004. The details of this are given in Table 4.5.

4.2.2.6 *Agarose gel electrophoresis and purification of amplicons*

All PCR products were analysed using gel electrophoresis. DNA samples were mixed with 6X loading dye (Thermo Fisher Scientific) in ratio 1:4 and loaded into the gel wells. A DNA ladder of 100 bp (Promega Corporation, US) was loaded for reference. The samples were run for 20 minutes, and the separated products were examined in GelDoc system (Syngene, USA) for the presence of bands. Purification of the positive amplicons was done using Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, USA). Purified samples were stored at -20°C for sequencing purpose.

4.2.2.7. *Sequencing and phylogenetic analysis*

Purified gene amplicons were sequenced with an ABI 3130XL genetic analyser. The primers used for sequencing were nirS1F, nirS3F, and nirS4F for *nirS*, norB2F for *norB* and nosZF for *nosZ* genes. The similarity between the sequences was compared with sequences in

GenBank using NCBI BLAST (http://blast.ncbi.nlm. nih.gov). The EXPASY tool was used to translate DNA sequences into amino acid sequences. Comparative sequence analyses were conducted on the nucleotide sequences. BLASTp similarity searches were also carried out to compare translated gene sequences with those from GenBank, to be certain that the gene sequences recovered from this study indeed represent the right protein. The sequences were aligned with known sequences from NCBI using CLUSTALW. Partial gene sequences from known denitrifiers such as *Pseudomonas stutzeri, Pseudomonas aeruginosa*, *Paracoccus* denitrificans, were used as reference strains in the construction of a phylogenetic tree. All of these sequences were then used for constructing phylogenetic trees following the maximum likelihood protocol in MEGA6 software (Tamura *et al*., 2013). The trees were produced using distance matrix methods. Bootstrap values indicate the reliability of phylogenetic reconstruction (100 replicates).

4.3. Results and discussion

4.3.1. *Screening of bacterial cultures for their nitrate utilisation capacity*

The accumulation of nitrite, the first intermediate of denitrification, provides an analytical tool for its occurrence within the OMZ (Bange *et al*., 2005). The nitrate reduction test qualitatively determines the ability of bacteria to reduce nitrate.. The distribution of denitrification among prokaryotes does not follow a distinct pattern. The reaction is carried out by taxonomically diverse bacteria mainly belonging to proteobacteria.

Qualitative analysis of bacterial isolates for denitrification potential/nitrate reducing capacity yielded 111 cultures that reduced nitrate to nitrite or further. *Pseudomonas stutzeri*, a known denitrifier surviving in low oxygen waters, was used as a positive control (Table 4.2). The isolates were differentiated as complete nitrate reducers (NO_3^- to $NO/N_2O/N_2$), partial nitrate reducers (NO_3^- to NO_2^-) and non-reducers. Among the tested isolates, *Alcanivorax dieselolei, Alteromonas macleodii, Glutamicibacter nicotianae, Halomonas aquamarina, Marinobacter hydrocarbonoclasticus Nitratireductor aquibiodomus, Nitratireductor kimnyeongensis, Pseudoalteromonas arabiensis, Pseudoalteromonas lipolytica, and Pseudoalteromonas*

prydzensis, Sulfitobacter dubius, Sulfitobacter faviae, Vibrio campbellii, Vibrio splendidus were able to reduce nitrate completely under oxic conditions at 28°C and 1 bar pressure. As seen in chapter 3, the dissolved oxygen levels varied from 0.6 to 0.2 mL L^{-1}, NO_2 from 0.01 to 4.01 μM and NO_3^- from 17.23 to 36.85 μM. Complete denitrifiers like *Marinobacter hydrocarbonoclasticus, Alteromonas macleodii,* and *Alcanivorax dieselolei* were obtained from regions with high nitrate (~17-32 μmol) and nitrite (1-3 μmol) values, suggesting their active role in nitrite utilisation. *Bacillus firmus, Bacillus infantis, Bacillus megaterium, Brevibacterium casei, Halomonas meridiana, Idiomarina zobelli, Marinobacter litoralis, Micrococcus luteus, Nesterenkonia lacusekhoensis, Staphylococcus saprophyticus, and Vibrio parahaemolyticus* were negative for nitrate reduction and mostly found at depths where nitrite values were ~0.02 μmol or lower. Most clades of gamma-proteobacteria are known to denitrify using nitrate as an electron acceptor (Miller *et al*., 2010). Based on the results of the Griess test, 29 representative isolates belonging to 9 genera namely; *Alcanivorax, Alteromonas, Bacillus, Glutamicibacter, Halomonas, Marinobacter, Pseudoalteromonas, Sulfitobacter* and *Vibrio* were positive for NO_3^- /NO_2^- reduction. Bacteria belonging to these genera are reported to contain genes involved in the nitrogen cycle (Jones *et al*., 2011; Cai and Jiao, 2008). Among gamma-proteobacteria, the predominant Alteromonodales in the AS OMZ appears to be a proficient denitrifier group (Bandekar *et al*., 2018). In our study, 25 isolates of *Marinobacter hydrocarbonoclasticus* were obtained. This species of bacteria has been previously shown to demonstrate efficient denitrifying ability (Li *et al*., 2013). Also, 21 isolates of *Pseudoalteromonas* were obtained and the presence of *nasA* gene (called *narB* genes in cyanobacteria) in *Pseudoalteromonas* sp. has been well documented in marine environments (Allen *et al*., 2001). Verbaendert *et al*., 2011 have reported many different species of *Bacillus* to be efficient denitrifiers. Reviewing marine bacteria involved in nitrate reduction is useful to note rates of nitrate utilisation. Gomes *et al*., 2018 have reported nitrate reduction rates from pure cultures of *M. hydrocarbonoclasticus* and *A. macleodii* to be 0.28 and 0.17 μM day^{-1}. Takaya *et al*., 2003 also reported N_2O production from *Pseudomonas stutzeri* to be in the range of 1.5×10^{-2} to 6.75×10^{-5} μmol min^{-1}.

Table 4.2. Nitrate reduction test of bacterial isolates obtained from Arabian Sea OMZ. Key: (- -) No nitrate reduction, (+-) Incomplete nitrate reduction (Reduction of NO_3^- to NO_2^-), (++) Complete nitrate reduction (Reduction of NO_3^- to $NO/N_2O/N_2$)

Sr. no.	Closest match in NCBI	Reduction of NO_3^-
1	*Alcanivorax dieselolei*	+ +
2	*Alteromonas macleodii*	+ +
3	*Bacillus firmus*	- -
4	*Bacillus flexus*	+ -
5	*Bacillus infantis*	- -
6	*Bacillus megaterium*	- -
7	*Brevibacterium casei*	- -
8	*Brevibacterium epidermidis*	+ -
9	*Brevibacterium sanguinis*	+ -
10	*Glutamicibacter nicotianae*	+ +
11	*Halomonas aquamarina*	+ +
12	*Halomonas axialensis*	+ -
1 3	*Halomonas denitrificans*	+ -
14	*Halomonas halodenitrificans*	+ -
15	*Halomonas meridiana*	- -
16	*Halomonas venusta*	+ -
17	*Idiomarina zobelli*	- -
18	*Marinobacter algicola*	- -
19	*Marinobacter alkaliphilus*	+-
20	*Marinobacter hydrocarbonoclasticus*	+ +
21	*Marinobacter flavimaris*	+-
22	*Marinobacter litoralis*	- -

23	*Micrococcus luteus*	- -
24	*Nesterenkonia lacusekhoensis*	- -
25	*Nitratireductor aquibiodomus*	+ +
26	*Nitratireductor kimnyeongensis*	+ +
27	*Pseudoalteromonas arabiensis*	+ +
28	*Pseudoalteromonas lipolytica*	+ +
29	*Pseudoalteromonas nigrifaciens*	+ -
30	*Pseudoalteromonas prydzensis*	+ +
31	*Pseudoalteromonas ruthenica*	+-
32	*Pseudomonas stutzeri*	+ +
33	*Staphylococcus arlettae*	+ -
34	*Staphylococcus cohnii*	+ -
35	*Staphylococcus saprophyticus*	- -
36	*Sulfitobacter dubius*	++
37	*Sulfitobacter faviae*	++
38	*Vibrio campbellii*	+ +
39	*Vibrio harveyi*	+ -
40	*Vibrio splendidus*	+ +
41	*Vibrio parahaemolyticus*	- -

4.3.2. *Functional gene analysis*

Molecular studies on denitrification are based on the detection and activity of functional marker genes which code for key enzymes in the denitrification process. Targeting these marker genes for their presence or activity of the enzymes gives a clear understanding of the denitrifying community existing in the water column. Several enzymes are involved in the complete denitrification process and these present targets for detection of denitrifiers in environmental studies that may be simpler to interpret from the perspective of functional diversity. The

presence and diversity of the *nirS, norB* and *nosZ* gene homologs among cultured nitrate reducing bacteria was investigated using the polymerase chain reaction and DNA sequencing. This work is a necessary precursor to studying the expression of the genes involved in the nitrogen cycling pathway.

A consistent methodology was applied throughout the study, allowing a robust relative comparison of samples. *Pseudomonas stutzeri* is an active denitrifying heterotrophic bacterium and considered as a model organism for studying the denitrification process (Zumft, 1997; Lalucat *et al*., 2006). Hence, its application as a positive denitrifier strengthens the conclusions drawn from this study. All the cultures with positive amplification for the denitrification genes and the closest match in the NCBI database are presented in Table 4.6.

4.3.2.1 *Nitrite reductase genes (nir)*

Combinations of three primer sets showed positive amplification with the DNA of *P. stutzeri*. All other isolates obtained from this study were hence tested with these sets of primers and standardised PCR conditions (Table 4.3). The amplification products obtained with the above primer combinations were analysed by electrophoresis on 1% agarose gel. Amplicons of ~550 bp, ~600 bp, and ~320 bp were obtained as depicted in Figure 4.2.

Most of the isolates showed multiple bands when tested for *nir* S gene probably because of the presence of multiple domains (Throback *et al*., 2004). However, sections of the *nirS* gene were successfully amplified from seven nitrate-reducing bacteria, belonging to gamma proteobacteria and actinobacteria group. These strains include *Marinobacter hydrocarbonoclasticus, Nitratireductor aquibiodomas, Pseudoalteromonas arabiensis, Pseudoalteromonas nigrifaciens, Pseudoalteromonas prydzensis, Nesterenkonia lacusekhoensis,* and *Brevibacterium sanguinis.* The *nirS* gene from *Marinobacter hydrocarbonoclasticus* was positively amplified using all three combinations of primers whereas primer combination 3F:6R worked well for *Brevibacterium sanguinis* and *Nitratireductor aquibiodomus* and 1F:4R for the rest of the samples.

Table 4.3. Primers for the *nirS* gene, PCR conditions used for gene amplification and the desired amplification length of the gene product.

Primer combination	PCR conditions	Desired amplicon size
(a) nirS1F-nirS4R	30 PCR cycles including ten initial cycles of 30s of denaturation at 94°C, primer annealing for 1 min with a touch down from 52 to 48°C, primer extension at 72°C for 1 min, and finally 30 cycles with a constant annealing temperature (48°C).	~550 bp
(b) nirS3F-nirS6R	30 PCR cycles including ten initial cycles of 30s of denaturation at 94°C, primer annealing for 1 min with a touch down from 50 to 45°C, primer extension at 72°C for 1 min., and finally 30 cycles with a constant annealing temperature (48°C).	~600 bp
(c) nirS4F- nirS6R	30 PCR cycles including ten initial cycles of 30s of denaturation at 94°C, primer annealing for 1 min with a touch down from 48 to 43°C, primer extension at 72°C for 1 min., and finally 30 cycles with a constant annealing temperature (48°C).	~320 bp

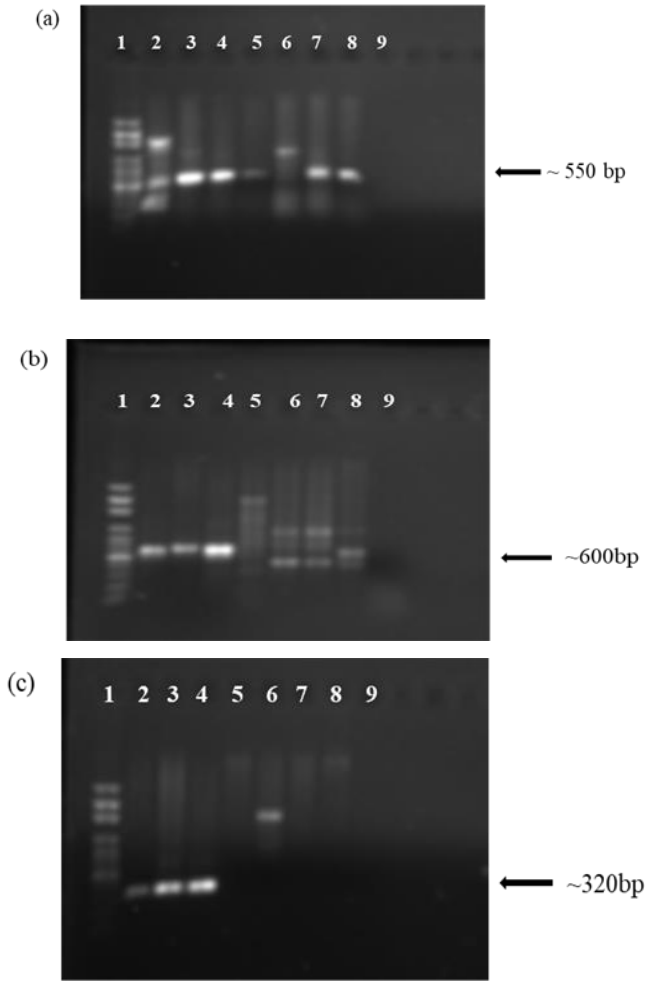

Figure 4.2. PCR amplicons of the *nirS* gene (a) amplification with primer pair nirS1F-nirS4R, lane 1: 100bp DNA ladder (100 to 3000 bp); Lanes 2 to 7 (representative strains of *Marinobacter hydrocarbonoclasticus* and *Pseudoalteromonas* sp. obtained from pure cultures from this study); Lane 8 (*Pseudomonas stutzeri*, MTCC 101) depict the PCR products amplified at ~700 bp; lane 9: PCR negative control (b) amplification with primer pair nirS4F-nirS6R, lane 1: 100bp DNA ladder (100 to 3000 bp); Lanes 2 to 7 (representative strains of *Marinobacter hydrocarbonoclasticus* and *Pseudoalteromonas* sp. obtained from pure cultures from this study); Lane 8 (*Pseudomonas stutzeri*, MTCC 101) depict the PCR products amplified at ~600 bp; lane 9: PCR negative control (c) amplification with primer pair nirS3F-nirS6R, lane 1: 100bp DNA ladder (100 to 3000 bp); Lane 2 (*Pseudomonas stutzeri*, MTCC 101); Lane 3 to 8 (representative strains of *Marinobacter hydrocarbonoclasticus*, *Pseudoalteromonas* sp. and *Halomonas* sp. obtained from pure cultures from this study); depict the PCR products amplified at ~320 bp; lane 9: PCR negative control

4.3.2.2. *Nitric oxide reductase genes (nor)*

The *norB* gene was successfully amplified from eight samples belonging to *M. hydrocarbonoclasticus, P. arabiensis, P. nigrifaciens*, and *P. prydzensis* species. Positive amplification of *norB* gene from the tested isolates was obtained with the primer pair; norB2F-norB7R (Table 4.4). Amplicons of ~440bp were obtained by gel electrophoresis technique (Figure 4.3).

Table 4.4. Primers for the *norB* gene, PCR conditions used for gene amplification and the desired amplification length of the gene product.

Primer combination	PCR conditions	Desired amplicon size
norB2F-norB7R	Initial denaturation of DNA at 95°C for 1 min, followed by 35 cycles of primer annealing at 50°C for 45 seconds, elongation at 72°C for 1 min with an extension (72°C, 10 min.)	~ 440bp

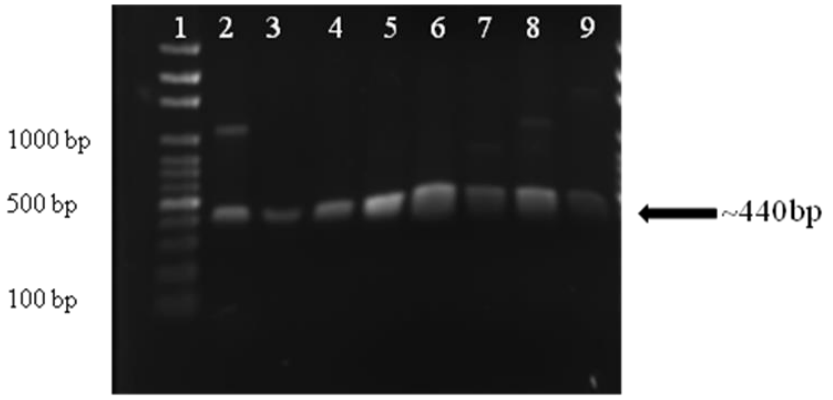

Figure 4.3. PCR amplicons of the *norB* gene, Lane 1: 100bp DNA ladder (100-3000 bp); lane 2 (*Pseudomonas stutzeri*, MTCC 101); lane 3, 4, 5, (amplified products of *Pseudoalteromonas arabiensis*), lane 6, 7 (amplified products of *Pseudoalteromonas nigrifaciens*), lane 8 (amplified products of *Pseudoalteromonas prydzensis),* lane 9 (amplified product of *Marinobacter hydrocarbonoclasticus)* isolates obtained from pure cultures from this study, amplified at ~440 bp.

4.3.2.3. *Nitrous oxide reductase genes (nos)*

Nitrous oxide reductase genes were amplified using primer pair nosZF-nosZR (Table 4.5). Amplicons of ~ 700bp were obtained (Figure 4.4). Most of the samples tested with these primers showed positive amplification. A total of 29 gene fragments were obtained belonging to *Marinobacter*, *Halomonas*, *Pseudoalteromonas*, *Idiomarina*, *Alcanivorax* and *Bacillus* genera.

Table 4.5. Primers for the *nosZ* gene, PCR conditions used for gene amplification and the desired amplification length of the gene product.

Primer combination	PCR conditions	Desired amplicon size
nosZF-nosZR	30 PCR cycles including ten initial cycles of 30 s of denaturation at 94°C, primer annealing, touch down from 55 to 50°C, primer extension (72°C,1 min.), 30 cycles with a annealing temperature of 50°C.	~ 700bp

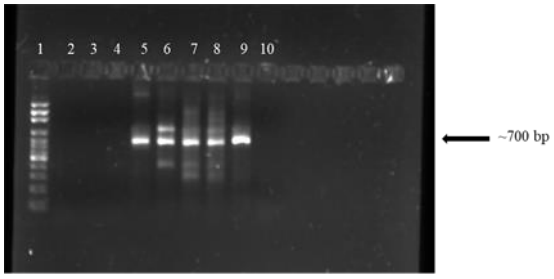

Figure 4.4. PCR amplicons of the *nosZ* gene from 5 samples, Lane 1: 100bp DNA ladder (100 to 3000 bp); Lane 5 to 8 (four strains of *Marinobacter hydrocarbonoclasticus* isolates obtained from pure cultures from this study); Lane 9 (*Pseudomonas stutzeri*, MTCC 101) depict the PCR products amplified at ~700 bp; lane 10: PCR negative control

Table 4.6. Bacterial strains used in this study, and PCR amplification of denitrifying genes (*nirS, norB and nosZ)* obtained with different sets of primers. Primers for *nirS*: nirS1F-nirS4R, nirS3F-nirS6R, nirS4F- nirS6R; Primers for *norB:* nor B2F:7R and primers for *nosZ:* nos ZF:ZR

Sr. no.	**Bacteria***	***nirS***	***norB***	***nosZ***
1	*Alcanivorax dieselolei*	ND	ND	NIOSSK056#77
2	*Alteromonas macleodii*	ND	ND	ND
3	*Bacillus firmus*	ND	ND	ND
4	*Bacillus flexus*	ND	ND	NIOSSK056#39
5	*Bacillus infantis*	ND	ND	ND
6	*Bacillus megaterium*	ND	ND	ND
7	*Brevibacterium casei*	ND	ND	ND
8	*Brevibacterium epidermidis*	ND	ND	ND
9	*Brevibacterium sanguinis*	NIOSSD026A#12nirS3F6R	ND	ND
10	*Glutamicibacter nicotianae*	ND	ND	ND
11	*Halomonas aquamarina*	ND	ND	NIOSSK056#81 NIOSSK079#5
12	*Halomonas axialensis*	ND	ND	ND
13	*Halomonas denitrificans*	ND	ND	NIOSSK056#83
14	*Halomonas halodenitrificans*	ND	ND	NIOSSK056#82

15	*Halomonas meridiana*	ND	ND	NIOSSK056#38
16	*Halomonas venusta*	ND	ND	
17	*Idiomarina zobelli*	ND	ND	NIOSSK056#84 NIOSSK056#85 NIOSSK069#2
18	*Marinobacter algicola*	ND	ND	NIOSSK056#86
19	*Marinobacter alkaliphilus*	ND	ND	
20	*Marinobacter hydrocarbonoclasticus*	NIOSSK069#3nirS1F4R NIOSSK069#4nirS1F4R NIOSSK069#5nirS1F4R NIOSSK056#87nirS1F4R NIOSSK056#86nirS1F4R NIOSSK069#3nirs4F6R NIOSSK069#4nirs4F6R NIOSSK069#5nirs4F6R NIOSSK069#3nirS3F6R NIOSSK069#5nirS3F6R NIOSSD026A#50nirS3F6R	NIOSSK056#88	NIOSSK056#88 NIOSSK056#89 NIOSSK056#90 NIOSSK069#4 NIOSSK069#3
21	*Marinobacter flavimaris*	ND	ND	ND
22	*Marinobacter litoralis*	ND	ND	NIOSSK079#66

23	*Micrococcus luteus*	ND	ND	ND
24	*Nesterenkonia lacusekhoensis*	NIOSSK069#8nirS1F4R	ND	ND
25	*Nitratireductor aquibiodomus*	NIOSSD026A#70nirS3F6R	ND	ND
26	*Nitratireductor kimnyeongensis*	ND	ND	ND
27	*Pseudoalteromonas arabiensis*	NIOSSK079#29 NIOSSK079#42 NIOSSK079#60	NIOSSK079#29 NIOSSK079#42 NIOSSK079#60 NIOSSK079#19	NIOSSK079#19 NIOSSK079#29 NIOSSK079#20 NIOSSK079#22 NIOSSK079#42 NIOSSK079#60
28	*Pseudoalteromonas lipolytica*	ND	ND	NIOSSK056#93
29	*Pseudoalteromonas nigrifaciens*	NIOSSK079#47	NIOSSK079#47 NIOSSK079#74	NIOSSK079#74 NIOSSK079#15 NIOSSK079#47
30	*Pseudoalteromonas prydzensis*	NIOSSK079#4 NIOSSK079#61	NIOSSK079#1	NIOSSK079#52 NIOSSK079#1 NIOSSK079#4
31	*Pseudoalteromonas ruthenica*	ND	ND	ND
32	*Pseudomonas stutzeri*	ND	ND	ND

33	*Staphylococcus arlettae*	ND	ND	ND
34	*Staphylococcus cohnii*	ND	ND	ND
35	*Staphylococcus saprophyticus*	ND	ND	ND
36	*Sulfitobacter dubius*	ND	ND	ND
37	*Sulfitobacter faviae*	ND	ND	ND
38	*Vibrio campbellii*	ND	ND	ND
39	*Vibrio harveyi*	ND	ND	ND
40	*Vibrio splendidus*	ND	ND	ND
41	*Vibrio parahaemolyticus*	ND	ND	ND

*Closest match in NCBI derived from 16S rDNA sequencing
ND: Not determined

4.3.2.4. *Phylogenetic analyses*

The NCBI BLAST analysis of gene sequences generated in this study showed similarity with genes from closely related cultured bacteria as well as uncultured bacteria. The phylogenetic relationship between these sequences is depicted in Figures 4.5 (a, b, c for *nirS*), 4.6 (*norB)* and 4.7 (*nosZ*).

The *nirS* sequences were successfully amplified with three different primer sets. Sequences generated with each primer s*et al*igned satisfactorily with sequences of known denitrifiers obtained from the NCBI database. A total of 7 sequences derived with primer combination nirS1F-4R clustered with *Marinobacter* sp. (cluster a1), *Halomonas* sp. (cluster a2) and *Pseudomonas* sp. (cluster a3). Clustering of sequences within the Gammaproteobacteria group was evident among these three clusters. Cluster a4 contains known denitrifiers belonging to the alpha-proteobacteria group (*Roseobacter denitrificans, Paracoccus denitrificans,* and *Paracoccus pantotrophus*) and the beta-proteobacteria group (*Ralstonia eutropha*). In all cases, the bootstrap values were higher than 84. Similarly, three sequences from the test isolates (primer set nirS4F-6R) clustered with the *nirS* gene of *Marinobacter hydrocarbonoclasticus* (bootstrap values, >90). For the 3rd set of nirS primers, i.e., nirS3F-6R, six sequences from the test isolates clustered with *Marinobacter* species (bootstrap values, >90). The *nirK* gene from *Blastobacter denitrificans* was used as outgroup in all three phylogenetic trees emphasising that the sequences generated indeed belonged to the *nirS* gene cluster. However, when *nirS* sequences from our study were compared with the *nirS* sequences from representatives of alpha-proteobacteria, beta proteobacteria or gamma proteobacteria, many of the sequences were closely related to one another than to the previously reported genes.

Among the isolates tested for the detection of the *norB* gene, eight sequences were obtained with the *norB* specific primer set. These sequences were aligned with complete or partial *norB* gene sequences obtained from the NCBI GenBank database. The phylogenetic tree generated revealed grouping of eight *norB* gene sequences from the cultures with *Halomonas halodenitrificans*, *Marinobacter hydrocarbonoclasticus,* and *Pseudomonas stutzeri*. These organisms are known denitrifiers belonging to the gamma-proteobacteria group. The bootstrap values were >70 and indicated the robustness of the analysis. The majority of known denitrifiers which harbour *norB* genes are α- and

β- subdivisions of the Proteobacteria (Casciotti and Ward, 2005), but the dominant group in the present study was gamma-proteobacteria. This phenomenon may be linked with environmental specificity as suggested by Philippot, in 2002. The previous study exploring the diversity of *nir* genes in environmental samples (Braker *et al*., 2000) suggested that the *cnorB* branch of *norB* sequences among known denitrifiers of the environmental sequences might be derived from proteobacteria.

The *nosZ* gene sequences (partial) were effectively amplified and sequenced from *Pseudoalteromonas* spp. (12 isolates), *Marinobacter* spp. (5 isolates), *Halomonas* spp. (3 isolates), *Idiomarina* spp. (3 isolates), *Bacillus flexus* and *Alcanivorax dieselolie*. Phylogenetic analysis of *nosZ* from 25 strains obtained in this study and six *nosZ* sequences selected from the NCBI database generated the tree presented in figure 4.6. The *nosZ* gene is known to be highly conserved among N_2O reductases such as *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, *Ralstonia eutropha*, *Pseudomonas denitrificans,* and *Sinorhizobium meliloti*. Hence, these strains were chosen for building the tree. The gene sequences obtained in this study clustered well with the *nosZ* gene from *Marinobacter hydrocarbonoclasticus* and *Pseudomonas aeruginosa*. N_2O reductase genes were recovered from the α, β and γ groups of proteobacteria but no putative nitrous oxide gene cluster has been identified in gram-positive or archaebacteria (Philippot, 2002).

Molecular studies undeniably provide information on the diversity of genes in a system, but in many cases, it is difficult to link the results obtained with functional genes to those from the rDNA approach. Even though numerous partial denitrifying sequences are available in the GenBank databases, a large number of them are from uncultured and unidentified strains. Not enough phylogenetic information (especially from *nirS*) is available to cluster these sequences among phylogenetically and physiologically characterised organisms (Goregues *et al*., 2003). The results presented here identified proteobacteria as the dominant denitrifier in the AS-OMZ. The isolates obtained from our study possess *nirS, norB* and *nosZ* homologs similar enough to published denitrifier genes that have been detected using degenerate PCR primers.

Phylogenetic analysis confirmed that PCR products from cultured strains were indeed *nirS*, *norB*, and *nosZ* gene fragments. Amongst the isolates tested for the presence of these genes, cultures of *Marinobacter hydrocarbonoclasticus* and *Pseudoalteromonas* sp. showed the presence of all three genes emphasising their role in denitrification processes in the Arabian Sea. Studies on *Marinobacter hydrocarbonoclasticus* by Tiedje, 1988 have shown that it is a true denitrifier since it can anaerobically reduce 90% of nitrate to N_2O in the presence of acetylene. Also, a novel strain of *M. hydrocarbonoclasticus* was shown to almost completely reduce NO_3^- under optimum conditions. This organism was also capable of rapid conversion of NO_3- into N_2 without NO_2- or N_2O accumulation (Li *et al*., 2013). Members of the genus *Pseudoalteromonas* are exclusively derived from marine waters, although representatives of most culturable bacterial genera can be isolated both from terrestrial and marine environments. *Pseudoalteromonas* is the largest genera in the gamma-proteobacteria and currently comprises more than 30 species (Ivanova & Mikhailov, 2001). It is a relatively recent genus revised from the previously designated *Alteromonas* genus based on phylogenetic comparisons performed by Gauthier *et al*., in 1995. These heterotrophic bacteria have received significant levels of attention because they are readily cultivated and frequently found in association with eukaryotic hosts in the marine environment (Holmstrom and Kjelleberg, 1999). Previous studies in the Arabian Sea (Bandekar *et al*., 2018) have shown members belonging to *Alteromonadales* to substantially contribute to a stable bacterial community structure in the OMZ depths. However, not many studies have reported the existence of this genus in the oxygen minimum zone. The novelty of sequences in these samples could be due to spatial and temporal differences between the sites in the Arabian Sea and the previously studied regions (Jayakumar *et al*., 2009)

An essential aspect in the study of microbial environmental diversity involves a critical analysis of primer design. Some of the work on this aspect published by Throback, *et al*., 2004 and Braker, *et al*., 1998, demonstrates the need to re-evaluate primers for PCR amplification of denitrifying bacteria. The absence of amplification in our cultured bacteria does not necessarily mean an absence of the denitrification function in those bacteria. This indicates the need for the development of better primer systems

and stringent amplification procedures. However, other molecular methods of gene analysis like DGGE proved to be an excellent tool for screening and comparing denitrifying communities in different types of environmental samples (Divya *et al*., 2011; Jain *et al*., 2014). Clustering of nir and nor functions may be common among denitrifiers and may facilitate genetic analysis as seen in the pseudomonads and in *Paracoccus denitrificans* where these genes are closely linked. It is crucial for the nitrite reductases and nitric oxide reductases to act concomitantly in order to keep free nitric oxide at a minimum level and prevent its accumulation in a bacterial cell (Zumft, 1993). Studies in *P. stutzeri* have also shown a link between the *nos* and the *nir, nor* genes (Zumft, 1997). Among the diversity studies in the Arabian Sea, Jayakumar *et al*., 2013 demonstrate the dominance of *nirS* in comparison to *nirK* genes in mid-depths of the Arabian Sea OMZ. These results support our observation of successful amplification of the *nirS* gene among our cultures. As mentioned earlier, not all the denitrification genes are present in one organism. The energy produced during denitrification decreases with the sequential reduction of substrates proportional to their oxidation number (Koike and Hattori, 1975). Likewise, the functional genes in each step of denitrification decrease in abundance (Bru *et al*., 2011) due to lower free energy available for liberation at each step along the pathway. Hence, the detection of bacterial cultures possessing all three genes is a significant finding and forms an integral part of the bacterial community structure in the Arabian Sea. Since most environmental sequences do not match the many sequences now available for isolated organisms, biodiversity studies provide little information on interactions and ecological roles of molecular isolates in their habitats. An understanding of the influence of environmental factors on denitrifier diversity and their impact on denitrification processes requires consideration of the physiological characteristics of different groups including clusters of not yet cultured strains, which in turn requires analysis of representative cultured strains.

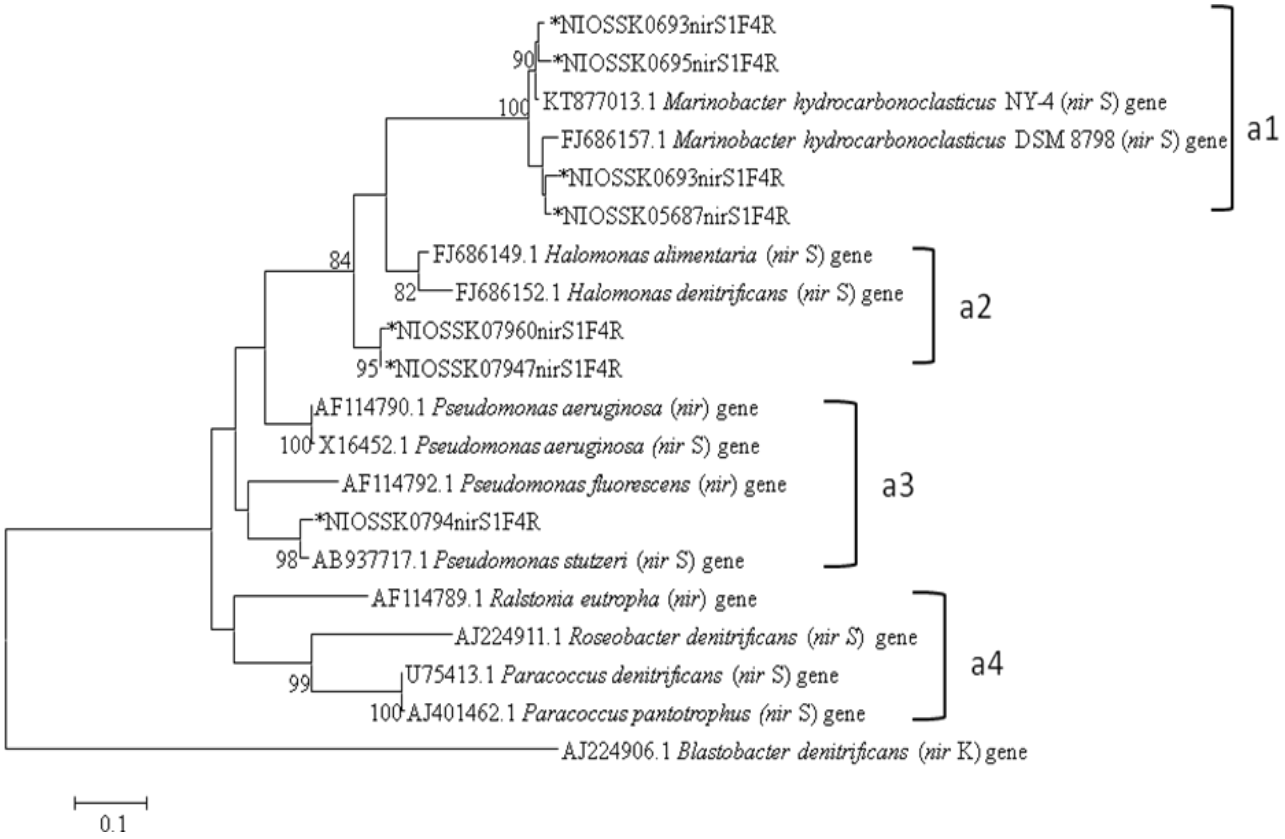

Figure 4.5A. Phylograms based on partial gene fragments of *nirS* gene. Phylogenetic positions of gene sequences generated from isolates obtained in this study (indicated by *) aligned with reference sequences derived from the GenBank are shown. Clusters a1, a2, and a3 represent sequences from *Marinobacter* spp. *Halomonas* spp., and *Pseudomonas* spp. respectively. Cluster a4 contains seqences from known denitrifiers of the α and β proteobacteria groups. The tree was generated with primer pairs nirS1F:4R based on maximum likelihood protocol. The scale bar indicates the percentage of nucleotide substitutions. Bootstrap values have been reported at the nodes. The sequence of *nirK* from *Blastobacter denitrificans* (Accession No. AJ224906) was used as outgroup to root the phylogram.

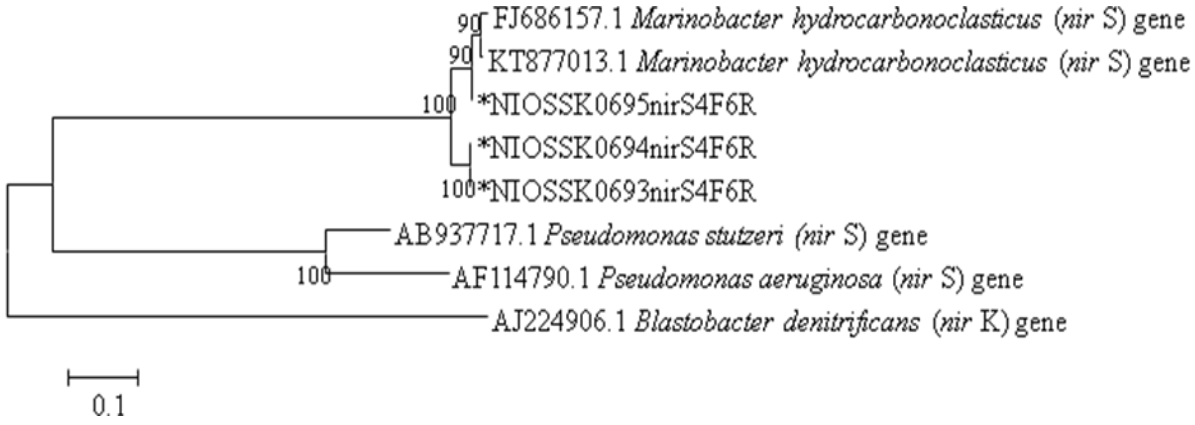

Figure 4.5B. Phylograms based on partial gene fragments of *nirS* gene generated with primer pair nirS4F:6R. Phylogenetic positions of gene sequences generated from isolates obtained in this study (indicated by *) aligned with reference sequences derived from the GenBank are shown. The tree is based on maximum likelihood protocol. The scale bar indicates the percentage of nucleotide substitutions. Bootstrap values >70 have been reported at the nodes. The sequence of *nirK* from *Blastobacter denitrificans* (Accession No. AJ224906) served as outgroup to root the phylogram.

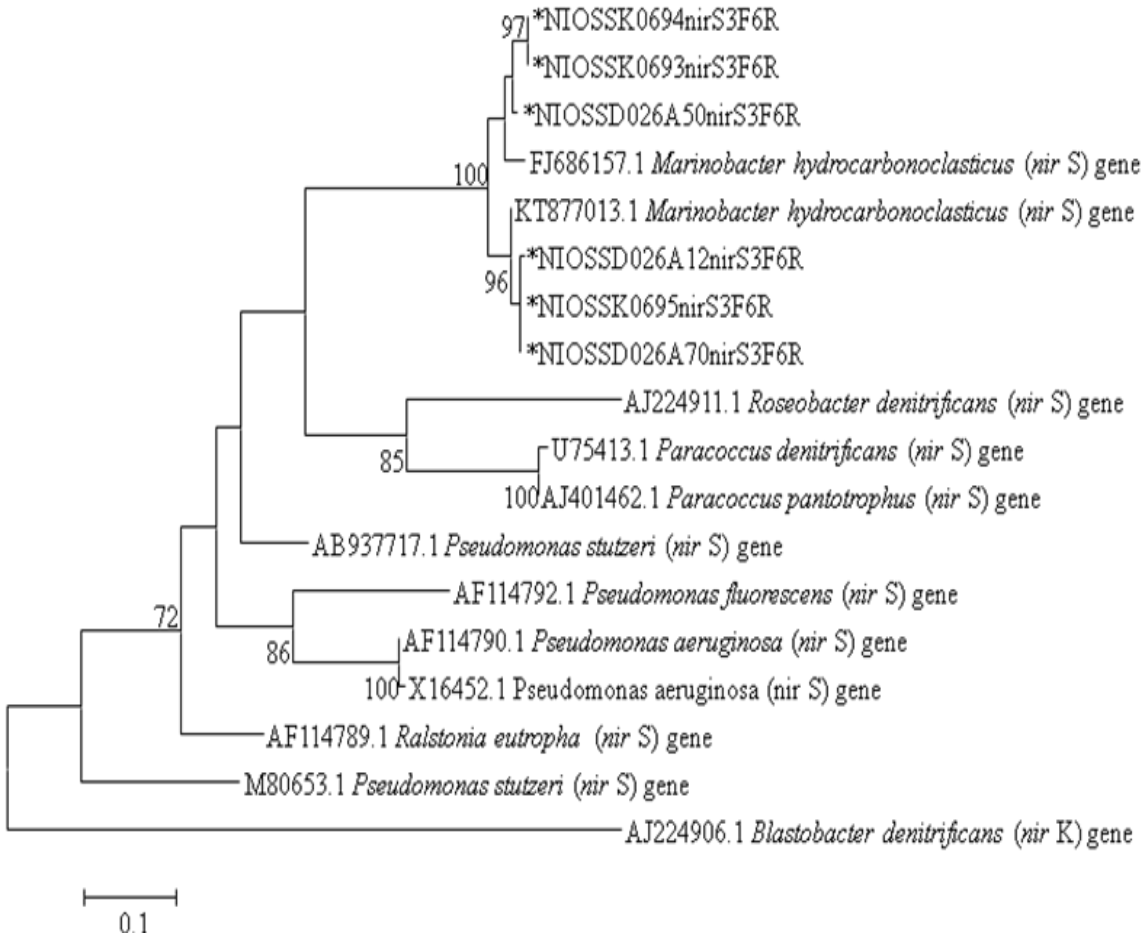

Figure 4.5C. Phylograms based on partial gene fragments of *nirS* gene generated with primer pair nirS3F:6R. Phylogenetic positions of gene sequences generated from isolates obtained in this study (indicated by *) aligned with reference sequences derived from the GenBank are shown. The tree is based on maximum likelihood protocol. The scale bar indicates the percentage of nucleotide substitutions. Bootstrap values >70 have been reported at the nodes. The sequence of *nirK* from *Blastobacter denitrificans* (Accession No. AJ224906) served as outgroup to root the phylogram.

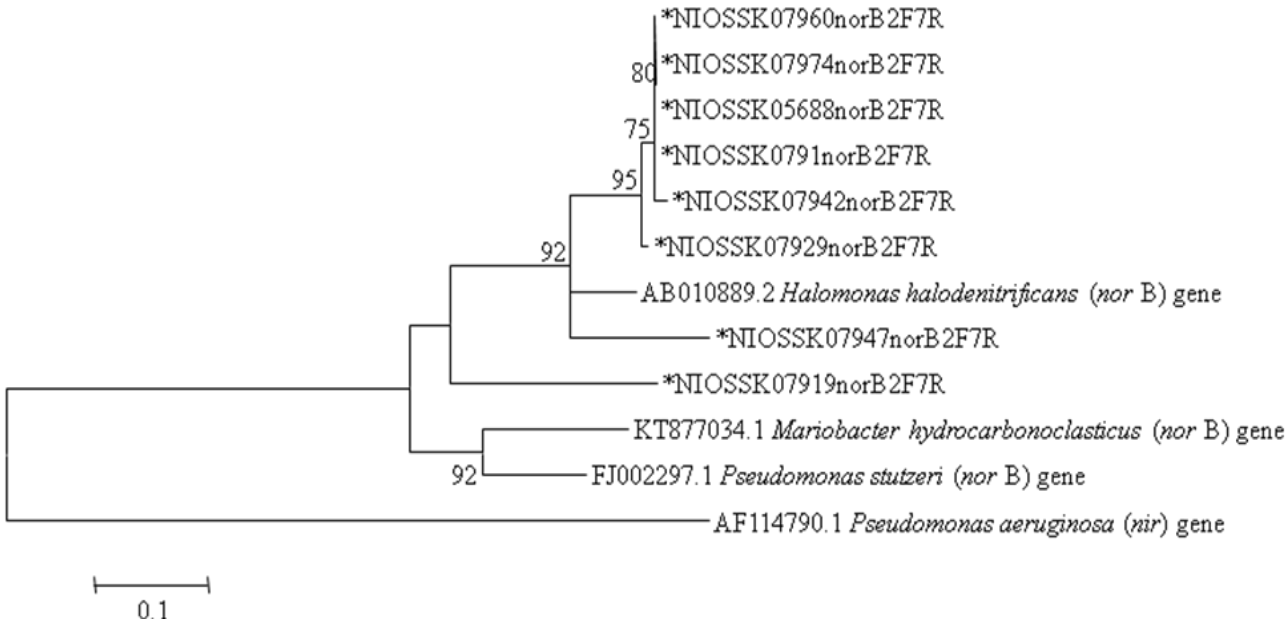

Figure 4.6. Phylograms based on partial gene fragments of *norB* gene generated with primer pair norB2F:7R. Phylogenetic positions of gene sequences generated from isolates obtained in this study (indicated by *) aligned with reference sequences derived from the GenBank are shown. The tree is based on maximum likelihood protocol. The scale bar indicates the percentage of nucleotide substitutions. Bootstrap values >70 have been reported at the nodes. The sequence of *nir* gene from *Pseudomonas aeruginosa* (Accession No. AF114790) served as outgroup to root the phylogram.

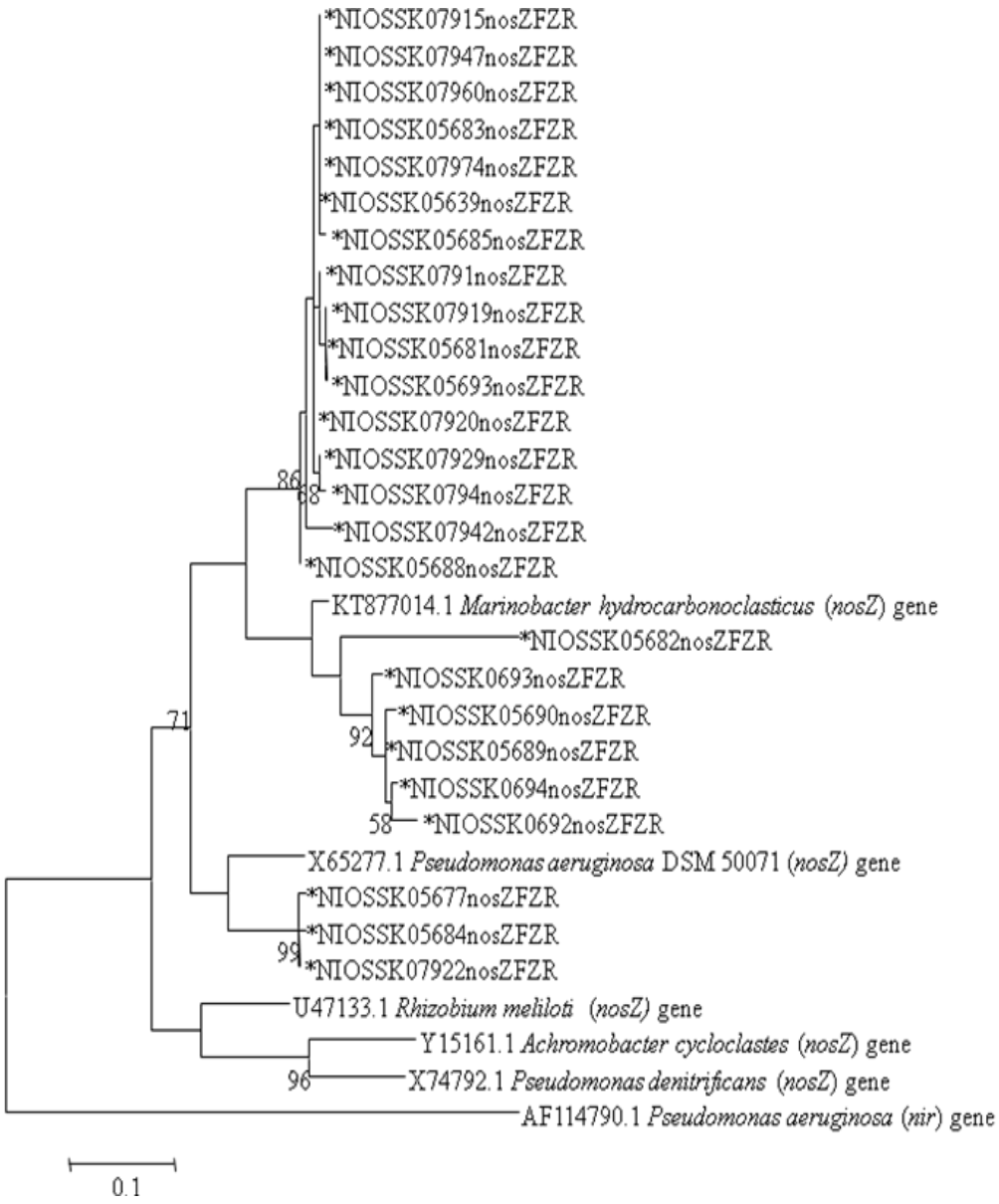

Figure 4.7. Phylograms based on partial gene fragments of *nosZ* gene generated with primer pair nosZF: ZR. Phylogenetic positions of gene sequences generated from isolates obtained in this study (indicated by *) aligned with reference sequences derived from the GenBank are shown.The tree is based on maximum likelihood protocol. The scale bar indicates the percentage of nucleotide substitutions. Bootstrap values >50 have been reported at the nodes. The sequence of *nir* gene from *Pseudomonas aeruginosa* (Accession No. AF114790) was used as outgroup to root the phylogram.

Chapter 5

Summary

Summary

The Arabian Sea has unique features governed by the geographical setting of the region. The behaviour of the Arabian Sea is different from the conventionally understood tropical basins due to its high ambient salinity, reversal of monsoons, coastal and open ocean upwelling and winter cooling effects (Kumar *et al*., 2000). Besides being a highly productive basin for most parts of the year, it harbours one of the most intense subsurface oxygen minimum zones in the world contributing to ~40% of the global pelagic N_2 production (Bange *et al*., 2005). In the marine environment, the nitrogen budget is believed to be imbalanced due to higher rates of consumption of N_2 than its fixing from the atmosphere. The biogeochemical system of nitrogen cycling is comprised of multi-step pathways involving the continuous microbial oxidation and reduction of organic and inorganic nitrogen intermediates. Majority of these conversions are carried out by bacteria and yield significant control over the bioavailability of nitrogen and the biological productivity of ecosystems. The process of denitrification appears to be the major pathway for fixed nitrogen loss in the Arabian Sea. Bacterial denitrification is nearly exclusively facultative. A taxonomically differing group of bacteria can reduce nitrate into nitrite, wherein low levels in oxygen concentration and availability of nitrogen oxide governs its expression. Heterotrophic micro-organisms remineralise most of the organic matter in the world oceans via aerobic respiration. However, when oxygen becomes deficient, micro-organisms use NO_3^- for the oxidation of organic matter. In canonical denitrification pathway, nitrate is reduced to dinitrogen in a series of four reductions, i.e., Nitrite reductase (encoded by the *nir* gene), followed by nitric oxide reductase (encoded by *nor* gene) and later by nitrous oxide reductase (encoded by *nos* gene).

Some bacteria contain genes encoding all the enzymes in the denitrification pathway, while others have a condensed mechanism performing fewer reactions (Hartig, 1999). Studies on denitrifying genes have been carried out in only a few species despite the great diversity of bacteria capable of denitrification. Besides their role in the nitrogen cycle, the denitrifying genes also provide a good model for studying the evolutionary relationship of functional genes. High rates of denitrification occur in low oxygen portions of the water column in the Arabian Sea and release a significant amount of N_2O

into the atmosphere besides being a sink for fixed nitrogen. Research on the biogeochemistry of nitrogen aims to elucidate the distribution of various nitrogen compounds in the environment by characteristics of the bacteria that produce and consume them. Hence, it is of utmost importance to understand what controls the abundance and activities of the particular kind of bacteria involved in these processes. With this background of the importance of bacterioplankton in the oxygen minimum zone of the Arabian Sea, the following objectives were framed with an attempt to address some of the lacunae existing in the previous studies. The objectives, methodology in brief and the salient findings of each objective are given as follows:

Objective I. Estimation of the abundance and distribution of bacterioplankton.

- The abundance and distribution of bacterioplankton were studied in response to the physical characteristics of the coastal and open ocean in the Arabian Sea. The data included physical oceanographic observations carried out onboard during four cruises and microscopy and flow cytometry based bacterioplankton abundance.
- Upwelling in the coastal waters was evident from the hydrographic properties of the water column which changed mostly in the upper 200 m. An upsloping of isotherms in the coastal region and the decrease in the MLD in the station farthest to the one nearest to the coast confirmed the occurrence of wind-induced eastern boundary upwelling phenomenon. A well-stratified salinity structure was observed below 50 m depth of the water column possibly due to the intrusion of high saline waters from the Red Sea and Persian Gulf waters. Likewise, the coastward upsloping of low oxygen water (<2 mL L^{-1}) from the offshore region was observed. Apart, a seasonal variation in the temperature, salinity and dissolved oxygen profiles within the central Arabian Sea indicated the occurrence of upwelling during summer monsoon and winter cooling and convective mixing during winter monsoon.
- Spatial variation among three groups of autotrophic picoplankton was seen between the coastal and offshore stations. *Synechococcus* abundances were generally higher at the coast (67-98 x 10^3 cells mL^{-1}) than in the offshore region (5.9-20 x 10^3 cells mL^{-1}). Abundances of *Prochlorococcus* in the coastal transect ranged between 0 and 20x10^3 cells mL^{-1}, much lower than the offshore stations (>50 x 10^3 cells mL^{-1}). The

abundances of picoeukaryotes were comparable between the coastal and the offshore stations, ranging between 0 and $13.8x10^3$ cells mL^{-1}. Abundance maxima in all the picoplankton groups were seen at the base of the thermocline and usually coincided with the chlorophyll maxima depth at each station. Overall, *Prochlorococcus* tends to be an important component of the picoplankton community at subsurface depth in the off-shore stations while *Synechococcus* contributed substantially to total abundance in the coastal stations. Picoeukaryotes had a uniform distribution across locations.

- Latitudinal differences in bacterioplankton abundance were observed in the central Arabian Sea. *Synechococcus* presented higher abundance in the northern stations ($44\text{-}202x10^3$ cells mL^{-1}) as compared to southern stations ($14\text{-}92x10^3$ cells mL^{-1}). *Prochlorococcus* generally ranged from $1.5\text{-}73x10^3$ cells mL^{-1} in the northern stations and between $60\text{-}63x10^3$ cells mL^{-1} in southern latitudes. Higher *Synechococcus* abundances in the northeastern AS perhaps due to the influence of convective mixing, while *Prochlorococcus* dominated southeastern AS known to experience a warm core eddy.
- No apparent latitudinal variation was observed between the picoeukaryotic distributions. The depth distribution of picoeukaryotes abundance at oligotrophic offshore stations is usually associated with a subsurface maximum near the base of the mixed layer. The maximum abundance of picoeukaryotes was seen between 65-90 m depth at the II6 and II4 stations (47, $101x10^3$ cells mL^{-1}) during SSK 79 and at II12 station ($23.9x10^3$ cells mL^{-1}) during SSD 26 cruise.
- Bacterial densities varied between 0.18 and $0.58x10^9$ cells L^{-1} during the late summer monsoon and between 0.21 and $0.78x10^9$ cells L^{-1} during the winter monsoon season. An increase of bacterial abundance from northern (21°N) to southern (9°N) the Arabian Sea was observed. Maximum bacterial abundance was observed to the south of 17°N during both cruises (SSK 79, II4; 0.78 $x10^9$ cells L^{-1} and SSD 26, II8; $0.58x10^9$ cells L^{-1}) perhaps supported by high DOC in the region (Hansell and Peltzar, 1998).
- The distribution of bacterioplankton in relation to the oxygen and nitrite in the oxygen minimum zone at the ASTS station within the central Arabian Sea depicted an overlap of heterotrophic bacteria maximum abundance with that of secondary nitrite maxima in the water column pointing the role of heterotrophic bacteria in denitrification

at this zone unlike autotrophic picoplankton involved in nitrate consumption in the upper layers.

- In terms of carbon biomass estimates of bacterioplankton, heterotrophic bacteria had the highest percentage contribution (23-97%) to the total biomass as compared to the autotrophic component (5-75%) in the euphotic zone of the water column. Within the autotrophic community, Picoeukaryotes contributed the most (2-40%). Spatially, *Synechococcus* biomass was higher in the northern stations (II14) while *Prochlorococcus* dominated the southern stations (II4).

Objective II. Identification of denitrifying bacteria using a molecular approach

- The diversity of nitrate-reducing bacteria was studied following a culturable approach from the permanent oxygen minimum zone of the Arabian Sea. During the study period, the water column DO values were below 2 mL L^{-1} that varied in the range of 0.09-1.6 mL L^{-1} (SSK 56, October), 0.04 mL L^{-1} (SSK 69, October), 0.04-0.79 mL L^{-1} (SSK 79, February) and 0.01-2 mL L^{-1} (SSD 26, September). High nitrite concentrations were particularly found at a depth of 200-350 m. The nitrite concentration in this water column varied between 0.01-3.8 µmol (SSK 56), 0.03-3.7 µmol (SSK 69), 0.01-3.2 µmol (SSK 79) and 0-2.9 µmol (SSD 26). Whereas, nitrate concentrations varied between 24.49 and 48.61 (SSK 56), 17.17 and 29.68 (SSK 69), 9.38 and 27.12 (SSK 79), 15.06 and 36.95 (SSD 26).
- A total of 152 nitrate-reducing bacterial isolates were obtained, whose identities were confirmed by *16S rDNA* gene sequencing and NCBI BLAST analysis. The isolated bacteria belonged to Proteobacteria (total isolates, 134), Firmicutes (total isolates, 9) and Actinobacteria (total isolates, 9). Diversity analysis at the genera level revealed a total of 16 genera namely, *Alcanivorax, Alteromonas, Bacillus*, *Brevibacterium, Glutamicibacter, Halomonas, Idiomarina, Marinobacter*, *Micrococcus, Nesterenkonia, Nitratireductor, Pseudoalteromonas, Pseudomonas, Staphylococcus, Sulfitobacter*, and *Vibrio*. Out of these, isolates belonging to *Halomonas* were the most abundant (38 bacteria) followed by *Marinobacter* (37 bacteria) and *Pseudoalteromonas* (21 bacteria). Our study reveals that culturable bacterial community in the AS-OMZ was dominated by Proteobacteria.

- Statistical analysis revealed maximum species diversity at ASTS (20) and least at II4 (7). Also, high diversity value at ASTS (H'=4.23) was associated with high species richness (d=5.7). The highest diversity of culturable nitrite reducers was found at depths with high nitrite levels (ASTS, 1.4-3.4 μM) considered to be the zones of high denitrification activity. Alpha diversity between all the nitrate reducers obtained in this study was deduced using Cluster analysis. Isolates belonging to the upper interface of the OMZ and the lower interface of the OMZ clustered together and showed around 50% similarity, while the bacteria isolated from the core of the OMZ were 70% different from the rest of the isolates. The CCA analyses denote an influence caused by DO (R=0.56). Also, a positive correlation between the isolated bacteria and DO, NO_3^-, NO_2^- concentrations were observed, and temperature/ salinity did not have any specific influence on the bacterial community in this region.

Objective III. Investigating the denitrifying potential of selected species of marine bacteria.

- Qualitative analysis of bacterial isolates for denitrification potential/nitrate reducing capacity yielded 111 cultures that reduced nitrate to nitrite or further. The isolates were differentiated as complete nitrate reducers (NO_3^- to $NO/N_2O/N_2$), partial nitrate reducers (NO_3^- to NO_2^-) and non-reducers. Among the tested isolates, *Alcanivorax dieselolei, Alteromonas macleodii, Glutamicibacter nicotianae, Halomonas aquamarina, Marinobacter hydrocarbonoclasticus Nitratireductor aquibiodomus, Nitratireductor kimnyeongensis, Pseudoalteromonas arabiensis, Pseudoalteromonas lipolytica, and Pseudoalteromonas prydzensis, Sulfitobacter dubius, Sulfitobacter faviae, Vibrio campbellii, Vibrio splendidus* were able to reduce nitrate completely under oxic conditions.
- Complete denitrifiers like *Alteromonas macleodii, Alcanivorax dieselolei* and *Marinobacter hydrocarbonoclasticus,* and were obtained from regions with high nitrate (~17 to 32 μmol) and nitrite (1-3 μmol) values, suggesting their active role in nitrite utilisation. *Bacillus firmus, Bacillus infantis, Bacillus megaterium, Brevibacterium casei, Halomonas meridiana, Idiomarina zobelli, Marinobacter litoralis, Micrococcus luteus, Nesterenkonia lacusekhoensis, Staphylococcus saprophyticus, and Vibrio*

parahaemolyticus were negative for nitrate reduction and mostly found at depths where nitrite values were ~0.02 μmol or lower.

- Based on the results of the Griess test, 29 representative isolates belonging to 9 genera namely; *Alcanivorax, Alteromonas, Bacillus, Glutamicibacter, Halomonas, Marinobacter, Pseudoalteromonas, Sulfitobacter* and *Vibrio* were positive for NO_3^- reduction.

Objective IV. Quantification of some functional genes (nir, nos, nor genes) involved in denitrification.

- The denitrifying ability of isolated bacterial cultures was correlated with the presence of functional genes (*nirS, norB,* and *nosZ* gene homologs) using the polymerase chain reaction (PCR) and DNA sequencing techniques.
- Functional gene analysis of the cultured isolates was carried out with primers specific for denitrification genes. Most of the isolates showed multiple bands when tested for the *nirS* gene probably because of the presence of multiple domains (Throback *et al*., 2004). However, single bands of the desired length were obtained for *norB* and *nosZ* genes. PCR analysis of these cultures with gene-specific primers revealed products of the expected size of *nirS* (550 bp, 600 bp, 320 bp), *norB* (440 bp) and *nosZ* (700bp) gene fragments. Gene for nitrite reductase (*nirS*) was amplified from pure cultures of *Brevibacterium sanguinis, Marinobacter hydrocarbonoclasticus, Nesterenkonia lacusekhoensis, Nitratireductor aquibiodomas, Pseudoalteromonas arabiensis, Pseudoalteromonas nigrifaciens* and *Pseudoalteromonas prydzensis.* Gene for nitric oxide reductase *(norB)* was amplified among cultures belonging to *Marinobacter hydrocarbonoclasticus, Pseudoalteromonas arabiensis, Pseudoalteromonas nigrifaciens,* and *Pseudoalteromonas prydzensis.* Nitrous oxide reductase genes were amplified from most of the isolates tested for it. A total of 29 gene fragments were obtained belonging to *Alcanivorax, Bacillus, Halomonas, Idiomarina, Marinobacter,* and *Pseudoalteromonas* genera.
- A NCBI BLAST analysis of gene sequences obtained in this study showed similarity with genes from closely related cultured bacteria as well as uncultured bacteria. Phylogenetic analysis confirmed that PCR products from cultured strains were indeed

nirS, *norB*, and *nosZ* gene fragments. Amongst the isolates tested for the presence of these genes, cultures of *Marinobacter hydrocarbonoclasticus* and *Pseudoalteromonas* sp. showed the presence of all three genes emphasising their role in denitrification processes in the Arabian Sea. The detection of bacterial cultures possessing all three genes is a significant finding and forms an integral part of the bacterial community structure in the Arabian Sea.

Conclusion and future prospects

Although the prevalence of oxygen minimum zone and the processes governing its intensification are known, very little has been understood about the contribution from microbes which are the driving force behind this phenomenon. Results presented here, document the species composition of bacterioplankton that forms an integral part in marine nitrogen and carbon cycling and forms the basis to conduct further research of these communities in the dynamic system of Arabian Sea. Through a culturable approach, bacterial diversity, particularly the nitrate reducers from AS OMZ was examined. A substantial number of cultivable bacteria capable of utilising available nitrate in the system are present in the AS OMZ, which can be further explored for their biochemical significance. For the first time, this study provides insights into the yet uncultured and ecologically significant bacterial communities. The present study shows that both the biochemical characterisation of denitrifiers and the functional gene phylogeny give a fair understanding of the major bacterial groups involved in nitrogen transformations. However, an in-depth study of sequences of nitrogen cycling biomarker functional genes will be beneficial to assess their potential in processes associated with N loss. Owing to the high degree of taxonomic diversity among denitrifiers and its role in the N cycle, the denitrifying community might serve as a good model for investigating the value of microbial biodiversity in ecosystem functioning.

While ubiquity among many of the key players within the microbial community of the OMZ enhances our understanding of important biogeochemical processes, patterns of endemism appear among closely related species. Combining the diversity studies with process rate analysis or targeted gene surveys will uncover novel modes of metabolic integration involved in carbon, nitrogen and sulphur cycles. Experimental analysis using

cultured organisms will help ascertain the influence of environmental parameters on the denitrification gene content within a population of denitrifiers. This information will be important in determining the nutrient and energy flow patterns in the expanding OMZs.

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Cruises:

Cruise ID	Month/Year	Region	Duration
SSK 69	October/2014	Northeastern Arabian Sea	25 days
SSK 79	February/2015	Northeastern Arabian Sea	25 days

Conference/Workshops/Training:

1. Poster presentation at International Symposium on Microbial Responses to Ocean Deoxygenation, December 3-5, 2016. *Nitrate-reducing bacterial diversity from the Arabian Sea oxygen minimum zone*
2. Poster presentation at Seminar on New perspectives in Biosciences, Department of Microbiology, Goa University, December 7, 2017. *Detection of denitrification marker genes in axenic bacterial cultures isolated from OMZ's of Arabian Sea*.
3. Workshop- South Asia Thematic School in Aquatic Microbial Ecology at NIOT, Port Blair; 6th -18th November 2017

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Diversity of culturable nitrate-reducing bacteria from the Arabian Sea oxygen minimum zone

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ABSTRACT

The subsurface waters of the northern Arabian Sea display a pronounced oxygen minimum layer associated with high nitrite maxima which provide an ideal niche for organisms that can respire nitrate. Culture-based studies elaborate the physiological characteristics of the organisms and their metabolic activities in biogeochemical cycles. In this study, the bacterial diversity and nitrate utilizing activity of the culturable heterotrophic bacteria inhabiting the water column oxygen minimum zone of Arabian Sea were investigated. Nitrate-reducing bacteria were isolated from the water column in the central Arabian Sea. Genotypic characterization of the isolates using 16 S rDNA gene sequencing grouped them into three phylogenetic groups i.e. Proteobacteria, Firmicutes, and Actinobacteria. Out of the 56 isolated bacteria, 45 strains belonged to Proteobacteria, 6 to Firmicutes and 5 to Actinobacteria. The nitrate reducing ability of the isolates was tested using Griess test. Thirty-six species belonging to genera *Alcanivorax, Alteromonas, Halomonas, Pseudoalteromonas, Marinobacter, Bacillus,* and *Vibrio* were positive for NO_2^-/NO_3^- reduction. Our results imply that cultivable bacteria capable of utilizing NO_3^- available in the system are present in the Arabian Sea oxygen minimum zone and the conditions existing therein must be favorable for their growth and functionality.

1. Introduction

Arabian Sea (AS) shows considerable variation in physical, chemical and biological processes because of the range of environmental conditions that exist in a year. One of the important features of the northern AS is the extremely well-developed oxygen minimum zone (OMZ) associated with high nitrite maxima and extensive mid-depth denitrification (DeSousa et al., 1996; Naqvi, 1994; Naqvi et al., 1998). The open ocean OMZ in the AS occurs permanently between depths of 150–1500 m (Wyrtki, 1971), where the oxygen concentration drops to almost 0.1 ml L^{-1} (4 μmol L^{-1}) or lower, due to intense mineralization of surface-derived organic matter and limited supply of oxygen to intermediate waters (Naqvi, 1994; Naqvi et al., 1998). Low oxygen in this marine system facilitates the utilization of nitrate (NO_3^-) as an electron acceptor, and its reduction to nitrous oxide (N_2O) or dinitrogen (N_2) via canonical denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) or to N_2 via anammox (anaerobic ammonium oxidation) (Lam and Kuypers, 2011). Denitrification in the AS is estimated to be 1/3rd of the global ocean denitrification (Naqvi, 1987) and varies between northeast (winter) and southwest (summer) monsoon seasons with higher nitrate deficits during the former (Naqvi et al., 1990). The subsequent loss of nitrogen from oceans to the atmosphere creates an imbalance in the oceanic N budget (Codispoti, 1995).

In spite of the global biogeochemical processes occurring within the OMZ, the phylogenetic and metabolic activities of microorganisms inhabiting it are poorly known (Riemann et al., 1999; Fuchs et al., 2005; Stevens and Ulloa, 2008). Knowledge of the activity of heterotrophic microbes is required to complete an understanding of the mineralization (Pomroy and Joint, 1999). The nearly complete dissolved oxygen depletion coupled with the high concentration of nitrate provides an ideal niche for microbes that can respire nitrate. Most of the research on microbial diversity conducted in OMZ of the AS is based on culture-independent methods which provides taxonomic classification (Castro-Gonzalez et al., 2005; Stewart, 2012; Jayakumar et al., 2004, 2009; Ward et al., 2009; Kuypers, 2005). Culture-based studies, however, provide information on the physiological characteristics of the organisms living in these regions, elaborating their metabolic activities in biogeochemical cycles and allow the description of new species. Although, the seasonal anoxic system in the coastal regions of India have been investigated for bacterial indications of nitrification and

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denitrification using a culturable approach, but the studies are confined to sediments (Krishnan et al., 2008; Divya et al., 2010). There is a paucity of studies on the diversity and isolation of culturable bacteria from the water column of AS.

The present study focuses on denitrification, wherein we report the bacterial diversity of nitrate reducing bacteria from permanent, open ocean OMZ of the AS where the oxygen levels are close to zero and have remained the same since the time of its discovery during the International Indian Ocean Expedition (Wrytki, 1971; Naqvi et al., 2006). Hence, we describe here the diversity, phylogenetic relationship and nitrate-reducing activity of heterotrophic bacteria inhabiting the water column OMZ following a culturable approach.

2. Methodology

2.1. Sampling

The water samples were collected onboard RV Sindhu Sankalp during three cruises in the Arabian Sea, SSK 56 (October 2013), SSK 69 (October 2014) SSK 79 (February 2015) to study the bacterial diversity within OMZ. Samples were collected from 6 different locations in transect along 68°E in the central Arabian Sea (Fig. 1) at varying depths in the oxygen minimum zone. Stations chosen were II14 (21°N 68°E), ASTS (17°N 68°E), II8 (15°N 68°E), II6 (12°N 68°E), II4 (11°N 68°E) and II2 (8°N 68°E). Arabian Sea Time Series (ASTS) location was sampled during all cruises while II14, II6, II2 were sampled during SSK 79 cruise and II8, II4 and II2 were sampled during SSK 56 cruise. The sampling stations selected from the study area were situated within a permanent oxygen minimum region in the central AS (Naqvi et al., 2006). The depths were chosen based on the in situ measurements of the physicochemical parameters of seawater (dissolved oxygen, temperature and salinity) which were recorded from different sensors fitted on to a CTD rosette. Dissolved oxygen (DO) was measured on-board within few hours of sampling following Winkler titration method modified by Carpenter (1965). The nutrients (nitrate and nitrite) were measured from frozen samples carried to the on-shore laboratory using Skalar Autoanalyser (Skalar Analytical, The Netherlands) following standard methods (Grasshoff et al., 1983).

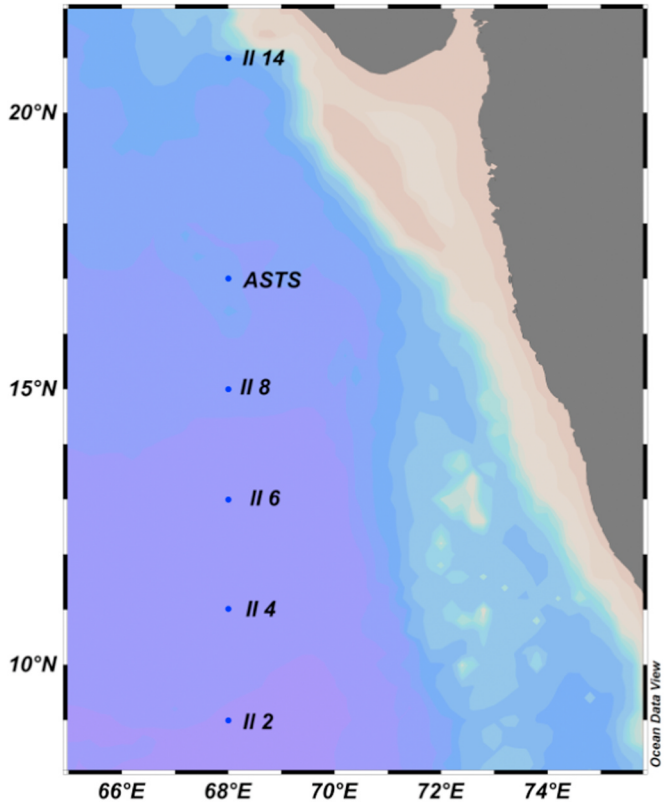

Fig. 1. Map showing sampling locations in Arabian Sea permanent OMZ.

2.2. Isolation of bacteria

Seawater (50 ml) was filtered through 0.22 µm nitrocellulose membrane and placed on Zobell's marine agar (ZMA) medium (Zobell, 1941). Water sample (200 µL) was also spread plated on ZMA to obtain higher number of isolates. All the plates were incubated at 28 °C for 5–7 days in incubator to allow even the slow growing organisms to appear. After incubation, plates were examined, and colonies of different morphology were selected, purified and transferred to nitrate agar medium for selectively isolating bacteria capable of utilizing nitrate for their growth.

2.3. DNA extraction and PCR amplification

For DNA extraction, each bacterial culture was grown for 2–4 days in 5 ml nitrate broth, incubated at 28 °C. The total genomic DNA was extracted using ZR Fungal/Bacterial DNA Kit (Zymo Research, CA) following instructions from the manufacturer. The 16 S rDNA gene was amplified using universal primers 27 F (5′-AGAGTTTGATCMTGGCT CAG-3′) and 1492 R (5′-TACGGYTACCTTGTTACGACTT-3′). PCR amplification was performed in a reaction volume of 50 µL containing 5 µL of 10X PCR buffer (750 mM Tris-HCL (pH-9), 500 mM KCl, 200 mM (NH_4) SO4), 20 mM $MgCl_2$, 2.5 mM of each dNTP, 0.6 µL of Taq polymerase (Genaxy Scientific, India), 1 µL each of forward and reverse oligonucleotide primers (Eurofins Genomics India) and 3 µL of template DNA. The final volume was adjusted to 50 µL with sterile distilled water. PCR cycle included initial denaturation at 95 °C for 1 min, followed by 35 cycles of primer annealing at 54 °C for 45 s, elongation at 72 °C for 1 min with a final extension at 72 °C for 10 min. DNA in the PCR product was quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific). The amplicon was visualized on an agarose gel (1%) under UV light after ethidium bromide staining.

2.4. 16S rDNA gene sequencing

The amplified PCR products of the bacterial gene fragments were purified using Wizard® SV Gel and PCR Clean-Up System (Promega Corporation, USA) and sequenced using 3130xl Genetic Analyzer (Applied Biosystems, USA). The sequences obtained were compared with NCBI database through BLAST searches. In this comparison, sequences of type strains most closely related to the sequences of isolates were examined. The phylotypes were defined based on sequence similarity of 97%. The 16 S rDNA gene sequences obtained in this study have been deposited in the NCBI GenBank under accession numbers KY679583-KY679590, KY678488-KY678496, KY678470-KY678485, KY678487, KY926902 and KY926903 (Supplementary material).

2.5. Nitrate reduction test

The isolates obtained were tested using the nitrate reduction test to detect their capacity to utilize available nitrate in the medium. This was determined by using the Griess test, which is based on the activity of the enzyme nitrate reductase (Smibert and Krieg, 1994). The nitrate test was performed by addition of the Griess reagent to the culture supernatant. At the end of an incubation time of 2 min, the presence of nitrite formed as a result of nitrate reduction is indicated by the formation of pink color. After checking for coloration, zinc dust was added to test for the presence of residual nitrate. The isolates that showed no nitrate reduction and tested positive for residual nitrate were categorized as non-reducers (–). The isolates that were positive for nitrite production but still had some residual nitrate were marked as incomplete nitrate

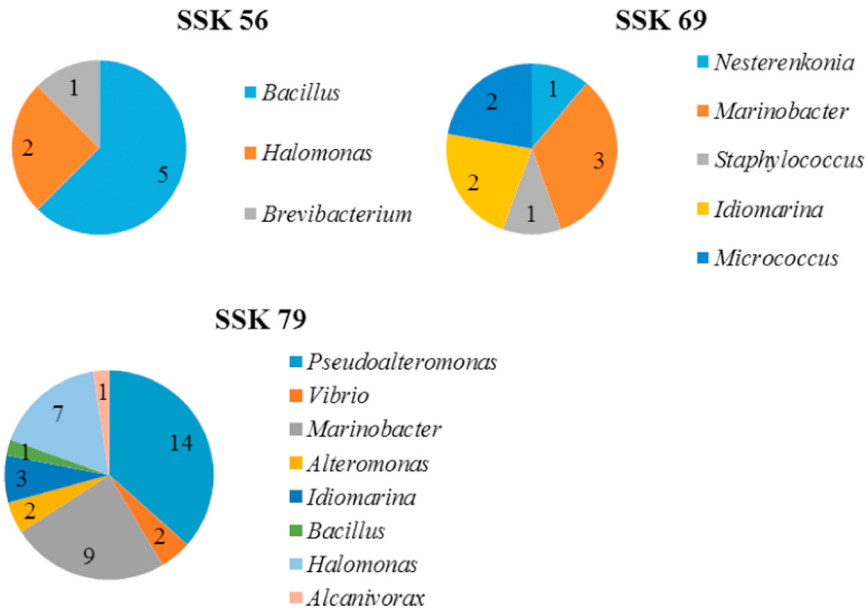

Fig. 2. Cruise wise distribution of bacteria (Genus level).

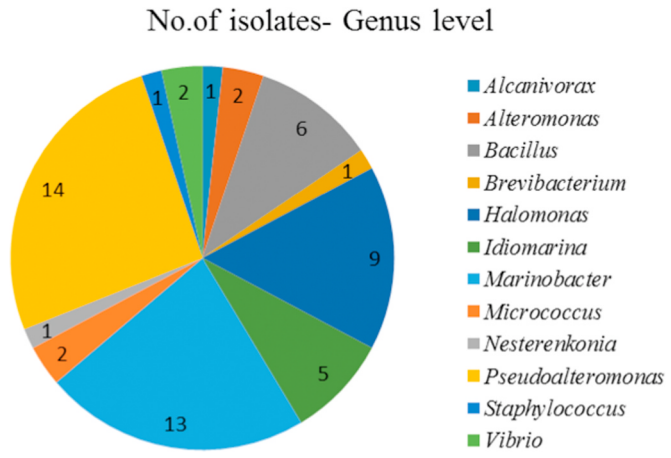

Fig. 3. Number of isolates in each genera.

reducers (+), while the isolates that were positive for nitrite accumulation and (or) negative for residual nitrate are grouped as nitrate reducers (++) (Manohar et al., 2014).

2.6. Construction of phylogenetic tree and statistical analysis

A phylogenetic tree was constructed to elucidate the evolutionary history of the isolates using the Neighbor-Joining method in MEGA6 (Tamura et al., 2013). The evolutionary distances indicated by the scale bar were computed using the Tamura-Nei method (Tamura and Nei, 1993). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. The species abundance and range of diversity indices at each station was calculated using the statistical software primer 6, version 6.1.9 (PRIMER-E).

3. Results and discussion

3.1. Culturable bacterial diversity from OMZ

In the current study, the community composition of culturable bacteria was studied by 16 S rDNA sequencing. A total of 56 bacterial isolates were obtained from 3 cruises in the AS from depths of oxygen minima (~150–1000 m). According to the partial sequencing of the 16 S rDNA gene, the isolated bacteria were identified belonging to three phylogenetic groups; Proteobacteria, Firmicutes and Actinobacteria and represented 25 phylotypes. Most of the isolates (45) belonged to Proteobacteria, which was the most abundant group at all the stations studied, while six belonged to Firmicutes and five to Actinobacteria. This high abundance of Proteobacteria is not surprising considering that bacteria from this phylogenetic group are among the most known and readily cultivable microorganisms from the marine environment (Fuhrman and Hagström, 2008; Da Silva et al., 2013). Also, Proteobacteria is the most diverse group of the microbial assemblage and hence is of biological significance (Gupta, 2000). The dominance of Gammaproteobacteria, has also been reported earlier from a variety of pelagic marine environments (Rappé et al., 2000) including the OMZ of the ETSP (Stevens and Ulloa, 2008; Ganesh et al., 2014; Stewart et al., 2012; Castro-Gonzalez et al., 2005) and Arabian Sea (Fuchs et al., 2005; Jain et al., 2014; Jayakumar et al., 2004), although some of the phylotypes identified belonged to not yet cultivated species. Studies on sediments of Arabian Sea revealed the dominance of Gammaproteobacteria from clone libraries as well as retrieval of *Micrococcus* sp., *Halomonas* sp. and *Alteromonas* sp. from the culturable fraction of bacteria from coastal AS (Divya et al., 2010, 2011). Other studies by Ye et al. (2016) in the east China Sea and Walsh et al. (2016) also displayed the bacterial diversity and community composition in the sediments as well as water column which mainly comprised of Gammaproteobacteria, particularly *Pseudoalteromonas* sp. Analyses of 16 S rRNA and *nas*A genes from bacterial communities in the Indian Ocean revealed 63 OTUs which clustered into Gammaproteobacteria (Jiang and Jiao, 2016).

Cultivation-independent studies in AS OMZ revealed high bacterial diversity (Fuchs et al., 2005; Stevens and Ulloa, 2008). However, culture-based studies help establish a link between the species and their functions. The total number of isolates obtained per cruise was dependent on the number of stations and depths sampled during each cruise and reflected clearly in the total number of strains isolated. The majority of bacterial cultures were obtained during the SSK 79 cruise, followed by SSK 69 and SSK 56 cruises (Fig. 2). Oxygen deficient waters in the intermediate depths is a permanent feature of the northeastern Arabian Sea which gets intensified during winter or northeast monsoon (NEM) season due to poor water circulation and high surface productivity (Naqvi et al., 1990; Prasanna Kumar and Prasad, 1996). Lower DO level and higher nitrite concentration at the average depth during NEM season signifies intensive denitrification processes (Sen Gupta

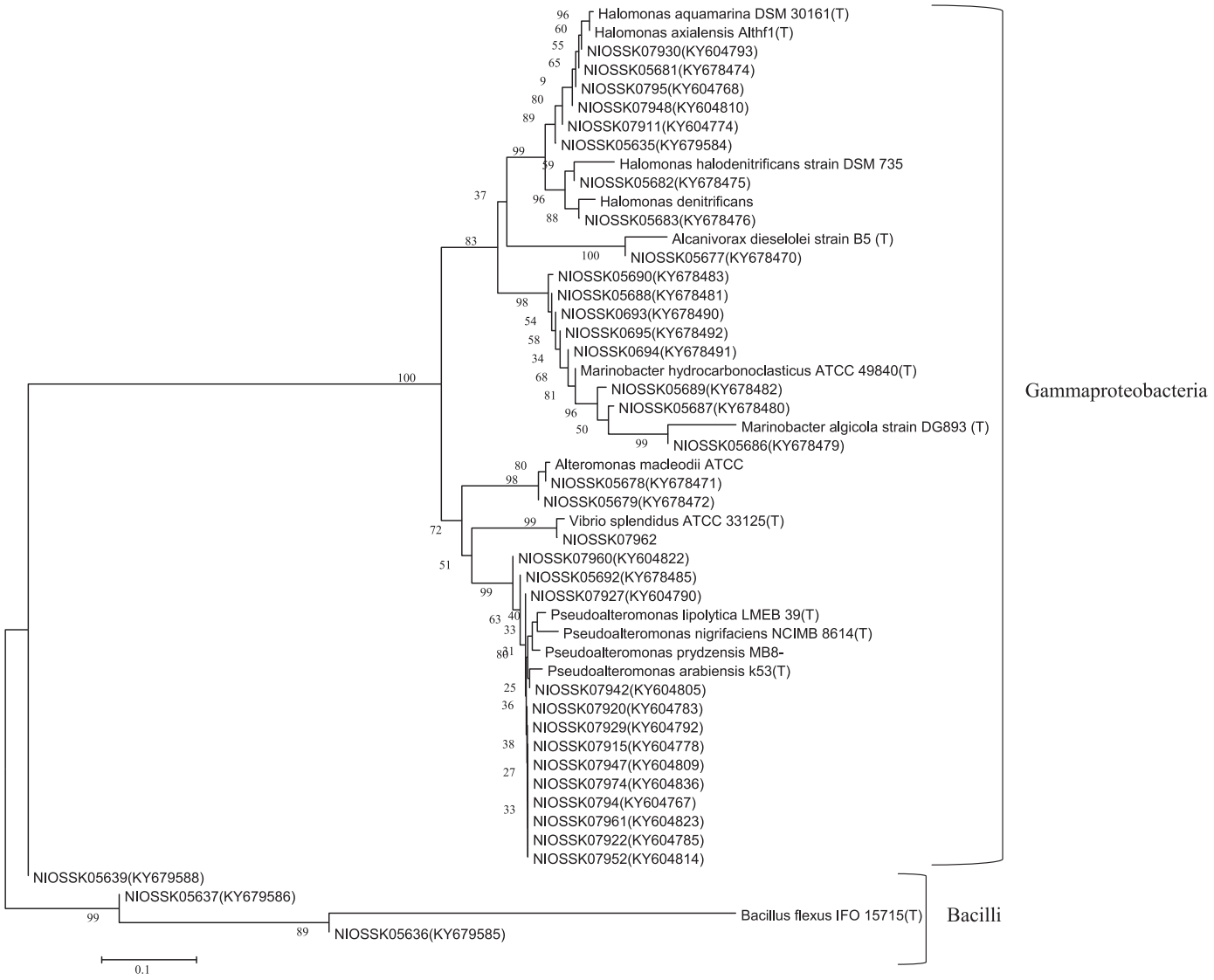

Fig. 4. Molecular Phylogenetic analysis using Neighbor Joining method MEGA 5.

Table 1
Species diversity indices.

Station	S (species)	d (richness)	J' (evenness)	H' (diversity)
II14	7	3.08	1	2.80
ASTS	15	4.82	0.98	3.83
II8	1	0	0	0
II6	5	2.269	0.9908	2.3
II4	1	0	0	0
II2	10	3.643	0.986	3.276

et al., 1976). This phenomenon could potentially explain the maximum recovery of bacteria during SSK 79 cruise which was carried out in February 2015, northeast monsoon season. A total of 12 genera of bacteria from OMZ water column were isolated and identified as *Alcanivorax, Alteromonas, Bacillus, Brevibacterium, Halomonas, Idiomarina, Marinobacter, Micrococcus, Nesterenkonia, Pseudoalteromonas, Staphylococcus and Vibrio* (Fig. 3). *Pseudoalteromonas* was the dominant genus followed by *Marinobacter* in our study both of which belong to the order Alteromonadales. Metagenomic studies by Bandekar et al. (Personal communication) suggest that Alteromonadales contribute substantially to the temporally stable bacterial community structure in the AS OMZ. Alteromonodales may be utilizing their ability to use abundantly available nitrate as a terminal electron acceptor for energy generation. Their persistence throughout the year in the AS OMZ may also be due to its microaerophilic metabolism and due to its ability to degrade recalcitrant compounds as well as to be able to degrade large sinking particulate matter (Ramaiah et al., 2005). Sun et al. (2017) studied the community composition of nitrous oxide consuming bacteria in the ETSP sediments and found *Marinobacter* sp. to be abundant. Paleoclimatic studies done by Orsi et al. (2017) suggested that the genera *Marinobacter*, *Alcanivorax* and *Pseudoalteromonas* being relatively abundant indicator taxa for strong OMZ periods.

3.2. Construction of phylogenetic tree and statistical analysis

The phylogenetic tree was created for the positive isolates using the 16 S rDNA gene sequences which revealed the evolutionary distances when compared with reference strains from the NCBI database (Fig. 4).

The species diversity was calculated according to the number of isolates obtained at each station. Table 1 represents the number of species (S), species richness (d), evenness (J') and species diversity (H'). A maximum number of species were recorded at ASTS (15) and least at II8 (1) and II4 (1). Also, high diversity value at ASTS (H' = 3.83) was associated with high species richness (d = 4.82).

Table 2
Nitrate reduction test.

Isolates	Closest match in NCBI	Reduction of NO_3^-
(NIOSSK056#77)	*Alcanivorax dieselolei*	+ +
(NIOSSK056#78, NIOSSK056#79)	*Alteromonas macleodii*	+ +
(NIOSSK056#95)	*Bacillus firmus*	– –
(NIOSSK056#37, NIOSSK056#38, NIOSSK056#39)	*Bacillus flexus*	+ -
(NIOSSK056#34)	*Bacillus infantis*	– –
(NIOSSK056#41)	*Bacillus megaterium*	– –
(NIOSSK056#40)	*Brevibacterium casei*	– –
(NIOSSK056#82)	*Halomonas halodenitrificans*	+ -
(NIOSSK056#35, NIOSSK079#30, NIOSSK056#81, NIOSSK079#5, NIOSSK079#48)	*Halomonas aquamarina*	+ -
(NIOSSK079#11)	*Halomonas axialensis*	+ -
(NIOSSK056#83)	*Halomonas denitrificans*	+ -
(NIOSSK056#38)	*Halomonas meridiana*	– –
(NIOSSK069#1, NIOSSK069#2, NIOSSK056#84, NIOSSK056#80, NIOSSK056#85)	*Idiomarina zobelli*	– –
(NIOSSK069#3, NIOSSK069#4, NIOSSK069#5, NIOSSK056#87, NIOSSK056#88, NIOSSK056#89, NIOSSK056#90)	*Marinobacter hydrocarbonoclasticus*	+ +
(NIOSSK056#86)	*Marinobacter algicola*	+ +
(NIOSSK079#53, NIOSSK079#75, NIOSSK079#66, NIOSSK056#91, NIOSSK056#96)	*Marinobacter litoralis*	– –
(NIOSSK069#6, NIOSSK069#7)	*Micrococcus luteus*	– –
(NIOSSK069#8)	*Nesterenkonia lacusekhoensis*	– –
(NIOSSK056#92, NIOSSK079#29, NIOSSK079#20, NIOSSK079#22, NIOSSK079#42, NIOSSK079#60)	*Pseudoalteromonas arabiensis*	+ +
(NIOSSK079#27)	*Pseudoalteromonas lipolytica*	+ +
(NIOSSK079#74, NIOSSK079#15, NIOSSK079#47)	*Pseudoalteromonas nigrifaciens*	+ -
(NIOSSK079#52, NIOSSK079#4, NIOSSK079#61)	*Pseudoalteromonas prydzensis*	+ +
(NIOSSK069#9)	*Staphylococcus saprophyticus*	– –
(NIOSSK079#62)	*Vibrio splendidus*	+ +
(NIOSSK056#94)	*Vibrio parahaemolyticus*	– –
Control	*Pseudomonas stutzeri*	+ +

Note: - - No nitrate reduction.
+- Incomplete nitrate reduction (Reduction of NO_3^- to NO_2^-).
+ + Complete nitrate reduction (Reduction of NO_3^- to NH_3^-).

3.3. Denitrification potential of the isolates and their co-relation with nitrate and nitrite values in water column

Representative isolates of each species were tested for nitrate utilization using Griess test. *Pseudomonas stutzeri*, a known denitrifier surviving in low oxygen waters, was used as a positive control (Table 2). Among the tested isolates, *Alcanivorax dieselolei, Alteromonas macleodii, Marinobacter hydrocarbonoclasticus, Marinobacter algicola, Pseudoalteromonas arabiensis, Pseudoalteromonas lipolytica, Pseudoalteromonas prydzensis, Vibrio splendidus* along with the positive control were able to reduce nitrate completely under oxic conditions at 28 °C and 1 bar pressure. As seen in Fig. 5, the DO levels varied from 0.6 to 0.2 ml L^{-1}, NO_2 from 0.01 to 4.01 μM and NO_3 from 17.23 to 36.85 μM. Complete denitrifiers like *Marinobacter hydrocarbonoclasticus*, *Alteromonas macleodii* and *Alcanivorax dieselolei* were obtained from regions with high nitrate (~ 17–32 μmol) and nitrite (1–3 μmol) values, suggesting their active role in nitrite utlisation. *Bacillus firmus, Bacillus infantis, Bacillus megaterium, Brevibacterium casei, Halomonas meridiana, Idiomarina zobelli, Marinobacter litoralis, Micrococcus luteus, Nesterenkonia lacusekhoensis, Staphylococcus saprophyticus, and Vibrio parahaemolyticus* were negative for nitrate reduction and mostly found at depths where nitrite values were ~0.02 μmol or lower. Most clades of Gammaproteobacteria are known to denitrify using nitrate as an electron acceptor (Miller et al., 2010). Based on the results of Griess test, 36 species belonging to *Alcanivorax, Alteromonas, Halomonas, Pseudoalteromonas, Marinobacter, Bacillus,* and *Vibrio* were positive for NO_2^-/NO_3^- reduction. Bacteria belonging to these genera are reported to contain genes involved in nitrogen cycle (Jones et al., 2011; Cai and Jiao, 2008). Among Gammaproteobacteria, the predominant Alteromonodales in the AS OMZ appears to be a proficient denitrifier group (Bandekar et al. personal communication). In our study, seven isolates of *Marinobacter hydrocarbonoclasticus* were obtained. This species of bacteria has been previously shown to demonstrate efficient denitrifying ability (Li et al., 2013). Also, 13 isolates of *Pseudoalteromonas* were obtained and the presence of *nasA* gene (called *narB* genes in cyanobacteria) in *Pseudoalteromonas* sp. has been well documented in marine environments (Allen et al., 2001).

4. Conclusions

Though the prevalence of OMZ and the processes governing its intensification are known, very little has been understood about the contribution from microbes which are the driving force behind this phenomenon. In the present study, the culturable approach was used to examine bacterial diversity, particularly the nitrate reducers from AS OMZ. Out of 56 isolates obtained, grouped into Proteobacteria, Firmicutes and Actinobacteria, 22 species were capable of complete reduction of nitrate while 14 species showed an incomplete/partial reduction in the Griess test. Our results imply that cultivable bacteria capable of utilizing NO_3^- available in the system are present in the AS OMZ and that conditions therein must be favorable for their growth and function. However, an in-depth study of sequences of nitrogen cycling biomarker functional genes will be beneficial to assess their potential in processes associated with N loss.

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Conflict of interests

The authors declare that they have no conflict of interest in this work.

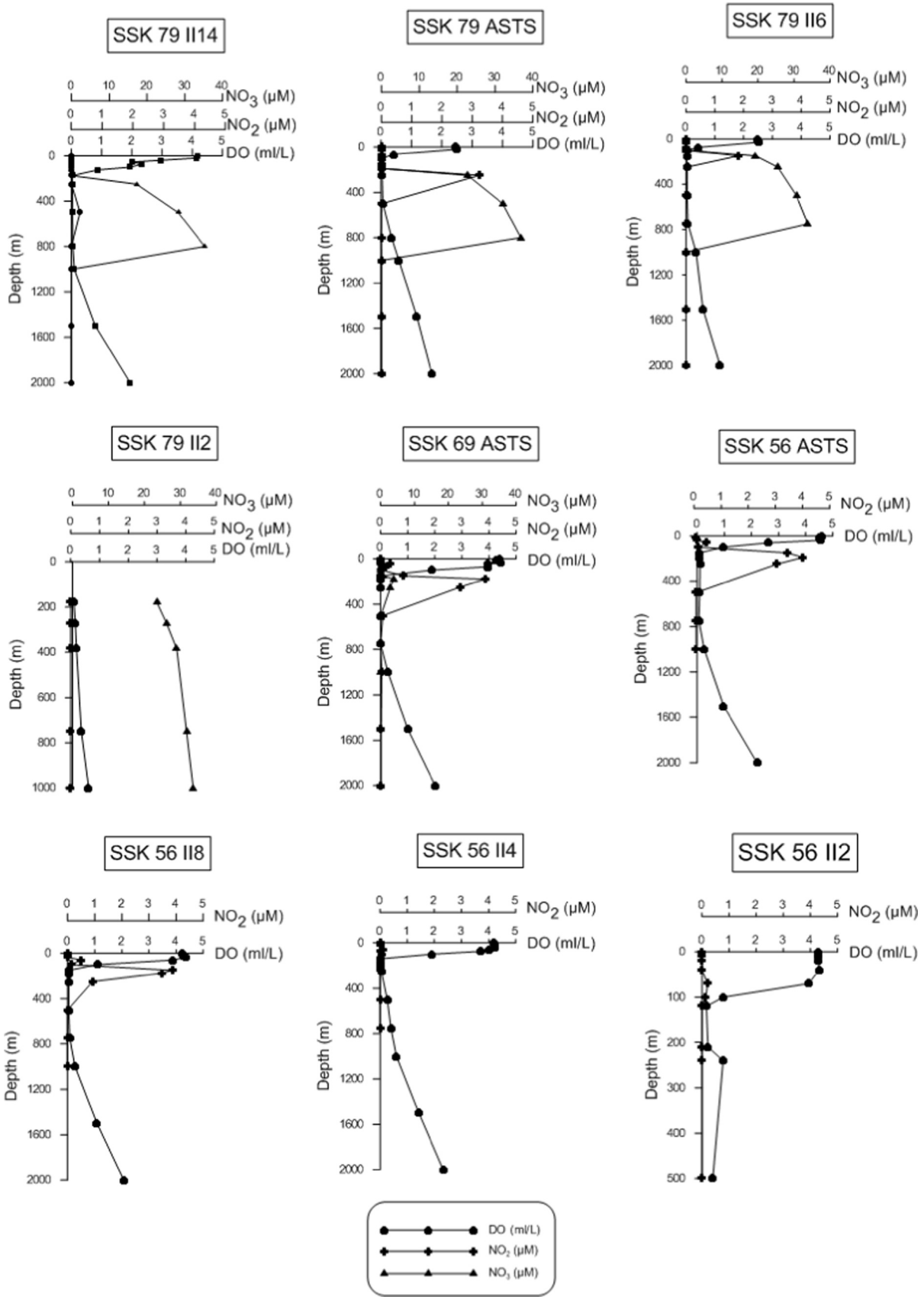

Fig. 5. DO, NO_3 and NO_2 values at some of the stations SSK, SSK and SSK 56 cruises.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.dsr2.2017.12.014.

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Phytoplankton response to the contrasting physical regimes in the eastern Arabian Sea during north east monsoon

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ABSTRACT

Phytoplankton abundance and composition in two contrasting physical regimes - convective mixing in the northeastern Arabian Sea (NEAS) and Arabian Sea mini warm pool (ASMWP) in the southeastern Arabian Sea (SEAS) - were investigated during the northeast monsoon (NEM) of 2015 and 2017. Observations in 2015 were carried out late during the season, and only one station in the north (at 21°N latitude) fell within the zone of convective mixing where microplankton was dominated by diatoms. In 2017, convective mixing occurred even at 16°N latitude, but the microplankton contribution was low, presumably due to low Si/N ratios. Within the convective mixing regime of the NEAS, chlorophyll (Chl) *a* concentrations were higher in 2015 (maximum 1080 ng L^{-1}; average 493 ng L^{-1}) than in 2017 (maximum 673 ng L^{-1}; average 263 ng L^{-1}). In contrast, pico-phytoplankton were dominant in the ASMWP of the SEAS with peak abundance associated with the subsurface chlorophyll maximum. A warm core eddy was present in 2015 in the SEAS where four times higher *Prochlorococcus* counts were found within the core of the eddy than at its periphery. This study provides the first description of the phytoplankton community in the ASMWP. Our results clearly demonstrate phytoplankton response to the contrasting physical conditions, highlighting the role of bio-physical coupling in the productivity of the Arabian Sea.

1. Introduction

The northern Indian Ocean is bounded by the Eurasian landmass at low latitudes (~25°N), which makes its oceanographic processes including biogeochemistry quite different from those in other ocean basins. For example, unlike the Atlantic and the Pacific Oceans, the most intense upwelling in the Indian Ocean occurs in its northwestern part - the Arabian Sea - in summer, when the winds blow from the southwest to the northeast. In winter, when the atmospheric and surface oceanic circulations are reversed, the northern Arabian Sea experiences moderate convective mixing due to cooling of surface waters. Together, these phenomena make the Arabian Sea one of the most productive regions of the world oceans (Madhupratap et al., 1996; Naqvi et al., 2006). High biological productivity along with restricted oxygen supply also result in the development of an intense oxygen minimum zone at intermediate depths (150–1200 m) (Wyrtki, 1971) providing suitable conditions for large scale nitrogen loss, mostly through denitrification (Sen Gupta and Naqvi, 1984; Naqvi, 1987, 1991; Ward et al., 2009). In contrast to the eastern Pacific Ocean, the denitrification zone is geographically separated from the high productive upwelling centres in the Arabian Sea (Naqvi, 1991).

The Arabian Sea has been extensively studied under two major international programmes – the International Indian Ocean Expedition (IIOE) in the 1960s and the Joint Global Ocean Flux Study (JGOFS) three decades later (Naqvi et al., 2003). Unfortunately, security concerns due to piracy constrained research in the region for about a decade but another major international project – IIOE-2 – has just been launched in recognition of the unique oceanography of this region and its sensitivity to human activities.

Among the best known features of the Arabian Sea is the response of the phytoplankton community to seasonal reversals of winds during the summer or southwest monsoon (SWM) and the winter or northeast monsoon (NEM) seasons (*e.g.* Sawant and Madhupratap, 1996; Madhupratap et al., 1996; Garrison et al., 1998, 2000; Tarran et al., 1999; Wood, 1999). These studies revealed that nutrient enrichment in euphotic zone arising from upwelling and convective mixing leads to extensive phytoplankton blooms contributing to the fishery potential of the region, not only in the open waters (~100 million tons of

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myctophids in OMZ; Madhupratap et al., 2001) but also to the coastal fishery (~2.3 million tons along the west coast of India; http://eprints.cmfri.org.in/11964/1/CMFRI%20Annual%20Report%202016-17.pdf). During winter, the cool dry continental air brought to the northern Arabian Sea by the trade winds promotes evaporation and cooling of surface waters. This drives convective mixing leading to the deepening of the mixed layer and entrainment of nutrients from the thermocline into the surface layer thereby supporting widespread winter blooms that are mostly dominated by diatoms (Madhupratap et al., 1996; Prasanna Kumar and Prasad, 1996; Prasanna Kumar et al., 2001). However, a recent study has reported a dramatic shift in the phytoplankton community over the past decade or so with the diatoms having been replaced by dinoflagellate *Noctiluca scintillans* (Gomes et al., 2014). This shift has been attributed to large-scale development of hypoxic conditions in the euphotic zone thereby favoring the growth of *N. scintillans* because the endosymbiont of this organism (*Pedinomonas noctilucae*) can fix carbon efficiently under hypoxic conditions. Such an ecosystem shift is expected to have huge impact on fisheries as the major consumers of *N. scintillans* are salps and jellyfish, both minor components of fish diet (Gomes et al., 2014). However, subsequent work by Prakash et al. (2017), employing *in situ* measurements and Argo floats data collected in winter, did not find any indication of the reported incursion of hypoxic waters into the surface layer in the NEAS.

Contrasting with the cooling of surface waters in the NEAS, the warm sea surface temperatures over the southeastern Arabian Sea (SEAS) are important for the onset of the SWM over western India during February–May (Vinayachandran et al., 2007). This warm region in the SEAS was defined as a "mini warm pool" (Rao and Sivakumar, 1999) or SST high (Shenoi et al., 1999). Later, Vinayachandran et al. (2007) called it the Arabian Sea mini warm pool (ASMWP). Warming of surface waters in this region begins in February and continues up to April–May. During this period, circulation in the SEAS consists of a quasi-permanent anti-cyclonic eddy (warm-core eddy) associated with a high sea level – the Lakshadweep High (Schott and McCreary, 2001). The eddies propagate westward and affect the hydrography of the region. Several studies (*e.g.* Fryxell et al. (1985) and Gould and Fryxell (1988) in the Gulf Stream; and Stramma et al. (2013) in the Pacific Ocean) found distinct phytoplankton communities associated with warm core eddies. Huang et al. (2010) observed the dominance of prochlorophyceae as well as haptophyceae in two different warm core eddies in South China Sea. The phytoplankton community structure within the warm core eddies of the Arabian Sea has not been investigated in detail so far.

In the present study, we highlight the response of phytoplankton to the contrasting physical regimes in the eastern Arabian Sea during NEM – the convective mixing that entrains nutrients from below the thermocline in the north, and stratification within the ASMWP that creates oligotrophic conditions in the south. Both processes lead to deepening of the mixed layer, but are expected to have different impacts. We employ HPLC based pigment analysis along with flow cytometry data to understand the phytoplankton community structure in the region, a tool found useful in several other recent studies (Mitbavkar et al., 2015; Rajaneesh et al., 2015; Roy et al., 2015; Ahmed et al., 2016). Based on the *in situ* measurements of physical and biological data, we explore the bio-physical coupling in the eastern Arabian Sea during the NEM season in 2015 and 2017.

2. Materials and methods

2.1. Sampling

Water samples for phytoplankton pigments and picophytoplankton counts were collected during the 79th cruise of R.V. *Sindhu Sankalp* (SSK-079; 7th to 15th March 2015) and 33rd cruise of R.V. *Sindhu Sadhana* (SSD-033; 5th to 15th February 2017). Samples were collected from nine stations in 2015 (Fig. 1a) and 13 stations (at every degree interval) in 2017 (Fig. 1b) from 21°N (II-14) to 9°N (II-2) along 68°E. Samples were taken from discrete depths using Niskin bottles mounted on a rosette frame fitted with a Sea-Bird Electronics conductivity-temperature-depth profiler (CTD). Temperature was measured using a pre-calibrated sensor attached to the CTD. Dissolved oxygen was measured onboard following the Winkler procedure using an autotitrator. Samples for nutrients were preserved at −20 °C and later analyzed using an Autoanalyzer (Skalar) following standard procedures (Grasshoff et al., 1983).

2.2. Pigment analyses

Samples for phytoplankton pigments were immediately filtered on glass fiber filter papers (GF/F, 0.7 μm pore size, 25 mm diameter) under dark and cold condition and preserved at −80 °C until analysis. The frozen filters were extracted with 100% methanol and analyzed using HPLC (Agilent HPLC 1200 Series) as detailed in Kurian et al. (2012). The calibration of the HPLC was performed using pigment standards procured from DHI Inc. Denmark. Since there was no standard available for divinyl chlorophyll *b* (divChl *b*), it was quantified using the response factor of chlorophyll *b*.

2.3. Picophytoplankton cell counts

Picophytoplankton samples fixed with glutaraldehyde (0.2% final concentration), were frozen in liquid nitrogen and stored at −80 °C until analysis. The analysis was carried out using a BD FACS Calibur Flow cytometer equipped with blue (488 nm) and red (633 nm) lasers, and absolute counts were obtained following Marie et al. (1997).

2.4. Satellite data

Sea surface temperature data were downloaded from http://apdrc.soest.hawaii.edu:80/dods/public_data/satellite_product/AMSR/AMSR-2/3dayIs with a spatial resolution of 0.25 × 0.25°. The daily mean sea level anomaly (MSLA) data were acquired from http://www.aviso.altimetry.fr/ with the same spatial resolution.

2.5. Statistical analysis

Prior to the statistical analysis, phytoplankton marker pigments (DP) were normalized and square root transformed. Spatial distribution of pigments was assessed through the non-metric-multidimensional scaling (nMDS) analysis based on Bray-Curtis index (Bray and Curtis, 1957). Moreover, the spatial association of maker pigments was explained by the distance-based redundancy (dbRDA) plot where the strength and direction of effect of the variable on the ordination plot can be clearly seen (Anderson et al., 2008). All the statistical analyses were performed in the module PRIMER V6 software.

3. Description of study area

Based on the SST distribution, the NEAS (SST < 26.5 °C) and the SEAS (SST > 28 °C) represented two distinct physical domains. While only the northernmost station (II-14; 21°N) experienced winter cooling in 2015 (Fig. 1a), six stations (II-14 to II-9; 21–14°N) were located within the zone of seasonal cooling in 2017 (Fig. 1b). The 28 °C isotherm has been used to identify the ASMWP in the SEAS. Vinayachandran et al. (2007) pointed out that ASMWP has the combination of some unique features such as warm SST (> 28 °C; Fig. 1a & b) and presence of warm core (anti-cyclonic) eddy known as the Lakshadweep High (LH). In March 2015, stations II-2 to II-6 were within the ASMWP (Fig. 2a), while only three stations (II-2 to II-4) fell within this feature in 2017 (Fig. 2b). Note that the satellite SST was averaged over 10 days (5–15 February 2017) in Fig. 1b; whereas stations II-2 to II-4 were sampled within three days (5–7 February), resulting in a slight

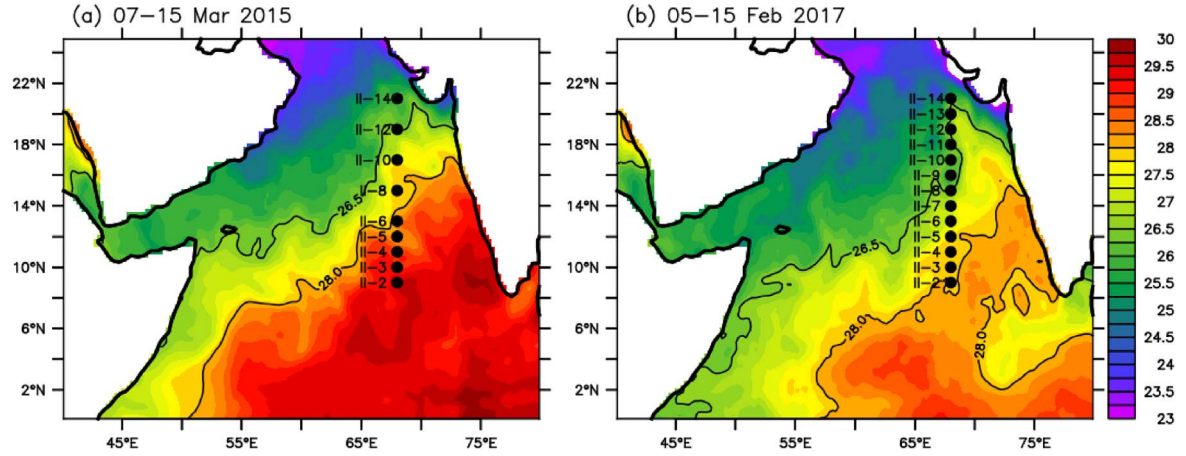

Fig. 1. Spatial maps of SST in the Arabian Sea during 7–15 March 2015 (a) and 5–15 February 2017 (b). Filled black circles represent the sampling locations at 68°E transect.

mismatch in SST between the satellite and *in situ* data. The distribution of temperature in the upper 200 m in March 2015 (Fig. 2a) clearly indicated cooler temperature (< 26 °C) in the NEAS (station II-14) and warmer temperature (> 28 °C) in the upper 100 m in the SEAS. The 27 °C isotherm showed distinct downward slope from NEAS to SEAS. This confirms mixing within the upper ~120 m of water column in NEAS (prevalence of convective mixing) and deepening of the thermocline in the SEAS. Similarly, the temperature distribution in February 2017 (Fig. 2b) demonstrates convective mixing in the NEAS at stations II-14 to II-9 and the ASMWP (stations II-2 to II-4) in the SEAS.

4. Results and discussion

4.1. Phytoplankton response to winter cooling in the NEAS

The NEAS experiences convective mixing during November to February and several earlier studies showed its influence on the phytoplankton community (Banse, 1987; Sawant and Madhupratap, 1996;

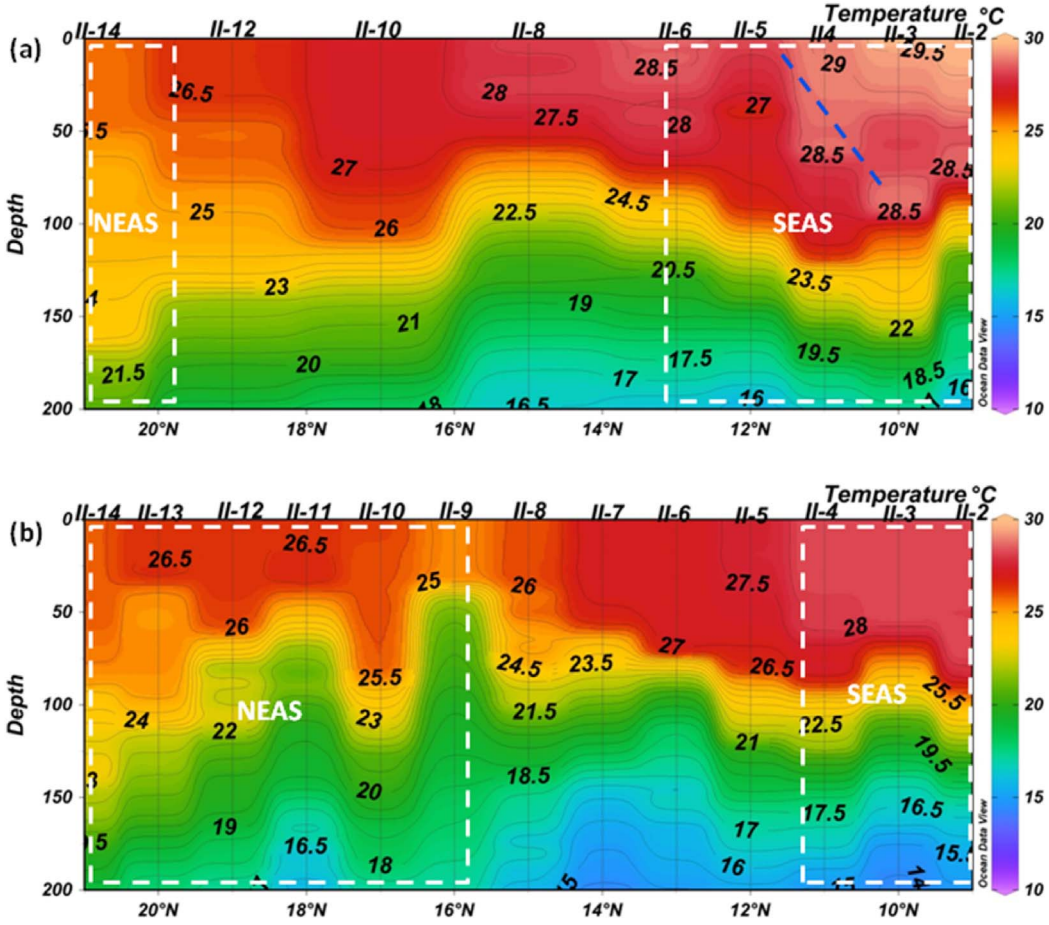

Fig. 2. Vertical section of temperature (°C) in the upper 200 m (21 to 9°N along 68°E) during March 2015 (a) and February 2017 (b). The dashed blue line indicates deepening of the isotherm representing the warm core eddy in the SEAS during 2015. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Chemotaxonomic association of phytoplankton marker pigments used in the present study (Gibb et al., 2001; Jeffrey et al., 1997).

Pigment	Abbreviation	Corresponding group
Chlorophyll *a*	Chl *a*	Total algal biomass (including cyanobacteria)
Chlorophyll *b*	Chl *b*	Chlorophytes, prasinophytes
19′-Butanoyloxyfucoxanthin	19′BF	Chrysophyceae, haptophyta
Divinyl chlorophyll *a*	DivChl *a*	*Prochlorococcus* sp.
Divinyl chlorophyll *b*	DivChl *b*	*Prochlorococcus* sp.
Fucoxanthin	Fuco	Diatoms, haptophyta, chrysophyceae
19′-Hexanoyloxyfucoxanthin	19′HF	Haptophyta
Peridinin	Per	Autotrophic dinoflagellates
Zeaxanthin	Zea	Cyanobacteria, *Prochlorococcus* sp.

Madhupratap et al., 1996). These studies revealed that the convective mixing leads to deepening of mixed layer enhancing nutrient availability in the euphotic zone, thereby supporting larger phytoplankton, mainly diatoms. Observations in 2015 were made in the first week of March starting from 21°N latitude and proceeding southward to 9°N latitude. SST data from satellite (Fig. 1a) and CTD (Fig. 2a) showed relatively low temperatures in the north (< 26 °C) associated with the well-mixed water column down to ~120 m due to convection.

Photosynthetic pigments are commonly used to understand the phytoplankton composition and their physiological status (Jeffrey et al., 1997). However, marker pigments are not exclusive for any one group of algae as seen in Table 1. Though having limitations, phytoplankton pigments (specifically diagnostic pigments; DP) are useful for representing the major groups contributing to the phytoplankton community and to calculate fractions of each size class with respect to total phytoplankton biomass (Uitz et al., 2006). The concentrations of phytoplankton pigments based on HPLC analysis are shown in Tables 2 & 3 and Figs. 3 & 4. Concentration of Chl *a* was higher in the NEAS (II-14; 1080 ng L^{-1}) in 2015 mainly contributed by diatoms (fucoxanthin - 360 ng L^{-1}), haptophytes (19′-hexanoyloxyfucoxanthin [19′HF] - 150 ng L^{-1}) and dinoflagellates (peridinin - 25 ng L^{-1}) in the subsurface waters. Similarly, Chl *a* concentrations were higher in the NEAS (up to 673 ng L^{-1} at II-9) in February 2017 contributed by diatoms (fucoxanthin - 101 ng L^{-1}), haptophytes (19′HF - 146 ng L^{-1}) and dinoflagellates (peridinin - 32 ng L^{-1}). *Prochlorococcus* marker pigments (divChl *a* and divChl *b*) were not found at II-14, but were present in low concentrations at station II-12 (Fig. 4c, d) in 2015. This is supported by picoplankton counts derived from flow cytometric analysis. Recently, flow cytometry has become an indispensable tool in marine microbial ecology for the identification and enumeration of picoplankton. However, *use of the small sample volumes (0.5–1.0 ml) from the open waters for picoplankton analyses using flow cytometer, for instance, could lead to underestimation of cells statistically (Dubelaar and Jonker, 2000). Consistent with marker pigments, flow cytometric data* also showed the absence of *Prochlorococcus* at station II-14, however, it was present in the subsurface waters (50–100 m) at station II-12 (Fig. 6a). In contrast, *Synechococcus* was abundant in NEAS (up to 37 × 10^3 cells ml^{-1} at II-14) as also evident from the presence of its marker pigment, zeaxanthin (Fig. 4b). DivChl *a* and divChl *b* were present in the NEAS in February 2017, though at lower concentrations (Fig. 4g, h). Flow cytometric count also revealed low abundance of *Prochlorococcus* in the NEAS as

Table 2
Concentration of phytoplankton pigments in ng L^{-1} during March 2015. "–" means concentration below detection limit.

SSK 79	Depth (m)	Per	19′BF	Fuco	19′HF	Zea	DivChl *b*	Chl *b*	DivChl *a*	Chl *a*
II-14	0	16	25	130	90	33	–	95	–	630
II-14	25	18	42	186	133	31	–	92	–	688
II-14	30	25	45	361	151	26	–	136	–	1084
II-14	70	–	–	14	9	0	–	–	–	37
II-14	100	–	–	6	–	–	–	–	–	26
II-12	0	–	–	–	17	76	–	19	80	131
II-12	25	–	–	–	32	81	17	–	83	142
II-12	50	–	64	40	151	42	–	75	60	398
II-12	80	–	64	61	108	10	–	59	–	347
II-12	100	–	30	37	41	5	–	–	–	127
II-10	0	–	–	–	12	62	–	–	61	62
II-10	20	–	–	–	9	40	–	–	38	30
II-10	40	–	9	–	27	70	20	–	95	156
II-10	60	15	70	60	146	25	–	79	29	460
II-10	80	–	55	18	51	5	–	21	–	166
II-10	100	–	15	5	15	–	20	–	–	22
II-8	0	–	–	–	11	66	–	–	38	38
II-8	25	–	103	41	267	21	–	193	57	584
II-8	55	–	8	5	24	76	–	–	47	80
II-8	80	–	54	16	82	8	87	–	54	175
II-8	100	–	18	5	33	–	54	–	25	53
II-6	0	–	0	0	8	78	0	–	46	48
II-6	30	–	7	0	22	73	0	–	61	64
II-6	75	–	88	19	125	20	191	–	89	320
II-6	100	–	35	8	39	5	100	–	43	89
II-5	0	–	–	–	18	73	–	–	32	51
II-5	44	21	113	20	19	78	157	–	151	415
II-5	105	–	21	17	–	–	–	–	–	–
II-4	0	–	–	–	10	90	–	–	57	45
II-4	30	–	10	–	26	86	15	–	70	148
II-4	68	13	125	25	163	52	187	–	127	401
II-4	100	–	34	9	38	4	97	–	40	78
II-2	0	–	–	16	6	76	–	–	44	61
II-2	20	–	–	10	15	88	–	–	57	56
II-2	40	–	8	–	23	74	15	–	80	65
II-2	60	–	33	6	64	62	29	–	106	134
II-2	85	–	97	19	132	27	103	–	78	283
II-2	120	–	21	–	19	–	24	–	–	38

Table 3
Concentration of phytoplankton pigments in ng L^{-1} during February 2017. "–" means concentration below detection limit.

SSD 033	Depth (m)	Per	19′BF	Fuco	19′HF	Zea	DivChl *b*	Chl *b*	DivChl *a*	Chl *a*
II-14	0	8	34	25	98	44	–	57	13	292
II-14	40	7	23	20	79	44	–	58	17	311
II-14	86	–	10	10	18	4	–	8	–	53
II-14	117	–	6	2	11	1	–	10	8	9
II-13	0	5	9	12	30	80	–	23	41	182
II-13	33	11	50	39	156	36	–	130	22	464
II-13	40	12	72	39	175	49	–	115	15	464
II-13	67	4	40	23	67	13	–	52	6	214
II-12	0	25	30	68	72	43	–	60	17	335
II-12	25	5	40	48	108	32	–	61	12	302
II-12	50	3	18	48	52	31	–	47	15	247
II-12	58	–	27	23	50	6	77	–	22	132
II-12	65	–	33	23	58	13	–	49	10	169
II-12	82	–	71	15	139	43	144	–	98	297
II-12	91	–	8	5	19	2	20	–	6	33
II-12	101	–	5	3	13	2	9	–	11	18
II-11	0	5	18	14	70	114	–	27	53	285
II-11	17	6	28	28	128	58	–	64	15	346
II-11	37	11	72	108	170	25	–	165	22	556
II-11	60	–	24	16	35	7	122	–	43	101
II-11	93	–	3	2	8	1	12	–	5	12
II-10	0	9	17	19	123	97	–	50	–	284
II-10	24	8	33	16	120	80	–	73	–	363
II-10	41	12	53	26	110	34	–	92	–	329
II-10	77	4	18	9	26	4	–	18	–	75
II-9	0	32	45	101	146	146	–	80	–	673
II-9	32	23	42	95	128	119	–	83	–	599
II-9	35	19	36	87	111	86	–	60	–	468
II-9	65	0	3	6	11	2	–	0	–	24
II-8	0	8	29	32	77	67	–	66	–	292
II-8	20	6	20	26	75	39	–	62	–	254
II-8	37	5	52	49	88	20	–	79	–	311
II-8	53	3	27	18	45	3	–	28	–	101
II-8	83	0	7	5	16	1	–	6	–	34
II-8	106	0	11	4	34	2	–	9	–	38
II-7	0	0	5	4	16	110	–	13	62	95
II-7	30	4	8	6	27	83	–	26	43	167
II-7	44	15	49	28	93	42	–	94	4	373
II-7	70	5	43	26	77	14	165	–	59	226
II-7	110	–	–	–	4	–	–	–	–	3
II-6	0	6	36	14	120	77	–	49	23	305
II-6	30	4	21	10	83	67	–	45	17	261
II-6	71	4	33	12	98	56	–	72	20	238
II-6	100	–	–	–	16	1	18	–	6	21
II-5	0	–	4	4	12	95	–	12	52	61
II-5	15	–	2	2	7	86	–	11	56	62
II-5	53	9	55	14	123	32	–	95	38	341
II-5	86	0	31	6	45	3	60	–	19	99
II-4	0	3	4	5	16	88	–	15	56	65
II-4	25	–	2	2	10	75	–	14	51	37
II-4	50	3	13	6	36	81	34	–	107	101
II-4	60	4	48	14	63	17	130	–	51	175
II-4	90	5	51	12	105	70	80	–	132	216
II-3	0	–	5	3	15	74	–	12	59	58
II-3	35	–	2	1	8	65	–	12	60	48
II-3	62	8	45	13	99	24	119	–	57	231
II-3	78	5	36	13	51	14	122	–	43	153
II-2	0	4	6	3	25	89	–	22	79	70
II-2	25	–	–	–	7	82	–	7	38	37
II-2	50	–	2	2	8	86	–	9	43	38
II-2	120	–	15	3	18	3	62	–	17	54

compared to the SEAS (Fig. 6b). Similar to the 2015 observations, *Synechococcus* was abundant in NEAS (up to 55.7×10^3 cells ml^{-1} at II-14) in 2017 as also evident from the presence of marker pigment, zeaxanthin (Fig. 4f).

In the NEAS, nutrients entrained into the euphotic zone from below the thermocline by convective mixing in winter support large phytoplankton as reported earlier by Sawant and Madhupratap (1996). These authors found diatoms to account for 70–90% of phytoplankton biomass both during winter and inter-monsoon seasons during the JGOFS survey in mid 1990s. More recently, it has been reported that a dramatic shift in the composition of winter phytoplankton blooms has occurred in the Arabian Sea over the past decade or so in that diatoms have now been replaced by the dinoflagellate, *Noctiluca scintillans* as the dominant group (Gomes et al., 2014). This shift has been attributed to massive influx of hypoxic water into the euphotic zone. However, in the present study, based on marker pigment fucoxanthin, we found diatoms were still the dominant phytoplankton group in the NEAS at the time of our observations. Dinoflagellates constituted a small fraction of the phytoplankton abundance (Fig. 3c), with the concentration of their marker pigment (peridinin) being an order of magnitude lower than

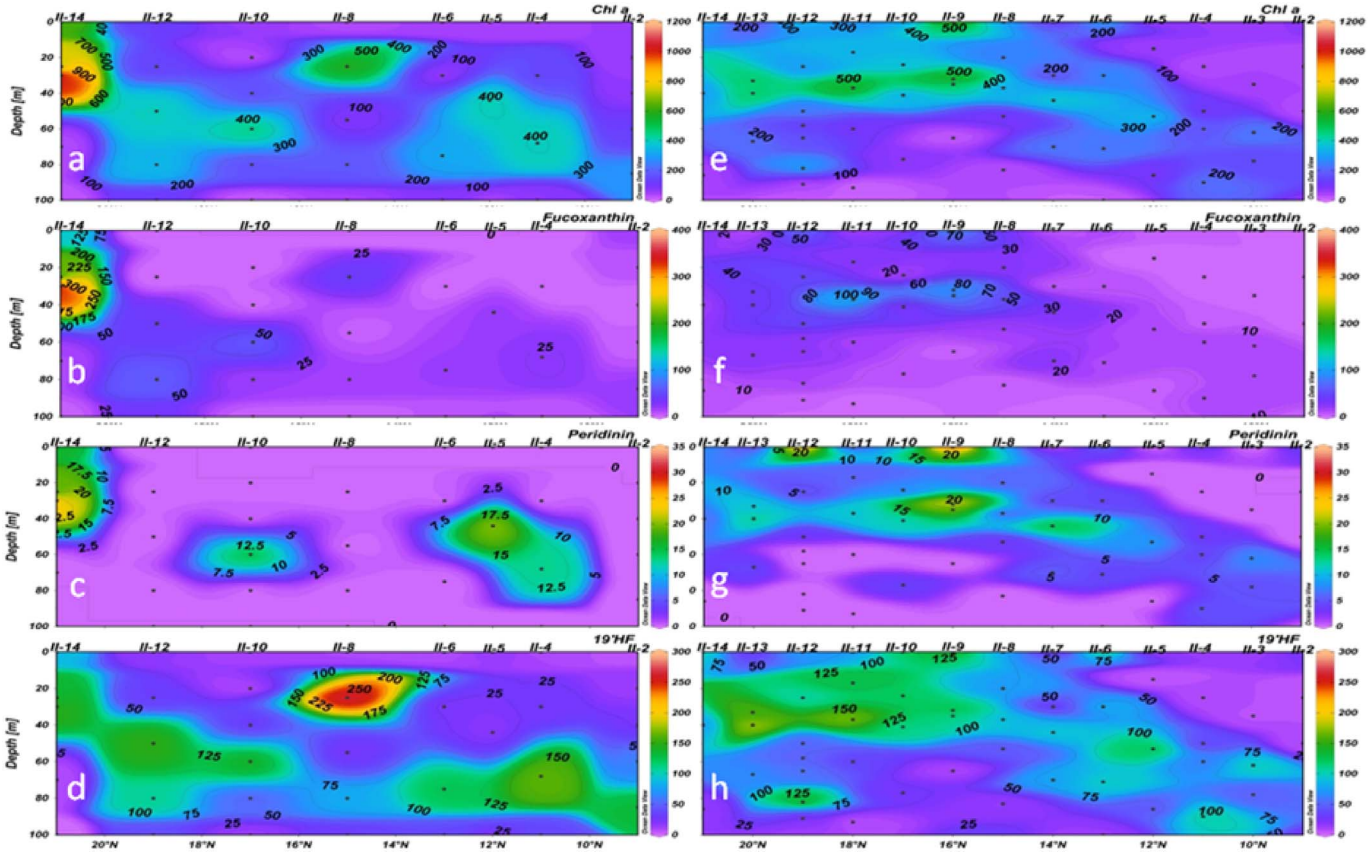

Fig. 3. Concentration of phytoplankton pigments (ng L^{-1}) from north to south (21 to 9°N) along 68°E in the eastern Arabian Sea. Panels on the LHS are during March 2015 and on the RHS during February 2017.

that of fucoxanthin. This is consistent with the results of microscopic analysis showing lesser abundance of dinoflagellates (Avg. 27% of total phytoplankton) than of diatoms (73%) in the upper 50 m of the water column at station II-14 in March 2015.

The water column was well oxygenated down to ~100 m depth as a result of convective mixing during both years (data not shown) in conflict with reported presence of hypoxic water within the euphotic zone by Gomes et al. (2014). Well oxygenated conditions within the photic zone were also observed by Prakash et al. (2017) in the NEAS in February 2015. The average nitrate concentration in the upper 100 m was low at station II-14 in 2015, which is as expected given that the observations were made towards the end of the convective mixing period. However, the silicate concentration was comparatively higher with an average Si/N ratio of 2.6 in the upper 100 m (Fig. 5) supporting the proliferation of diatoms (Fuco: 320 ng L^{-1}, Fig. 3b). On the other hand, the nutrient distribution was different in 2017 with lower Si/N ratios (averaging from 0.6 to 1.3 in the upper 100 m; Fig. 5) at stations II-9 to II-14. Thus, it appears that the Si/N ratio controlled the phytoplankton composition in the NEAS in 2017 with lesser contribution from microplankton (Fuco: max. 101 ng L^{-1}) and the nanoplankton dominating. This is also reflected by lower Chl *a* in 2017 (average 263 ng L^{-1}) than in 2015 (average 493 ng L^{-1}).

Prochlorococcus was not present at station II-14 (NEAS) going by the absence of divChl *a* and divChl *b*, which is also evident from the flow cytometric data. The absence of *Prochlorococcus* is consistent with the observations of Roy et al. (2015) who concluded that winter mixing in the northern Arabian Sea inhibits *Prochlorococcus* growth owing to changes in light and biogeochemical regimes. However, *Prochlorococcus* was present at station II-14 in 2017 in low abundance (Fig. 6b).

4.2. Phytoplankton in the Arabian Sea mini warm pool of the SEAS

Conditions observed in the southern part of the transect were quite different from those described above. As stated earlier, in 2015 the warm surface temperatures in the SEAS (stations II-6 to II-2) were due to the presence of ASMWP and the conditions were oligotrophic in nature. The low Chl *a* concentration (maximum 415 ng L^{-1}; 2.6 times lower than in NEAS) along with a well-defined subsurface chlorophyll maximum at ~80 m (Fig. 3a) indicated typical oligotrophic characteristics of the SEAS in 2015. Conditions were similar in 2017 as well with low Chl *a* concentration in the SEAS (up to 231 ng L^{-1} at station II-3) compared to the NEAS (up to 673 ng L^{-1}). Marker pigments of *Prochlorococcus* (divChl *a* and divChl *b*) and *Synechococcus* (Zea) along with haptophytes (19′HF and 19′BF) were higher in this region in both the years. Microplankton pigments were much lower (Fuco: up to 13.7 ng L^{-1} and peridinin: 7.6 ng L^{-1}) in the SEAS as compared to the NEAS (Fig. 3).

The deepening of isotherms to 100–200 m at stations II-5 to II-3 indicated downwelling associated with a warm core eddy (Fig. 2a) in 2015. The maximal deepening of the isotherms at stations II-4 and II-3 represents the core of the eddy whereas stations II-5 and II-2 were located at the periphery of the eddy. This is also supported by the map (Fig. 6c) of Sea Level Anomalies (SLA), which shows the presence of strong warm core eddy in the SEAS with stations II-4 and II-3 (+27 cm) located within the core of the eddy and stations II-5 and II-2 being at its periphery (+15 cm). Such anti-cyclonic eddies depress the isopycnals in the upper ocean significantly influencing phytoplankton composition and abundance (McGillicuddy et al., 1999; Sweeney et al., 2003). Among the picophytoplankton, *Prochloroccocus* showed highest abundance (up to 101.5×10^3 cells ml^{-1}; 4 times higher) within the core of the eddy (II-4) compared to that at station II-2 (up to

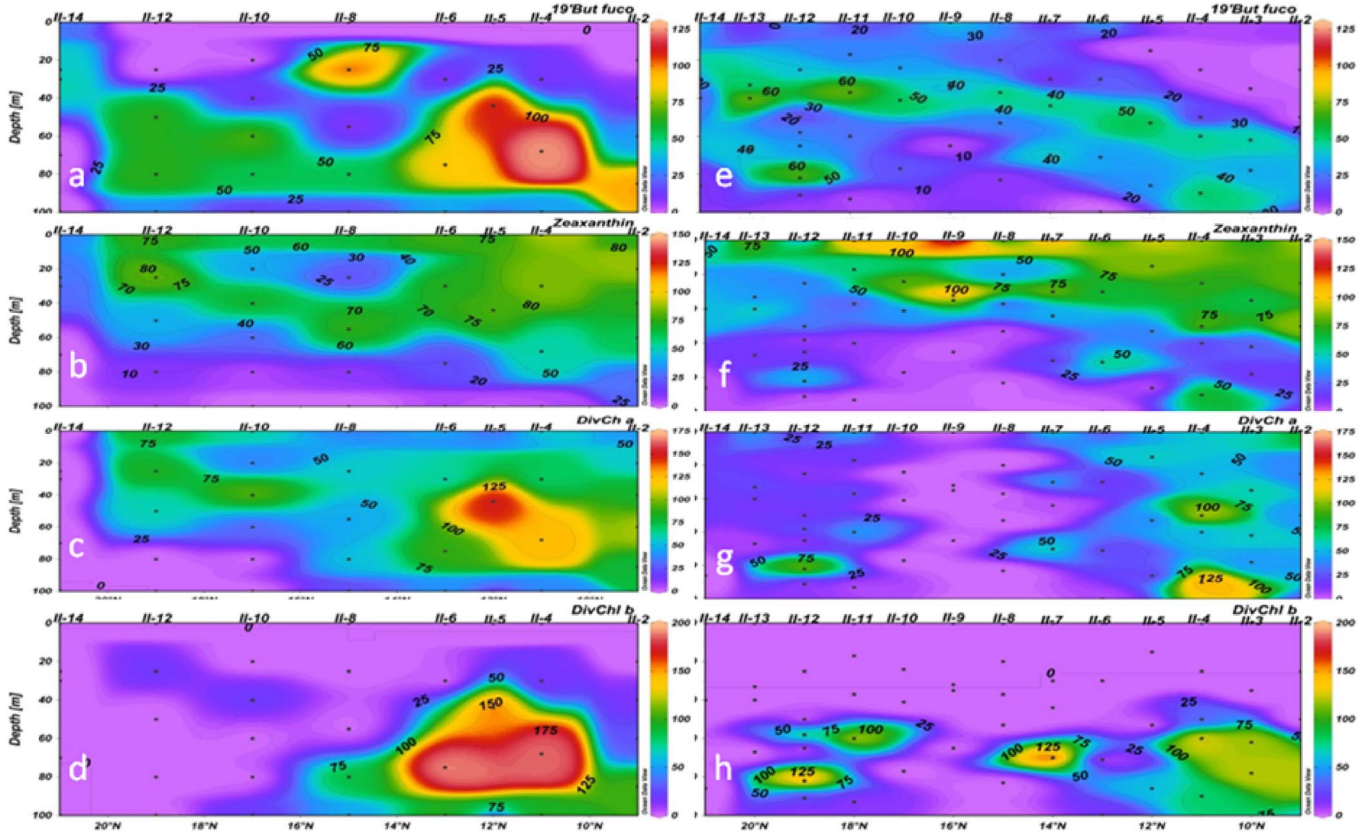

Fig. 4. Concentration of phytoplankton pigments (ng L^{-1}) from north to south (21 to 9°N) along 68°E in the eastern Arabian Sea. Panels on the LHS are during March 2015 and on the RHS during February 2017.

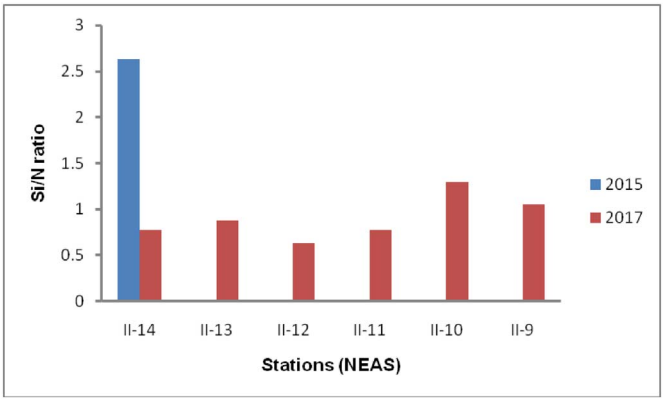

Fig. 5. Average silicate to nitrate (Si/N) ratios for the upper 100 m water column in the NEAS during 2015 and 2017.

23.8 × 10^3 cells ml^{-1}) which was located at the periphery of the eddy (Fig. 6a). This is also reflected in the marker pigments divChl *a* and divChl *b* which were higher within the core of the eddy than at its periphery (Fig. 4c, d). The *Prochloroccocus* counts and the concentration of marker pigments were lower in 2017 when no warm core eddy was detected in the sampling region. The processed (delayed mode) SLA data clearly indicated the absence of warm core eddy at the sampling locations (figure not shown). Hence our study provided an opportunity to compare the response of picophytoplankton within the ASMWP with the presence (2015) and absence (2017) of warm core eddy.

Synechococcus was also present in the SEAS (up to 5.2 × 10^3 and 9.4 × 10^3 cells ml^{-1} at stations II-4 and II-2 respectively) in 2015 and (up to 8.1 × 10^3 and 9.4 × 10^3 cells ml^{-1} at stations II-4 and II-2 respectively) in 2017. However, there was a reduction in its abundance from north to south. Among the picophytoplankton, *Prochlorococcus* was more abundant at the core of the eddy in response to the physical environment (well stratified warm water column) as compared to *Synechococcus*. Similar results were observed within the anti-cyclonic eddies of the Sargasso Sea (Sweeney et al., 2003; Ewart et al., 2008). Huang et al. (2010) also reported the dominance of Prochlorophyceae in one of the anti-cyclonic eddy located in the South China Sea whereas Haptophyceae dominated in the other. They attribute the difference in the phytoplankton community to the different origins and ages of the two eddies.

4.3. Statistical analysis

Statistical analysis was performed to support our observation of distinct phytoplankton groups in contrasting physical regimes. Non-metric-multidimensional scaling (nMDS) analysis was carried out for the marker pigments in NEAS and SEAS for 2015 and 2017 and the results are shown in Figs. 7a and 8a. For the year 2015, nMDS plot clearly showed the spatial partition of sampling sites in two major groups with 60% similarity. The assemblage of one group contains II-14 samples representing winter mixing in the NEAS; the other group includes sampling stations II-2, II-4, II-5 and II-6 representing ASMWP in the SEAS (Fig. 7a). In addition, dbRDA analysis showed that marker pigments fucoxanthin and peridinin (representing microphytoplankton) were mostly associated with station II-14 (Fig. 7b). This station falls within the winter convective mixing zone in the NEAS and dominated by diatoms (73%) followed by dinoflagellates (27%). By contrast, divChl *a* and divChl *b* were associated with stations II-4 and II-6 within the SEAS where *Prochlorococcus* was dominant (Fig. 6). *Synechococcus* marker pigment zeaxanthin was also closely associated with the SEAS stations as seen from the length of the vector (Fig. 7b). Thus, the statistical analysis clearly demonstrates that phytoplankton groups

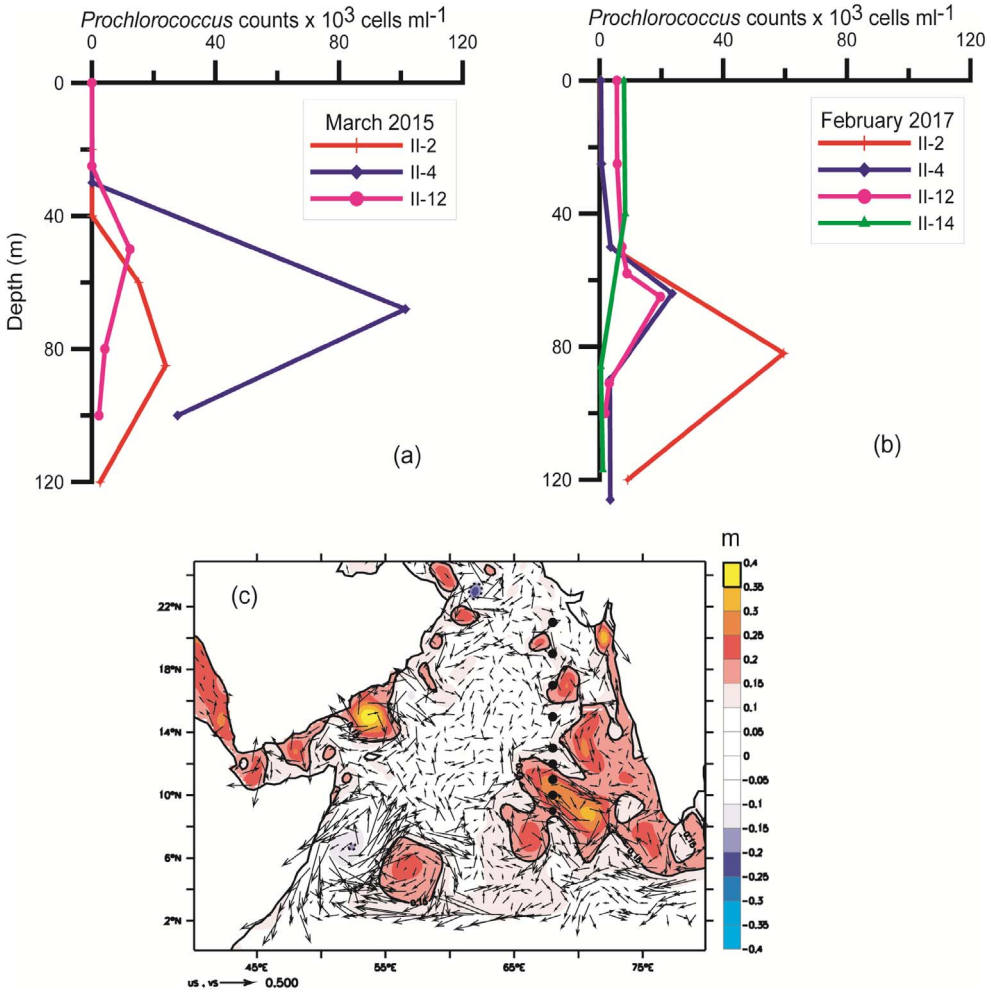

Fig. 6. Vertical profile of *Prochlorococcus* counts ($\times 10^3$ cells ml^{-1}) in the upper water column during March 2015 (a) and February 2017 (b). Please note that *Prochlorococcus* were absent at II-14 during March 2015. Spatial maps of Sea Level Anomaly in the Arabian Sea during 7–15 March 2015 (c). Positive values indicate warm core eddies and negative values indicate cold core eddies. Vectors represent the geostrophic currents and black dots represent sampling locations.

associated with contrasting physical regimes (winter convective mixing and ASMWP) in the Arabian Sea were quite different in March 2015.

The nMDS analysis of the 2017 data showed three major groups with 80% similarity, where most of the NEAS stations (II-9 to II-14) formed one cluster whereas the SEAS stations (II-4 to II-2) formed the second cluster. However few NEAS and SEAS stations together formed a third cluster (Fig. 8a). As compared to March 2015, the hydrographic conditions in the NEAS and SEAS were less different in February 2017. The dbRDA analysis also showed that the marker pigments of microphytoplankton and nanophytoplankton were mostly associated with all the NEAS stations, whereas picophytoplankton marker pigments (Zea, DivChl *a* and DivChl *b*) were more inclined towards the SEAS stations (Fig. 8b). In general, statistical analysis supported the presence of distinct phytoplankton groups in the two contrasting physical regimes as also seen from the concentration of marker pigments (Figs. 3 and 4).

4.4. Comparison with other regions

Our study demonstrates distinct north-south variation of phytoplankton pigments associated with contrasting physical conditions in the Arabian Sea. Convective mixing in the NEAS supports the dominance of microphytoplankton whereas strong stratification in the SEAS supports picophytoplankton. Although some earlier studies had described responses of the phytoplankton community to the winter mixing in the NEAS (Madhupratap et al., 1996; Sawant and Madhupratap, 1996), the composition of autotrophs in the ASMWP existing at the same time in SEAS was not known. However, several studies dealt with phytoplankton communities associated with contrasting oceanographic conditions in other oceanic areas. For example, Thompson et al. (2011) reported dynamics of phytoplankton communities on the east and west coasts of Australia associated with the seasonality of the two major boundary currents (East Australian Current and Leeuwin Current) and their influence on the vertical stability of the water column and availability of nutrients in the euphotic zone. Nitrate was found to limit new production along the southwest coast of Australia resulting in fewer bacillariophytes and dinophytes (microplankton) compared to the southeast coast. Armbrecht et al. (2014) studied the phytoplankton composition under contrasting (upwelling and downwelling) oceanographic conditions along the east coast of Australia. During the downwelling period, these authors observed cross-shelf transition in microphytoplankton composition from an offshore to inshore community. In contrast, under upwelling conditions elevated nutrient availability resulted in maximal microphytoplankton abundance (mainly diatoms) on the mid-shelf. In both cases diatoms were found to be the most sensitive phytoplankton class to changes in temperature and nutrients (especially silicate) across the continental shelf. We also found that the Si/N ratio controlled the microplankton composition in the NEAS with lower abundance in 2017 than in 2015. In the northern Indian Ocean, Pandi et al. (2014) reported dominance of microphytoplankton in the northern coastal Bay of Bengal (greatly influenced by the runoff from the Ganges-Brahmaputra system), whereas the southern coastal Bay of Bengal (influenced by the peninsular rivers) was dominated by nano-

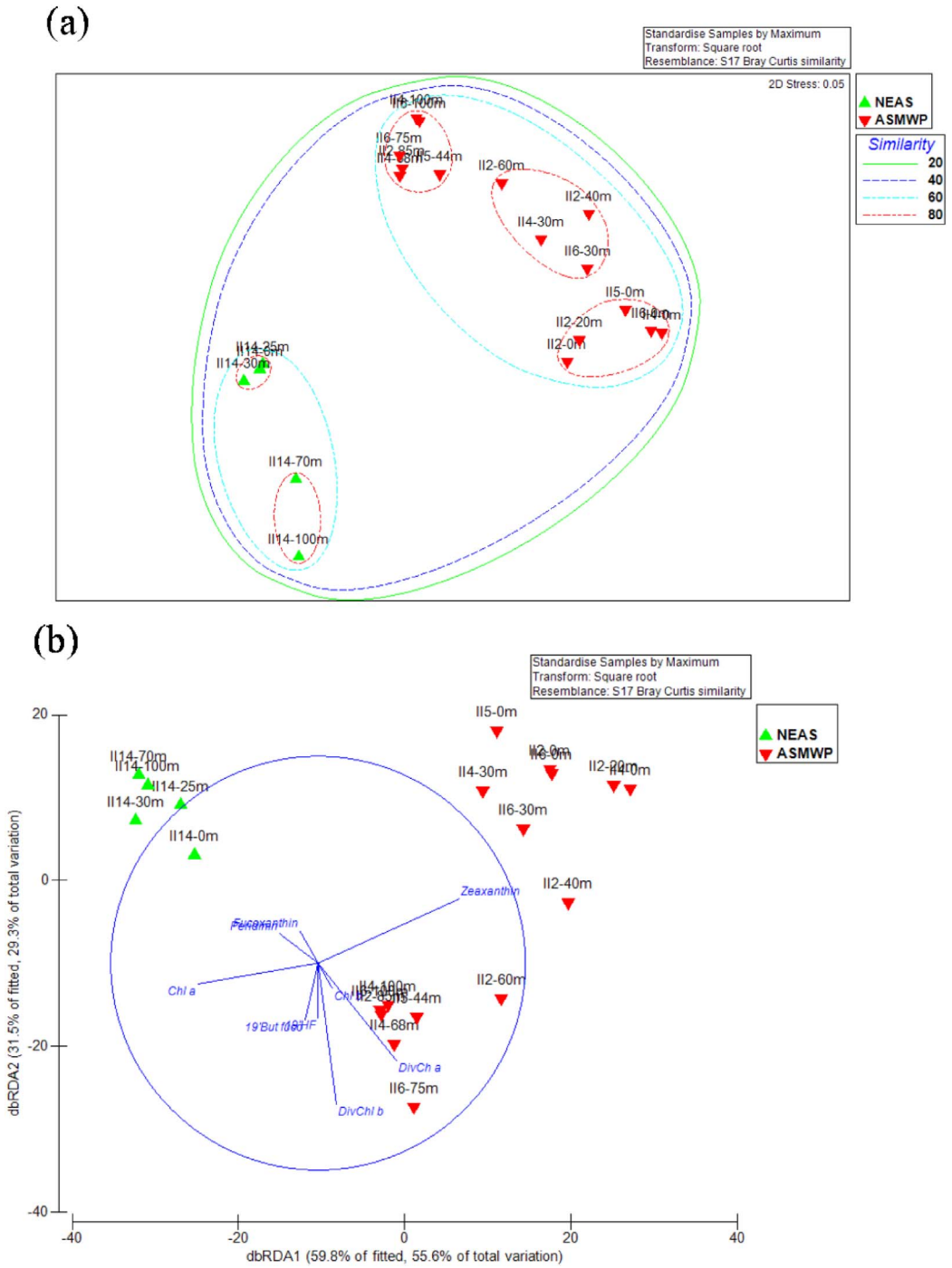

Fig. 7. (a) nMDS ordination based on phytoplankton marker pigments as per Bray–Curtis similarity index during March 2015. (b) Distance-based redundancy (dbRDA) plot representing sampling stations and fitted marker pigments with their vectors (strength and direction of effect of the variable on the ordination plot) during March 2015. Axis legends include percentage of variation explained by the fitted model and percentage of total variation explained by the axis.

and picophytoplankton. This difference was attributed to the contrasting optical and ecological conditions in the two coastal regions. Recently, Ahmed et al. (2016) highlighted the influence of environmental conditions in structuring phytoplankton composition in the eastern Arabian Sea during the upwelling period (June) with distinct north-south variation. The nutrient-rich upwelled waters in the south were dominated by diatoms and *Synechococcus*, while *Prochlorococcus* was dominant in the relatively oligotrophic northern part. The present study was carried out during late winter when convective mixing results in deepening of mixed layer and nutrient availability within the euphotic zone in the NEAS, thereby promoting the growth of microplankton, mainly diatoms. It also shows that the phytoplankton respond to the development of the ASMWP by increasing the fraction of picophytoplankton. Earlier studies from this region were mainly focussed on the physical characteristics of the mini warm pool (Rao and Sivakumar, 1999; Vinayachandran et al., 2007) and the bio-physical coupling was not addressed by earlier workers. Richardson and Jackson (2007) reported that despite their small size, picoplankton contributed significantly to carbon export from the surface ocean. The contribution of picophytoplankton to carbon export in the ASMWP warrants detailed study in the future.

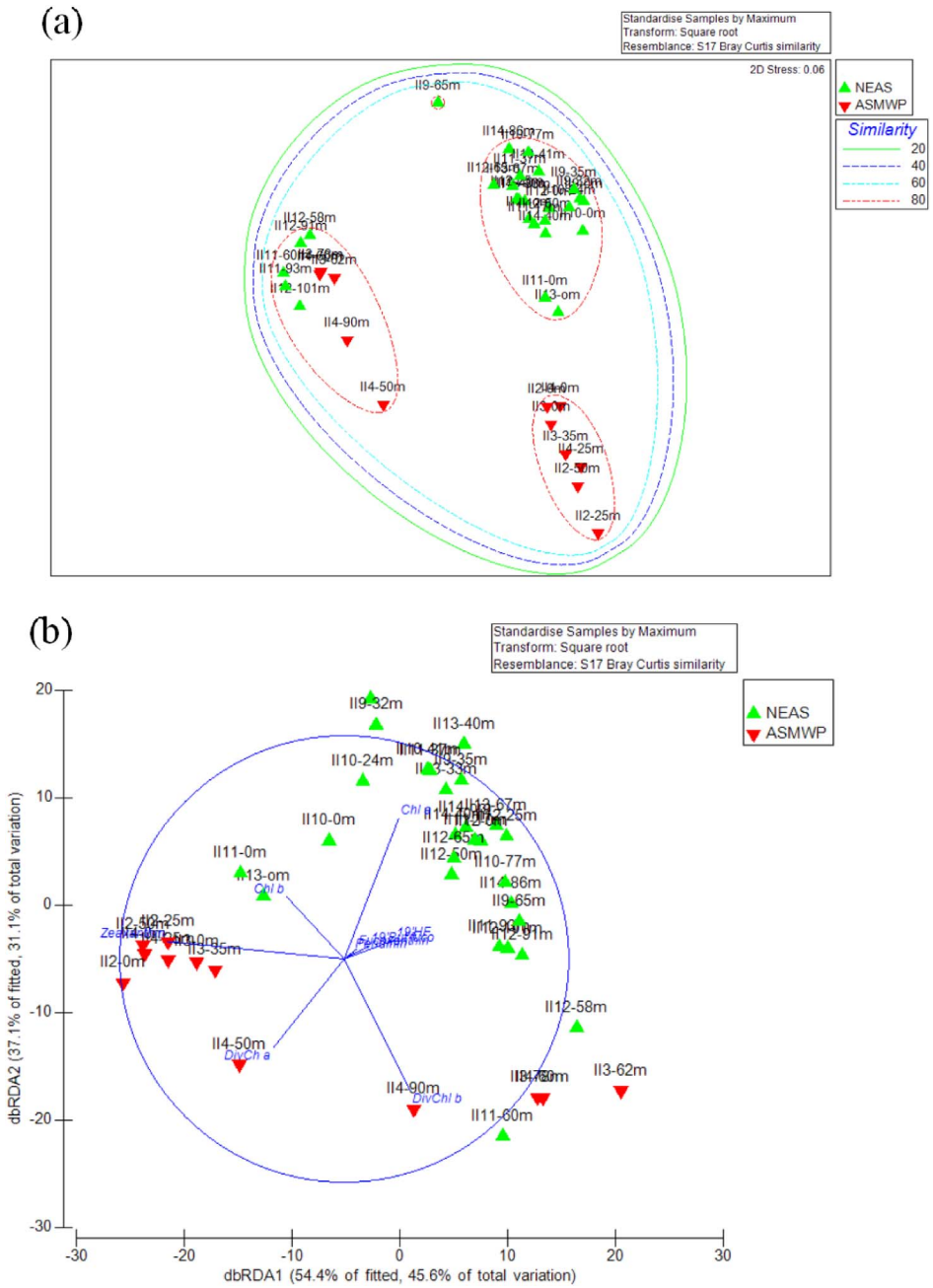

Fig. 8. (a) nMDS ordination based on phytoplankton marker pigments as per Bray–Curtis similarity index during February 2017. (b) Distance-based redundancy (dbRDA) plot representing sampling stations and fitted marker pigments with their vectors (strength and direction of effect of the variable on the ordination plot) during February 2017. Axis legends include percentage of variation explained by the fitted model and percentage of total variation explained by the axis.

5. Conclusion

Based on marker pigment analysis and cell counts by flow cytometry, our study demonstrates significant changes in phytoplankton composition associated with different physical regimes (convective mixing and ASMWP) prevalent in the northern and southern parts of the eastern Arabian Sea in late winter. Convective mixing in the north supports larger phytoplankton community with diatoms as the most important autotrophs. Although the contribution of diatoms was lower in 2017 than in 2015, presumably due to the variability of the Si/N ratio in the NEAS, our results do not support the reported shift from a diatom-dominated community to a dinoflagellate (*Noctiluca*)-dominated community forced by the development of hypoxic conditions in the euphotic zone, which were also not observed in 2015 or 2017. In the SEAS, oligotrophic conditions prevailed within the ASMWP where picoplankton (*Prochlorococcus* and *Synechococcus*) and nanoplankton dominated over the microplankton. Statistical analysis also supported the presence of distinct phytoplankton groups in the two contrasting physical regimes. In addition, the presence of a warm core eddy in the SEAS in March 2015 provided favorable conditions for the proliferation of *Prochlorococcus*. The dominance of picophytoplankton in the ASMWP, being reported for the first time, warrants further work to investigate in detail the associated food web and carbon export to the deep sea.

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Diversity of culturable Sulphur-oxidising bacteria in the oxygen minimum zones of the northern Indian Ocean

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ABSTRACT

Oxygen minimum zones (OMZs) are unique, widely spread and well-studied features of the global ocean, varying in seasonality and intensity. The Northern Indian Ocean contains OMZs in the Arabian Sea (AS-OMZ) and the Bay of Bengal (BB-OMZ) having unique biogeochemical features. OMZ water column harbours distinct microbial communities that play vital roles in ocean biogeochemical cycles. Sulphur cycling processes facilitated by OMZ microbial communities are poorly understood with regards to different microbial groups involved, spatially and temporally. Here we sampled different stations in the AS-OMZ and BB-OMZ across dissolved oxygen gradients, to obtain culturable sulphur-oxidising bacterial (SOB) diversity using modified agar-based media. Phylogenetic analysis of the isolates placed the majority of them in Phylum Proteobacteria, followed by Actinobacteria, Firmicutes, and Bacteroidetes. Analyses of thiosulphate oxidation potential of isolates revealed that an α-proteobacteria, *Citreicella thiooxidans* and a β-proteobacterial species, *Achromobacter xylosoxydans* oxidised thiosulphate to sulphate within 48 h, suggesting that this potential is present within a narrow group of bacteria. This study sheds light on the functioning of the microbial community in the sulphur cycle of the Indian Ocean OMZs.

1. Introduction

Upwelling of nutrient-rich waters promotes high primary production and oxygen consumption through microbial respiration (Wyrtki, 1962; Helly and Levin, 2004; Paulmier and Ruiz-Pino, 2009) due to which vast areas of the global ocean are characterized by naturally occurring oxygen minimum zones (OMZs). These OMZs contain dissolved oxygen concentrations as low as 20 μM at intermediate depths (100–1300 m) (Kamykowski and Zentera, 1990; Levin, 2003; Stramma et al., 2008; Gilly et al., 2013). Around 8% of the entire oceanic surface is covered by permanent OMZs which include Eastern Subtropical North Pacific (ESTNP), Eastern Tropical North Pacific (ETNP) and Eastern South Pacific (ESP) in the Pacific Ocean; Arabian Sea (AS), Bay of Bengal (BB), Persian Gulf and Red Sea in the Indian Ocean; Black Sea, Baltic Sea and southwest African continental margin (SWACM) in the eastern Atlantic Ocean. Two seasonal OMZs at high latitudes are identified in the West Bering Sea (WBS) and in the Gulf of Alaska (GA) (Paulmier and Ruiz-Pino, 2009).

The Indian Ocean covers around 59% of the total global OMZ area, inclusive of the Arabian Sea and Bay of Bengal (Helly and Levin, 2004). The Northern Indian Ocean OMZs are especially intense due to the geographic characteristics of being semi-enclosed and bounded by land restricting the ventilation of the thermocline from the north (Naqvi et al., 2006). The Arabian Sea is one of the major permanent global OMZs and is anomalously the most intense in terms of lowest dissolved oxygen reported, i.e. $< 0.05\ mL\ L^{-1}$ (~2 μM) (van Bennekom and Hiehle, 1994) observed between 100 m and 1000 m (Paulmier and Ruiz-Pino, 2009; Bianchi et al., 2012). The Arabian Sea experiences extreme seasonal changes during the monsoon and inter-monsoon periods. These lead to hydrographic and nutrient alterations producing dramatic and unique physical, chemical, and biological changes in the water column (Morrison et al., 1998). In comparison, the BB-OMZ contains DO levels below $0.1\ mL\ L^{-1}$ (~4 μM) within a similar depth range. The Bay of Bengal has a large seasonal freshwater in-flux from rivers ($1.625 \times 1012\ m^3\ yr^{-1}$, Subramanian, 1993) as well as excess precipitation over evaporation ($\sim 2\ m\ yr^{-1}$, Prasad, 1997), which leads to reduced salinity and increased stratification. The loss of fixed nitrogen from the Bay of Bengal is around 12% of the N_2 production rate in the Arabian Sea, and 2.5% of the global water-column production in OMZ settings (66 ± 6 Tg N yr − 1) (Bristow et al., 2016). The AS-OMZ has a DO lower by 2 μM as compared to that in the Bay of Bengal, a minute difference, and yet resulting in starkly contrasting biogeochemical cycling (Morrison et al., 1998; Naqvi et al., 2006). The contrast in OMZ intensity between the two seas is proposed to be due to

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variations in levels of primary productivity, differing intensities of mesoscale eddies, and a contrasting transport of organic matter and oxygen (McCreary Jr. et al., 2013), but mechanisms maintaining these contrasting intensities needs further exploration (Al Azhar et al., 2017).

OMZs are known to be dominated by microbial communities since much of the macrofauna of the ocean is unable to survive extended hypoxia (Diaz and Rosenberg, 2008; Gilly et al., 2013). Persisting low DO conditions majorly influence the active microbial community in OMZ waters and their role in cycling of carbon, nitrogen, sulphur and other elements (Lam et al., 2009; Canfield et al., 2010; Naqvi et al., 2010; Ulloa et al., 2012; Wright et al., 2012; Bristow et al., 2016). Since OMZs are expanding, the degree of anoxia is intensifying in the core of the OMZs (Stramma et al., 2008; Gilly et al., 2013). These steep oxygen gradients create a chemically complex habitat conducive to many types of microbial metabolisms (Wright et al., 2012). While OMZs are well-known sites of nitrogen cycling, especially the process of denitrification (Jayakumar et al., 2009; Lam and Kuypers, 2011) in the O_2-deficient waters, recent research indicates that 'cryptic' sulphur cycling occurs within the OMZ water column of the Eastern Tropical South Pacific Ocean (ETSP) (Canfield et al., 2010). Sulphur metabolism is well studied, and genes related to sulphur oxidation and reduction are found dispersed throughout the bacterial and archaeal domains (Ghosh and Dam, 2009).

Pelagic sulphur cycling in the OMZs has only recently been revealed by evidence based on genetic and biogeochemical studies demonstrating the role of sulphur-oxidising bacteria (Stevens and Ulloa, 2008; Lavik et al., 2009; Walsh et al., 2009; Canfield et al., 2010). Metagenomic analyses of oxygen-deficient waters in the ocean, coasts and inland seas have shown a high representation of pelagic sulphur-oxidising γ-proteobacteria (Fuchs et al., 2005; Stevens and Ulloa, 2008; Lavik et al., 2009; Walsh et al., 2009; Wright et al., 2012; Glaubitz et al., 2013; Marshall and Morris, 2013) and further phylogenetic analyses of this functional group revealed two closely related lineages viz. ARCTIC96BD-19 (Bano and Hollibaugh, 2002) and SUP05 (Sunamura et al., 2004).The ARCTIC96BD-19 cluster comprises autotrophic sulphur-oxidising bacteria capable of aerobic respiration (Swan et al., 2011; Marshall and Morris, 2013). Genomic analysis of the marine group SUP05, revealed enzymes for chemolithotrophic oxidation of reduced sulphur, as well as those for nitrate reduction to nitrous oxide, suggesting a mechanistic link between pelagic sulphur cycling and partial denitrification (Walsh et al., 2009). These Gammaproteobacterial sulphur-oxidisers (GSOs) belonging to the SUP05 clade were found to be present as 30% of the prokaryotic population in the oxic-anoxic interface of the Black Sea as analysed by CARD-FISH technique (Glaubitz et al., 2013). Other groups of bacteria including the δ-proteobacteria SAR324 (Swan et al., 2011; Sheik et al., 2014), and the Marine Group A SAR406 clade (Wright et al., 2014) have been prominently present in OMZ waters revealed by metagenomic studies. SAR324 has the potential for chemo-lithoautotrophic metabolisms that utilize sulphide and thiosulphate, as well as oxidise nitrogen, as sources of energy (Walsh et al., 2009; Canfield et al., 2010; Swan et al., 2011; Sheik et al., 2014). The bacterial oxidation of sulphur compounds is also known to take place extensively in sediments under OMZs (Schmaljohann et al., 2001), in various soil ecosystems (Tourna et al., 2014) as well as in mangrove soils (Behera et al., 2014). In pelagic OMZ environment, microbial communities involved in the cycling of sulphur compounds are not well identified. Some thiotrophic bacteria were found in the OMZ waters across the ETSP as free-living populations, as well symbiont forms found in Arabian Sea OMZ water using metagenomic studies. Canfield et al. (2010) demonstrated the presence of substantial sulphur-based metabolic pathways existing in the Chilean OMZ by metagenomic analyses and radio-isotopic studies. A closer investigation of the sulphur cycle in OMZs and the bacteria carrying out the conversion reactions could lead to new and unexpected discoveries about the biogeochemical role of sulphur within these unique habitats (Stevens and Ulloa, 2008). Zinger et al. (2011) examined the bacterial beta diversity of seafloor and seawater ecosystems and demonstrated that pelagic communities and epi-benthic communities differed significantly at all taxonomic levels. Bacterial community study carried out by Walsh et al. (2016) supported this observation and extended it to sub-seafloor sediment, as the abundance-weighted bacterial community compositions of the sediment are apparently distinct from those in the overlying water column.

There remain relatively few cultivated representatives of bacteria from OMZ waters, owing to the challenges associated with culturing environmental microorganisms (Ward et al., 1990). Many genetically distinct phenotypes of bacteria are phenotypically indistinguishable for example some bacteria are resistant to culture on conventional media, certain bacteria have fastidious growth requirements including the need for specific physical conditions like pH, incubation temperatures or oxygen in the atmosphere (Köpke et al., 2005; Boivin-Jahns et al., 1996). The major limitation of cultivation of bacteria is mainly related to the selectivity of the nutrient media and culture conditions which lead to favouring only a fraction of the bacterial community, and this dramatically underestimates the microbial numbers and composition in the samples under study. On the other hand, the major advantage of this approach over modern molecular techniques lies in the fact that it provides the researcher with the microbe "in hand" that can be used in further analyses and for physiological and ecological experimentations (Al-Awadhi et al., 2013). The culture-dependent approach does provide the best opportunity to dissect and demonstrate the functional capacity of the microbiota. Bacteria involved in active metabolic processes associated with the sulphur cycle remain under-studied with respect to culturable bacteria, despite their contribution to biogeochemical cycles (Boetius et al., 2000; Hedges et al., 2001). Divya et al. (2011) carried out studies on phylogenetic groups in the sediments underlying the OMZ of the eastern Arabian Sea using cloning technique. This method also yielded the highest number of sequences identified as Proteobacteria. In this study, we report the 16S rRNA-based phylogenetic diversity of some culturable sulphur-oxidising bacteria from the Northern Indian Ocean OMZ waters. The bacteria were examined for their potential to oxidise thiosulphate, however ecological inferences cannot be made since bacteria are present in different communities in their natural environment and have different interactions in situ. The obtained culturable SOB diversities from the Arabian Sea and Bay of Bengal OMZ water column were compared to decipher their possible ecological significance with varying geographical environments.

2. Methods

2.1. Sampling in both OMZs

Water samples from the OMZ water columns in the Arabian Sea and Bay of Bengal were collected during cruises onboard RV Sindhu Sankalp (SSK079) in March 2015 and FORV Sindhu Sadhana (SSD020) in March 2016, respectively. Four stations were sampled in both OMZs using CTD rosette system (Seabird Electronics). The dissolved oxygen was measured using modified Carpenter method (1965) using an auto-titrator (Tables 1, 2). Water was transferred from Niskin samplers into sterile plastic containers and processed immediately.

2.2. Bacterial isolation

Five litres of water from each depth was collected; 200 mL was individually filtered through 0.22 µm nitrocellulose membrane and placed aseptically onto seven different modified agar media viz. *Thiobacillus* medium (TTM), Denitrifying agar with nitrite or nitrate (DMA-NO_2, DMA-NO_3) and inorganic media with varying sources of sulphur, i.e. Thioox agar, Thiopar agar, Thiobac agar, Thiomicro agar (Table 3). During filtration and plating, one plate of each medium was kept open in the laminar airflow to check for possible contamination. One blank filter paper was placed on each media as a negative control.

Table 1
Arabian Sea OMZ sampling stations and DO values*.

Sampling station	Latitude and longitude	Sampling depth (m)	Dissolved oxygen (μM)
ASTS		250	BD
	17°N, 68°E	500	BD
		800	BD
		1000	3.0801
II14		250	3.7944
	21°N, 68°E	500	BD
		800	BD
		1000	9.2851
II6		150	BD
	13°N, 68°E	250	BD
		500	1.0267
		750	5.5354
II2		179	1.6963
		238	21.6504
	9°N, 68°E	384	12.276
		750	18.6595
		1000	27.6322

BD – Below detection limits (0.5 μM).
* Measured using modified Carpenter method (1965) using auto-titrator.

Table 2
Bay of Bengal OMZ sampling stations and DO values*.

Sampling station	Latitude and longitude	Sampling depth (m)	Dissolved oxygen (μM)
#6 (BoBTS)		156	1.2053
	18°N, 89°E	250	0.6249
		390	2.2320
		530	4.8211
#17		167	7.4995
	17°N, 87°E	300	1.1160
		391	1.5624
		546	3.3033
#18		147	3.6158
	17°N, 89°E	268	0.3124
		450	2.0981
		586	5.8478
#19		136	7.3209
		250	0.4910
	17°N, 90°E	373	0.7588
		466	5.2228
		1000	68.9688

* Measured using modified Carpenter method (1965) using auto-titrator.

All plates were incubated at 28 °C for 3–15 days. Bacterial colonies obtained were purified and maintained on their respective media at 4 °C. The isolates from control plates were also purified and maintained.

2.3. DNA extraction, PCR amplification, and sequencing of 16S rRNA

Bacterial isolates purified from the modified agar media were grown in Zobell marine broth (ZMB) for 24–48 h at 28 °C. The culture broth was centrifuged at 12000 rpm for 3 min to obtain a cell pellet, and genomic DNA was extracted from this using ZR Bacterial DNA MicroPrep™ Kit (Zymo Research, USA) following the manufacturer's instructions. Gene amplification of 16S rRNA was carried out using 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTG TTACGACTT-3′) primers (Lane, 1991). Each 50 μL PCR reaction consisted of 10× Taq DNA Polymerase Buffer (Genaxy) 5 μL, 10 mM dNTP (Genaxy) 1 μL, 20 μM forward and reverse primer (Eurofins) 1 μL each, DNase-free water 38.4 μL, 3 U μL^{-1} Taq polymerase (Genaxy) 0.6 μL and template DNA 3 μL. PCR amplification included denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C, 1 min, annealing at 54 °C, 45 s, elongation at 72 °C, 1 min and final elongation at 72 °C for 10 min. The PCR-amplified 16S rRNA gene was purified using PCR Clean-Up System (Promega Corporation, USA) and sequenced on 3130xl Genetic Analyser (Applied Biosystems, USA).

The BLASTn analysis was carried out against NCBI GenBank database, and EzTaxon e-database was used to identify similarity values between obtained isolates and closely related taxa. Isolates from the control plates were identified, and all other similarly identified isolates were considered contaminants and discarded from the isolate list. The 16S rDNA sequences of the isolates were deposited in NCBI and have accession numbers KY604765, KY604766, KY604769-KY604773, KY604775-KY604777, KY604779-KY604781, KY604784, KY604786-KY604789, KY604791, KY604794-KY604804, KY604806-KY604809, KY604811-KY604813, KY604816-KY604821, KY604825-KY604827, KY604829-KY604835, KY604838 (AS-OMZ) and KY616223-KY616257, KY616290-KY616355, KY616393-KY616398, KY616435-KY616501, KY616562-KY616622 (BB-OMZ).

2.4. Phylogenetic distribution of 16S rDNA

The 16S rDNA sequences were aligned, along with sequences of type strains of closest taxa, using Clustal W (Thompson et al., 1994) and phylogenetic trees were constructed using three algorithms which included neighbour-joining (Saitou and Nei, 1987), maximum likelihood and maximum parsimony method with MEGA version 5.0 (Tamura et al., 2011). Robustness of the trees was assessed with 1000 bootstrap replicates. The three trees were compared and maximum likelihood tree retained for discussion.

2.5. Qualitative analysis of thiosulphate oxidation

Representative genera from both OMZs were inoculated into mineral salts (MS) broth containing 8 mM sodium thiosulphate (Teske et al., 2000; Ghosh and Roy, 2007) and the culture broth tubes were incubated at 28 °C for 1–3 days. Phenol red indicator was added in a concentration of 0.02 g L^{-1} to detect acid formation from the oxidation of thiosulphate in the MS broth. False positive due to oxidation of other components in the media does not occur since the mineral salts are in minute quantities in comparison to the thiosulphate provided. An uninoculated control tube was maintained at same conditions, to account for any chemical oxidation of the components. The test is considered to be positive only when pH drops below 6.5 to cause a change in colour of phenol red from orange (pH 7) to yellow.

Table 3
Modified agar media used for isolation.

S. no.	Medium	S and N components	Reference
1	TTM	Sodium thiosulphate, ammonium sulfate	DSM 36, DSMZ 2015
2	DMA-NO_2	Sodium thiosulphate, Sodium nitrite	DSM 856, DSMZ 2007
3	DMA-NO_3	Sodium thiosulphate, Sodium nitrate	DSM 856, DSMZ 2007
4	Thioox	Sulphur sublimate, Potassium nitrate	Modified Waksman and Starkey medium, Collins, 1963
5	Thiopar	Sodium thiosulphate, Potassium nitrate	Modified Starkey medium, Collins, 1963
6	Thiobac	Sodium thiosulphate, Potassium nitrate	Modified Lieske's medium, Collins, 1963
7	Thiomicro	Sodium sulphide, Potassium nitrate	ATCC 1255 Thiomicrospira medium

3. Results

3.1. Isolation of Sulphur Oxidising bacteria (SOBs)

The agar media were modified especially for their sulphur and nitrogen sources to enable the isolation of potential sulphur oxidizers. Different colonies were observed on the modified agar media, and morphologically different bacterial isolates were picked and purified. Fifty-seven bacterial isolates were obtained from AS-OMZ while 233 bacterial isolates from BB-OMZ using the seven different media. These isolates were subcultured and maintained on the agar medium similar to that of their isolation. The growth of individual colony forming units was very sparse, and most of them were colourless or white, translucent and pinpoint colonies. Some were waxy, while others had a water drop texture.

3.2. Identification of the potential SOBs

The two hundred and ninety bacterial isolates were identified by carrying out BLAST analysis against NCBI database of 16S rDNA sequences. The closest match with type strain was considered as identification up to genus level. Two hundred and twenty-seven bacterial isolates were identified from the water columns of the Arabian Sea and Bay of Bengal OMZs.

3.3. Phylogenetic analysis of 16SrRNA gene sequences

Two hundred and ninety bacterial strains were identified in total. We were able to isolate diverse bacteria from the BB-OMZ water column that grouped into six classes, in comparison to bacterial isolates obtained from AS-OMZ that grouped into three classes. The potential SOBs in the AS-OMZ water column included 81.5% representatives from Gammaproteobacteria, 14.8% representatives from Alphaproteobacteria and 3.7% representatives from Bacilli (Fig. 1). In the BB-OMZ water, Gammaproteobacteria were represented by 64.4% of the isolates, Alphaproteobacteria by 28.3%, while Bacilli by 5.1% and the remaining 2.2% categorized into class Betaproteobacteria, Actinobacteria and Flavobacteria (Fig. 2). Phylogenetic analysis showed that all the isolates from both the OMZ water columns grouped into respective classes, none of them being novel or unclassified. The highest diversity of potential SOBs was observed in the core of the OMZ water columns in both Arabian Sea and Bay of Bengal.

3.4. Qualitative thiosulphate oxidation

Nine isolates from AS-OMZ and twenty-two isolates from BB-OMZ were tested for oxidation of thiosulphate to sulphate, using phenol red as an indicator. Two of the 31 isolates tested showed a change in phenol red indicator to yellow indicating pH drop due to sulphate production, within 48 h. A species belonging to α-proteobacteria, *Citreicella thiooxidans,* isolated from AS-OMZ demonstrated the oxidation of thiosulphate to sulphate, while the BB-OMZ yielded a β-proteobacterial species, *Achromobacter xylosoxydans* that showed the conversion of thiosulphate to sulphate (Tables 4, 5).

4. Discussion

The primary focus of this study was the isolation of culturable fraction of sulphur oxidising bacteria from the OMZ water column of the north Indian Ocean using different media. In this study, we showed that the culturable bacteria obtained by membrane filtration technique from the AS-OMZ and BB-OMZ regions belonged majorly to Gammaproteobacteria and Alphaproteobacteria indicating that these classes are possibly among the more abundant and widely distributed in both the Indian Ocean OMZs, although cultivation biases exist due to limitation of the media utilized for isolation. The dominance of these two proteobacterial classes was also reported from the Arabian Sea OMZ by metagenomic analysis of the water column carried out by Jain et al. (2014), with Alphaproteobacteria making up 45% of bacterial community while Gammaproteobacteria made up 15%. Comparably, metagenomic and phylogenetic analyses of the bacterial communities of the Eastern Tropical South Pacific (ETSP) OMZ water column, also a permanent OMZ like the Arabian Sea, revealed 53.7% Gammaproteobacteria population, 19.7% Alphaproteobacteria and 11.2%

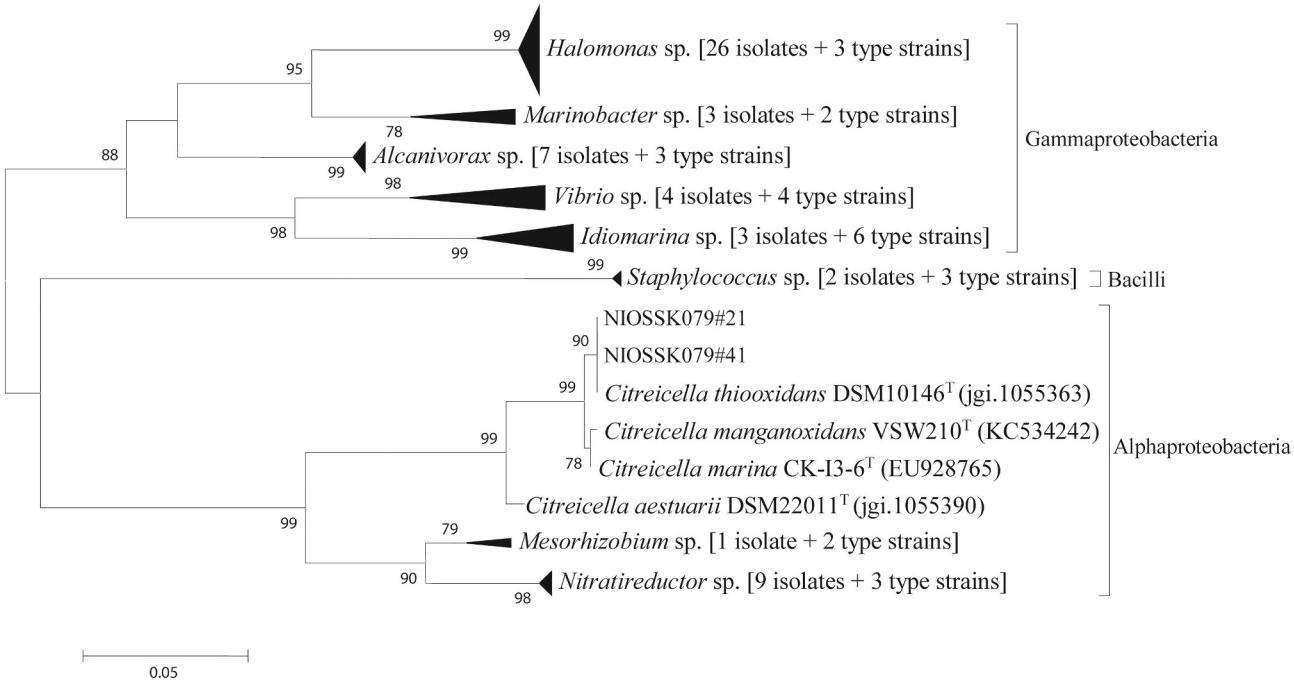

Fig. 1. Phylogenetic tree demonstrating evolutionary relationships between cultured bacteria from the Arabian Sea OMZ water column as determined by Maximum Likelihood method. Numbers at nodes represent bootstrap values > 50% (based on 1000 bootstrap re-samplings). The scale bar represents 0.05 fixed mutations per nucleotide position.

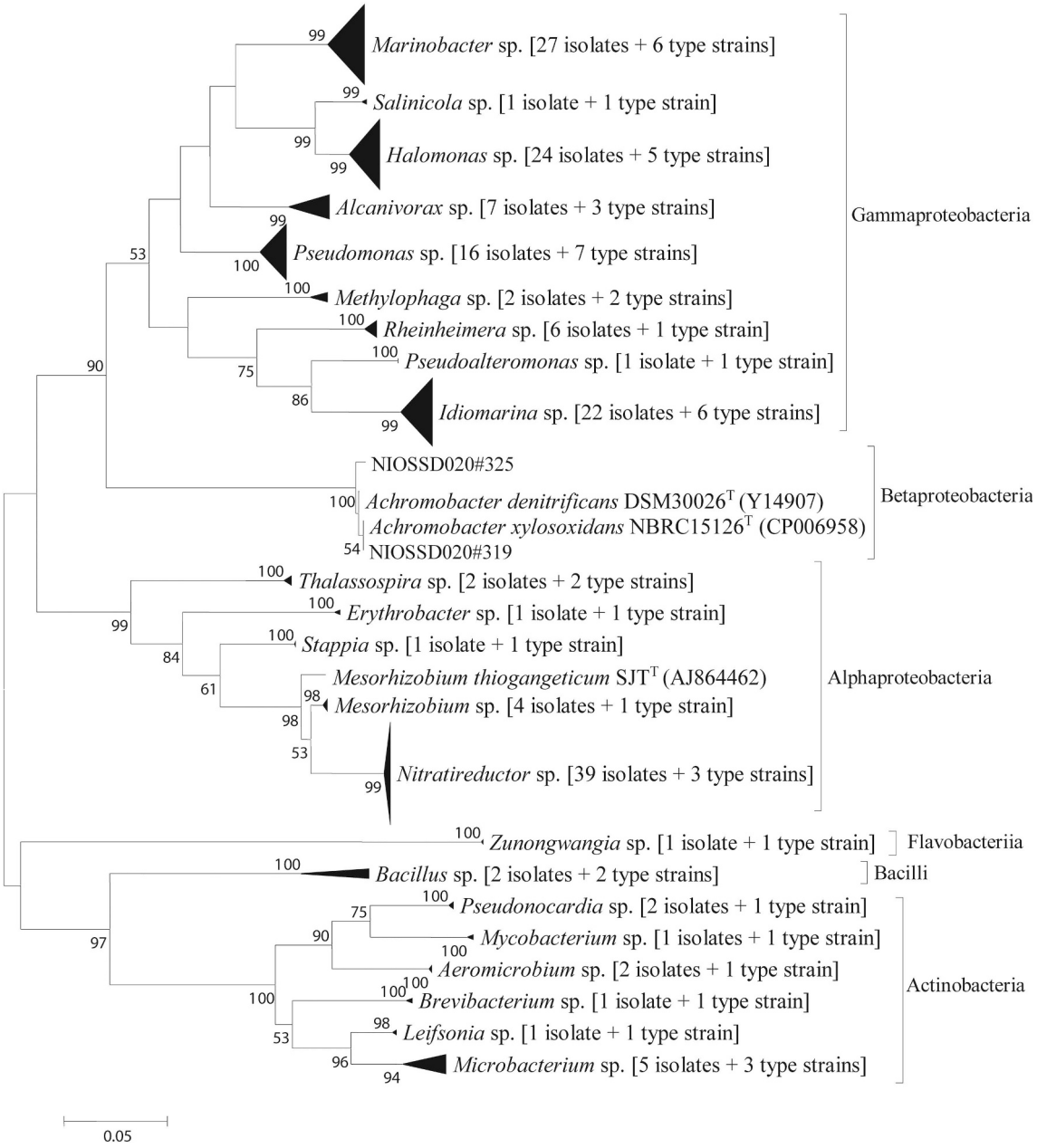

Fig. 2. Phylogenetic tree demonstrating evolutionary relationships between cultured bacteria from Bay of Bengal OMZ water column as determined by Maximum Likelihood method. Numbers at nodes represent bootstrap values > 50% (based on 1000 bootstrap re-samplings). The scale bar represents 0.05 fixed mutations per nucleotide position.

Table 4
Thiosulphate oxidation by isolates from AS-OMZ.

S. no.	Representative SOB isolate from Arabian Sea OMZ	Thiosulphate oxidation
1	*Alcanivorax dieselolei*	−
2	*Citreicella thiooxidans*	+
3	*Halomonas aquamarina*	−
4	*Idiomarina piscisalsi*	−
5	*Marinobacter hydrocarbonoclasticus*	−
6	*Mesorhizobium sediminum*	−
7	*Nitratireductor aquibiodomus*	−
8	*Staphylococcus haemolyticus*	−
9	*Vibrio harveyi*	−

Key: + pH indicator changed colour to yellow; − pH indicator did not change colour to yellow.

Bacteroidetes (Stevens and Ulloa, 2008).The culturable bacteria from BB-OMZ water column also consisted of the classes Actinobacteria, Flavobacteria, and Betaproteobacteria. Bacterial cultures from these classes if present in the AS-OMZ water column were unable to be cultured by the methods used in this study.

The availability of different forms of sulphur alters interactions among microbial groups and processes and influences carbon and nitrogen cycling in OMZs (Johnston et al., 2014). In contrast to the bacterial diversity values, particular analysis of sulphur oxidising bacteria from other ecosystems like sulphidic springs (Engel et al., 2004) revealed the presence of Gamma, Beta, and Delta proteobacterial classes, with the dominance of Epsilonproteobacterial class consisting of genera that are known sulphur oxidisers. Sulphur-oxidising bacteria play an important role in the detoxification of sulphide in water and sediments in mangrove ecosystems, for example, the symbiotic SOBs associated with the bivalve family are commonly found in muddy

Table 5
Thiosulphate oxidation by isolates from BB-OMZ.

S. no.	Representative SOB isolate from Bay of Bengal OMZ	Thiosulphate oxidation
1	*Achromobacter xylosoxidans*	+
2	*Aeromicrobium erythreum*	−
3	*Alcanivorax dieselolei*	−
4	*Bacillus okhensis*	−
5	*Brevibacterium casei*	−
6	*Erythrobacter citreus*	−
7	*Halomonas aquamarina*	−
8	*Idiomarina baltica*	−
9	*Leifsonia shinshuensis*	−
10	*Marinobacter alkaliphilus*	−
11	*Mesorhizobium sediminum*	−
12	*Methylophaga muralis*	−
13	*Microbacterium aquimaris*	−
14	*Mycobacterium cosmeticum*	−
15	*Nitratireductor aquibiodomus*	−
16	*Pseudoalteromonas shioyasakiensis*	−
17	*Pseudomonas flavescens*	−
18	*Pseudonocardia carboxydivorans*	−
19	*Rheinheimera aquimaris*	−
20	*Stappia indica*	−
21	*Thalassospira povalilytica*	−
22	*Zunongwangia profunda*	−

Key: + pH indicator changed colour to yellow; − pH indicator did not change colour to yellow.

mangrove areas (Liang et al., 2006).

Sulphur oxidation in the Arabian Sea and Bay of Bengal OMZs is not well known. In 2005, Fuchs et al. proposed that at certain times and locations, specific bacterial populations might catalyze sulphur reduction or oxidation in the water column of the Arabian Sea. In our study, culturable bacteria from both Indian Ocean OMZ water columns revealed the presence of genera belonging to Gammaproteobacteria which are significant in sulphur cycling. Comparably, the recent identification of uncultured Gammaproteobacteria, closely affiliated with sulphur-oxidising symbionts, in OMZ waters off the Chilean coast (Stevens and Ulloa, 2008) suggests that sulphur cycling may play a significant role in nitrate-rich OMZs. In 1977, Tuttle and Jannasch identified bacteria capable of oxidising thiosulphate in diverse inshore and offshore waters and demonstrated the potential for thiosulphate oxidation to enhance carbon fixation and glucose utilization. The two isolates, *Citreicella thiooxidans*, belonging to Alphaproteobacteria obtained from AS-OMZ and *Achromobacter xylosoxydans* belonging to Betaproteobacteria obtained from BB-OMZ, demonstrated thiosulphate oxidation to sulphate indicating their ability and the potential for similar conversions in situ, and probable influence on carbon cycling in the OMZ waters. Canfield et al. (2010) similarly demonstrated the inherent capacity for active in situ coupling between the sulphur and nitrogen cycles in OMZ zones of the marine water column off the Chilean coast.

To gain greater insight into sulphur oxidation in OMZs, Carolan et al. (2015), used qPCR to detect sulphur oxidation genes in Alpha- and Gammaproteobacterial sulphur oxidizers, such as SUP05. Putative sulphur oxidizers from the SAR324 and ARCTIC96BD-19 clades were among the most abundant microbial groups found in the Eastern Tropical North Pacific (ETNP) OMZ, comprising up to 25 and 16.5% of sequence libraries, respectively. SUP05 Gammaproteobacteria were found to be widely distributed, but not abundant in the ETNP OMZ, being implicated in detoxifying sulphide plumes. Marshall and Morris (2013) attempted to obtain a culturable form from the SUP05 clade and demonstrated the ability of the isolate to oxidise sulphur. Not many culturable representatives have been obtained from these clades to prove their inherent sulphur oxidation ability in vitro. The activity of SUP05 bacteria in the ETNP is consistent with anaerobic sulphur oxidation in the OMZ, and expression of *dsrA*, *rdsrA,* and *soxB* genes indicates that several sulphur-cycling pathways and processes may be active in this OMZ. Such information is yet to be generated with respect to the Indian Ocean OMZ. Using DGGE, the phylogenetic diversity of bacteria in the Arabian Sea OMZ water column has been determined by Jain et al. (2014). Isolation of sulphur oxidising bacteria from both the AS- and BB-OMZs in the present study demonstrates that the metabolic potential for sulphur oxidation exists in both systems.

The culturable fraction of bacteria that was obtained from both the OMZs using different media revealed a comparable diversity of sulphur oxidizers. The genus *Citreicella* was previously isolated from the oxygen-sulfide interface from the Black Sea, a seasonal OMZ (Sorokin et al., 2005). This bacterium was isolated for the first time from the Arabian Sea OMZ by the culturing method used in the present study. No representative species capable of sulphur oxidation from the genus *Achromobacter* have been isolated previously from any OMZ water. From the demonstrated potential of these two bacteria, we can hypothesize that these contribute to the functioning of the sulphur cycle in the north Indian Ocean OMZs. Further quantification of sulphur oxidation rates of these bacteria would shed light on the exact biochemical functioning and the processes they carry out in the sulphur cycle within the OMZs of the Indian Ocean.

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ORIGINAL ARTICLE

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Spatial variability of microzooplankton in the central Arabian Sea during spring intermonsoon

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Abstract

Spatial variability of microzooplankton (MZP; organisms in size 20–200 μm) along with total heterotrophic bacteria (THB) and picophytoplankton in the upper 120-m water column of the central Arabian Sea (AS) was studied during the spring intermonsoon period (March 2015). Six stations divided into three regions (northern, southern, and central) were sampled along 68°E transect, between 9°N and 21°N. A total of 25 species of MZP were recorded corresponding to loricate ciliates (11 species), aloricate ciliates (two species), and heterotrophic dinoflagellates (12 species). Along the transect, MZP total abundance that varied between 5 and 9 × 10^3 org./m^3 was largely contributed by ciliates, especially loricate ciliates (30%–62%) followed by heterotrophic dinoflagellates (23%–26%). MZP counts were lower in the central region (*SD* ± 0.4, 7–8 × 10^3 org./m^3) than in the northern and the southern regions (*SD* ± 0.7, 8–9 × 10^3 org./m^3) of the Arabian Sea. Heterotrophic bacterial counts were higher in the northern (28–202 × 10^3 cells/ml) and southern (16–92 × 10^3 cells/ml) and lowest in the central (13–89 × 10^3 cells/ml) region. In contrast, picophytoplankton showed higher counts in central (3–54 × 10^3 cells/ml) and southern (4–113 × 10^3 cells/ml) regions and lowest in the north (1–45 × 10^3 cells/ml). Strong positive coupling of MZP–THB ($0.05 > p > 0.01$) than that of MZP–picophytoplankton density and MZP–chlorophyll *a* concentration suggests the spatial scale variability and functioning of microbial loop in the central Arabian Sea during the spring intermonsoon period.

KEYWORDS
Central Arabian Sea, microbial loop, microzooplankton, picophytoplankton, spring intermonsoon

1 | INTRODUCTION

Microzooplankton (MZP) refers to a group of organisms defined by their size (20–200 μm), function, and limited swimming ability (Dussart, 1965). These are classified into two groups, mixotrophic and heterotrophic (Protozoans and Metazoans). Protozoans include dinoflagellates, ciliates, radiolarians, and foraminifera, whereas metazoans contain rotifers, meroplankton, and holoplankton larvae (Calbert & Alcaraz, 2007). They are overall capable of exploiting pico-/nanoplankton, which in turn are utilized by other large trophic components such as mesozooplankton (Stoecker & Capuzzo, 1990). Their intermediary role between bacterioplankton and large mesozooplankton through grazing on bacterioplankton thus controls the bacterial community (Azam et al., 1983; Bernard & Rassoulzadegan, 1993; Gifford & Dagg, 1991). This is particularly important in stratified oligotrophic water, which is seasonally experienced in the study region in the central Arabian Sea. Most tropical ocean regions experience strong stratification seasonally and remains well mixed for

the most part of the year (Cushing, 1989), thus supporting two possible food chains in these waters (Gauns, Mohanraju, & Madhupratap, 1996). A well-mixed water column supports mesozooplankton through a traditional food chain, whereas in a strongly stratified oligotrophic water column, zooplankton switch to a "microbial loop" wherein MXP play a significant role (Gauns et al., 1996; Madhupratap et al., 1996). The central Arabian Sea is known to experience a variably stratified condition particularly during the intermonsoon (spring and fall) period, stronger in the northern than southern part (Prasanna Kumar et al., 2000). The landlocked nature of AS in the north and opening of sea to the equatorial region in the south bring large-scale seasonal variability in the dynamics of the mixed layer depth (MLD). During intermonsoon, this is a resultant of prevailing Rossby wave-driven circulation in the southern part that leads to a deep MLD and, to the north, increasing surface temperature and weak winds cause a shallow MLD (Prasanna Kumar & Narvekar, 2005). Thus, in order to understand the response of a microbial component (microzooplankton along with heterotrophic bacteria and picophytoplankton) to these physical changes, work was undertaken to study the spatial pattern of MZP community composition and their numerical abundance in the upper 120 m of the water column of the central Arabian Sea during the oligotrophic spring intermonsoon period of 2015.

2 | MATERIALS AND METHODS

Samples for phytoplankton biomass (chlorophyll *a*), microzooplankton (MZP), total heterotrophic bacteria (THB), and picophytoplankton were collected during the spring intermonsoon (March 2015) onboard R.V. Sindhu Sankalp (Cruise no. SSK79) covering the transect between 9° and 21°N along 68°E. The upper 120 m of the water column of this transect in the central Arabian Sea was sampled at six stations: S1–9°N, S2–11°N, S3–13°N, S4–15°N, S5–17°N, and S6–21°*N* (Figure 1). At every station, profiles of water temperature, salinity, dissolved oxygen (DO), and fluorescence were obtained with a Sea-Bird Electronics (SBE) 9plus CTD unit equipped with pre-calibrated sensors for DO and fluorescence.

Chlorophyll *a* was processed following JGOFS protocol (JGOFS, 1994); wherein, 1 L of water was filtered through 47-mm GF/F filters (0.7 µm pore size). The samples were incubated in dark for 24 hr at −20°C for extraction purpose using 10 ml of 90% acetone and analyzed by fluorometry (JGOFS, 1994).

Sampling for MZP involved a slow vertical haul through a hydrographic winch to avoid cell damage using 20-µm mesh net (mouth diameter of 30 cm and length of 120 cm) within the upper 120 m covering the euphotic portion of the water column. The volume of water filtered through the net was calculated using a Hydrobios 438,110 calibrated digital flow meter (Lasternas et al., 2011). Roughly 3.7–4 m^3 of water was filtered through this process. Utmost care was taken while passing samples through 200 µm mesh to avoid/minimize damage to delicate forms of microzooplankton. Nonetheless, there is a fairly good chance that some cells may have been damaged during this process, leading to underestimation of

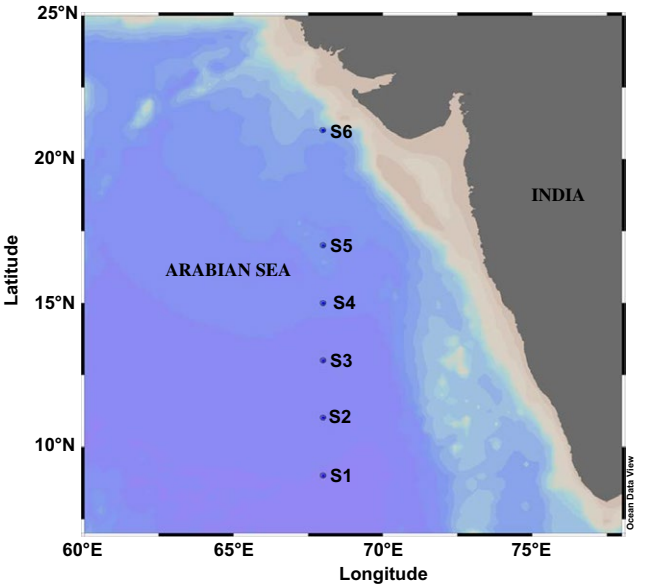

FIGURE 1 Map showing station locations in the central Arabian Sea

total density. The processed sample was then immediately concentrated to ~500 ml and preserved with 1% acid Lugol's iodine solution (Gauns et al., 1996). For MZP taxonomy, a known volume (1 ml from 500 ml) of concentrated sample was taken in a Sedgwick-Rafter counting chamber, and then, the entire chamber was examined under an inverted microscope (magnification of 400×, Nikon Eclipse t-u). The MZP species were identified using the standard identification keys (Hada, 1938; Jyothibabu, 2004).

Heterotrophic bacteria and picophytoplankton abundance were estimated using a flow cytometer (BD FACS Calibur) equipped with a blue and a red laser, for which 1.5 ml of water samples was preserved with glutaraldehyde (0.2% final concentration) in cryovials, flash frozen in liquid nitrogen, and stored at −80°C for laboratory analysis. The frozen samples were thawed to room temperature and then analyzed on the flow cytometer. Autotrophic picophytoplankton were detected based on autofluorescing and scatter properties of individual cells, while heterotrophic bacteria were stained with SYBR Green dye (1 part in 10,000) which binds to the DNA and absolute counts were obtained using Cell Quest Pro software (Marie, Partensky, Jacquet, & Vaulot, 1997).

3 | RESULTS

The hydrography of stations located in the central Arabian Sea along 68˚E transect between 9°N and 21°N implied variability in physicochemistry from north to south (Figure 2). The mixed layer depth (MLD) was relatively deep in the north (21°N; MLD ~ 30 m) and shallow in the south (9°N; MLD ~ 9 m) region. The water temperature and salinity in upper 120-m column ranged from 21.6°C to 27.3°C, 36–36.6 psu in the north; 16.3–28.4°C, 35.5–36.4 psu in central; and 20.9–29.9°C, 36–38.6 psu in southern regions. A gradual increase in

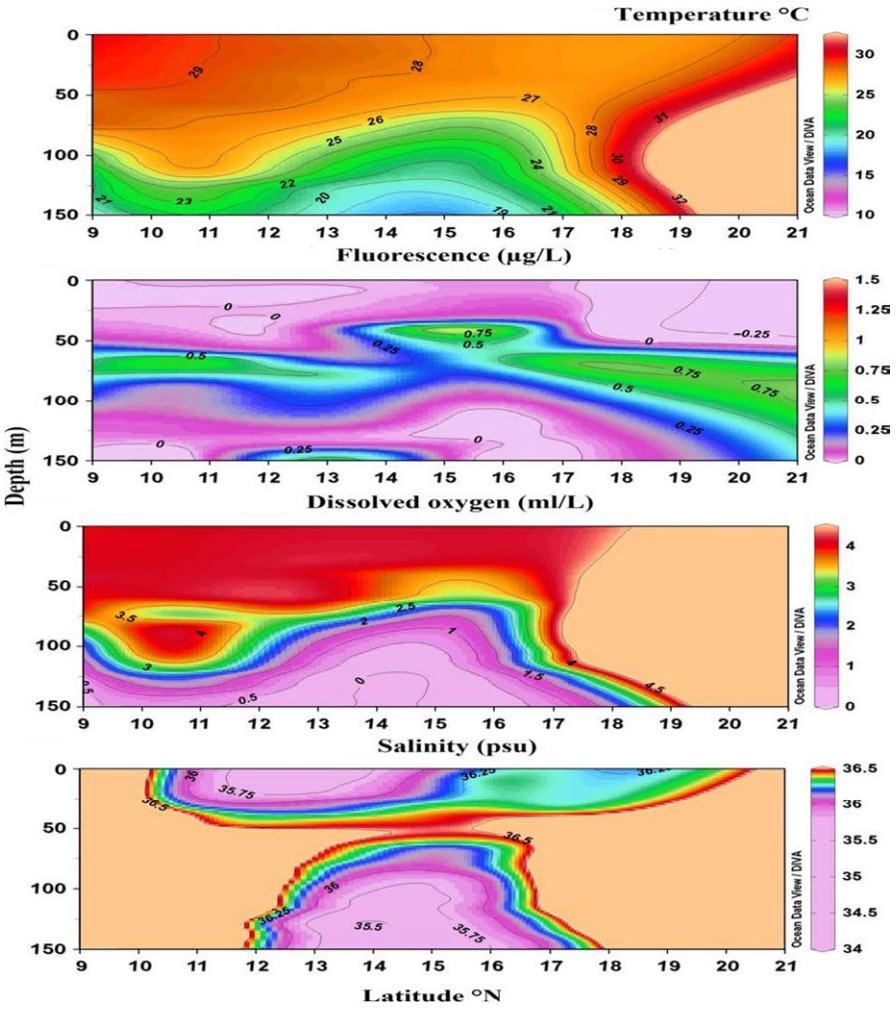

FIGURE 2 Vertical profiles of temperature (°C), salinity (psu), Do (ml/L), and fluorescence (µg/L) along the longitude 68°E transect (9° and 21°N)

temperature was observed from north to south. However, the DO values decreased from north to south and ranged from 0.7 ml/L to 4.3 ml/L in the north, 0.05–4.2 ml/L in central, and 0.96–4.1 ml/L in southern regions.

In addition, phytoplankton biomass (Chlorophyll *a*) in the upper 120 m varied between 0.02 and 0.75 µg/L in the northern region, between 0.01 and 0.65 µg/L in the central region, and between 0.01 and 0.71 µg/L in the southern region. Overall, the trend observed in the chlorophyll distribution showed high concentration in north and least in the central region. Depth-integrated (0–120 m) chlorophyll *a* ranged between 14 and 18 mg/m^2 (Figure 3). The sampling stations were hence grouped based on the above physicochemical conditions into three regions to emphasize biological variations (in MZP, THB, and picophytoplankton): southern region (included stations S1 and S2; 9° to 11°N), central region (stations S3 and S4; 13°–15°N), and the northern region (stations S5 and S6; 17°–21°N).

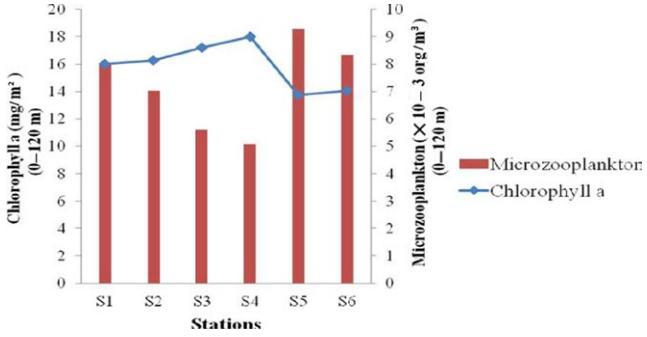

FIGURE 3 Spatial variation in microzooplankton density and depth-integrated chlorophyll *a*

3.1 | Microzooplankton abundance

The abundance of MZP varied in the range of 5–9 × 10^3 org./m^3 (Figure 3). The northern region displayed high abundance (8–9 × 10^3 org./m^3; Avg. 9 × 10^3 org./m^3) followed by the southern region (7–8 × 10^3 org./m^3; Avg. 8 × 10^3 org./m^3). The lowest abundance of MZP was observed in the central region (5–6 × 10^3 org./m^3; Avg. 5 × 10^3 org./m^3).

3.2 | Microzooplankton composition

The MZP community was represented by three different groups corresponding to ciliates (loricate and aloricate), heterotrophic

dinoflagellates, and copepod nauplii. A total of 25 species of MZP were identified from the region, of which 11 species belonged to loricate ciliates, two species to aloricate ciliates, and 12 species to heterotrophic dinoflagellates (Table 1). The dominant species were identified as loricate ciliates (*Streenostrupiella* sp., *Dadayiella gaymedes*, *Salpingella* sp., and *Eutintinnus lusus undae*) and heterotrophic dinoflagellates (*Protoperidinium breve*, *Podolamphus* sp., and *Protoperidinium latistriatum*).

In all the regions, MZP composition was majorly contributed by loricate ciliates (44%–62%; Avg. 40%–53%) followed by copepod nauplii (11%–33%; Avg. 22%–30%) and heterotrophic dinoflagellates (23%–27%; Avg. 24%–28%), whereas the aloricates were found only

TABLE 1 List of microzooplankton species from the central Arabian Sea (0–120 m) during spring intermonsoon

Microzooplankton (Org./m^3)	S1	S2	S3	S4	S5	S6
Loricate ciliates						
Amphorella acuta	68	541	68	68	757	108
Ascambelliella sp.	0	0	0	0	0	162
Codonellopsis sp.	68	135	0	0	0	162
Codonellopsis morchella	0	0	0	0	0	54
Codonellopsis ostenfeldi	270	0	0	0	0	0
Dadayiella gaymedes	338	541	68	135	973	865
Dictyocysta sp.	0	135	68	0	216	162
Epiplocyclis undella sp.	203	270	0	0	0	54
Epiplocyclis sp.	135	0	0	0	0	0
Eutintinnus lusus undae	135	811	68	811	216	54
Metacylis sp.	68	135	0	68	0	432
Proplectella perpusilla	338	135	0	0	0	108
Protorhabdonella simplex	405	135	203	203	324	486
Rhabdonella elagans	135	811	68	68	108	0
Rhabdonella poculum	0	0	0	0	0	216
Rhabdonella spiralis	68	135	0	0	432	270
Salpingella sp.	676	270	338	473	216	108
Stenosemella sp.	0	0	0	0	0	162
Streenostrupiella sp.	405	270	811	473	432	0
Xystonellopsis sp.	203	0	0	270	649	324
Aloricate ciliates						
Lohmaniella spiralis	0	0	0	135	0	0
Strombidium conicum	0	0	135	0	0	0
Heterotrophic dinoflagellates						
Dinophysis apicata	405	270	68	0	216	0
Dinophysis caudate	135	0	0	0	0	0
Gymnodinium sp.	0	0	68	0	0	0
Ornithocerus magnificus	135	0	68	135	324	0
Ornithocerus thumii	0	0	0	135	0	0
Podolamphus sp.	473	541	541	338	216	162
Protoperidinium breve	405	270	135	270	757	865
Protoperidinium depressum	0	0	0	0	0	270
Protoperidinium divergens	135	0	0	0	216	0
Protoperidinium granii	68	0	68	0	0	216
Protoperidinium latistriatum	135	270	676	270	0	108
Protoperidinium longicollum	0	135	0	0	0	0
Protoperidinium ovatum	0	270	0	0	0	216
Protoperidinium stenii	0	135	203	0	649	54
Copepod nauplii	2,635	811	1959	1,216	2,595	2,703

in the central region with minor (3%) contribution to the MZP composition (Figure 4).

3.3 | Diversity indices

In the upper 120 m of the water column, the number of MZP species (S) varied between 17 and 22. The number of species recorded was 22 in the southern region, 20 in the northern region, and 17 in the central region. Species diversity (H′) in the southern region (2.7) was higher than in the northern (2.5) and central (2.3) regions. Evenness (*J*) in the southern region (0.9) was higher than in the north and central (0.8) regions, whereas no variation in species richness (*d*) was observed among regions (0.1).

3.4 | Heterotrophic bacteria and picophytoplankton

Total heterotrophic bacterial (THB) showed higher abundance in the northern (28–202 × 10^3 cells/ml) region and lower in the southern (16–92 × 10^3 cells/ml) and the central (13–89 × 10^3 cells/ml) regions. The column-integrated (0–120 m) cell abundance of THB (×1,012 cells/m^2) followed a similar trend wherein abundance varied in the range of 7–16 in the northern, 5–8 in the southern, and 2-6 in the central regions.

Picophytoplankton cell abundance in the water column varied from 1 × 10^3 cells/ml to 113 × 10^3 cells/ml. Cell counts were lower in the northern region (1–45 × 10^3 cells/ml) and higher in the southern (4–113 × 10^3 cells/ml) and central (3–54 × 10^3 cells/ml) regions. The column-integrated (0–120 m) cell abundance of picophytoplankton (×10^{12} cells/m^2) varied in the range of 2–3 in the north, 2–5 in southern, and 1–3 in central regions.

4 | DISCUSSION

In the present study, the MZP community in the upper 120 m of the central Arabian Sea was mostly dominated by loricate ciliates (30%–62%) followed by heterotrophic dinoflagellates (23%–33%). Loricate ciliates are known to dominate the MZP community in coastal and

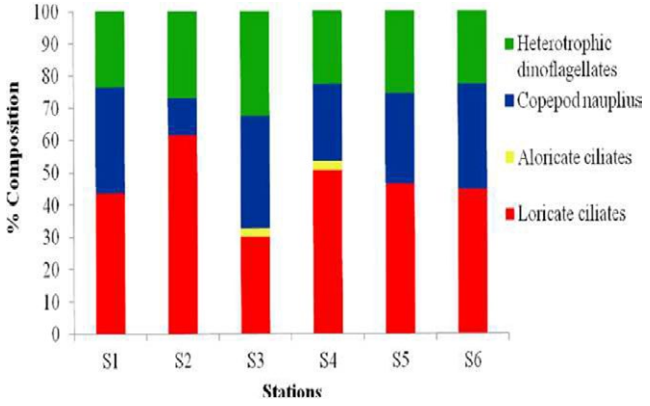

FIGURE 4 Percentage composition of microzooplankton in the central Arabian Sea

open ocean waters due to their euryhaline and eurythermal nature (Edwards & Burkill, 1995; Godhantaraman, 2001; Leakey, Burkill, & Sleigh, 1996; Saab 1989; Sanders, 1987). Likewise, the dominance of heterotrophic dinoflagellates is possibly due to their adaptive nature to different modes of feeding (mixotrophic) which compete with other predators for prey (Jyothibabu et al., 2003). The density profile of MZP revealed that southern and northern regions of the central Arabian Sea were denser (8–9 × 10^3 org./m^3) than those of the central region (5–6 × 10^3 org./m^3). During the intermonsoon, the MZP biomass in the Arabian Sea ranged between 0.4 and 7.6 mg C/m^3 (Stelfox, Burkill, Edwards, Harris, & Sleigh, 1999) and the higher biomasses found in the northern (0.43 g C/m^2) and southern (0.25 g C/m^2) regions (Dennett et al., 1999). The Arabian Sea is a tropical ocean characterized by a restricted circulation and seasonal changes by monsoon effect (Leakey et al., 1996). During the intermonsoon, the Southern Arabian Sea harbors a deep mixed layer due to Rossby wave propagation (Prasanna Kumar & Narvekar, 2005), whereas the Northern Arabian Sea experiences increasing sea surface temperature and weak winds which lead to shallow mixed layer depth and less primary production controlled by availability of nutrients in the surface water (Prasanna Kumar & Narvekar, 2005). However, in the present study, this trend was reversed, with a deep MLD in the north and shallow in the south suggesting a prolonged winter season during the study period.

Mixed layer depths are known to control the biological productivity in the ocean through the availability of nutrients and light (Jang, Park, & Park., T & Yoo, S., 2011). In our study, depth-integrated chlorophyll *a* value showed a negative correlation with MZP density (Figure 3). The low density of MZP during high chlorophyll condition could be attributed to the phytoplankton species composition which is probably dominated by larger chain-forming and colonial diatoms such as *Chaetoceros* sp. followed by an inability of MZP to graze on this larger phytoplankton. A similar observation was recorded during a diatom bloom in the East China Sea and Andaman Coastal waters (Elangovan et al., 2012; Lee & Kim, 2010). In earlier studies, the phytoplankton composition from the same region is well reported with the chain-forming species such as *Chaetoceros* sp., *Thalassiosira* sp., *Rhizosolenia* sp., and *Bacillaria* sp. being most dominant (up to 90%) in the coastal and open ocean of the Arabian Sea during the intermonsoon season (Devi et al., 2010; Padmakumar et al., 2017; Sawant & Madhupratap, 1996). MZP are known to feed on suspended detritus and its associated bacterial microflora (Moritz, Montagnes, Carleton, Wilson, & Mckinnon, 2006). On the other hand, ciliates consuming bacteria (several fold higher than flagellates) have been shown previously through grazing studies (Epstein & Shiaris, 1992). A study from the eastern Bering Sea reported that in the early spring season, MZP do not increase in abundance until phytoplankton biomass is high enough to support their growth (Diane, Alison, Dean, & Michael, 2014).

The present data show that MZP density is positively correlated ($0.05 > p > 0.01$) with the column-integrated value of THB ($p = 0.013$) and picophytoplankton ($p = 0.011$) (Figure 5). Besides,

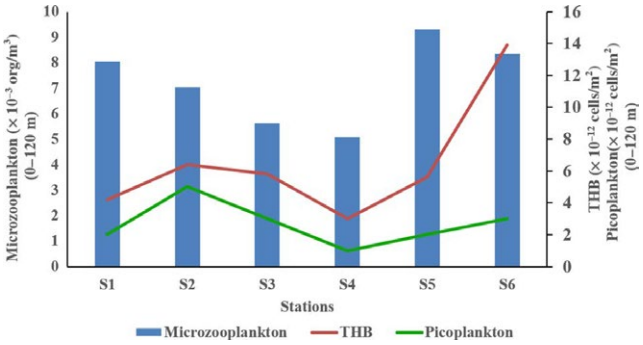

FIGURE 5 Depth-integrated value of microzooplankton, THB, and picophytoplankton in the upper 120-m water column of central Arabian Sea

Dennett et al (1999) reported that the biomass of heterotrophic nanoflagellates (HNF) was higher in the northern (0.49 g/cm^2) and southern (0.43 g/cm^2) regions during intermonsoon. The high bacterial abundance in the southern and northern regions could be due to the availability of total organic carbon (TOC) in the water column which ranged between 90 µM in the north and 77 µM in the south and lower (60 µM) in the central region (Suhas Shetye, unpublished data). Work of Hansell and Peltzer (1998) earlier reported a similar trend of TOC during the spring intermonsoon in the range of >80 µM from the northern and 75–90 µM from the central and southern regions. Bacterial abundance is not related to seasons of high primary production, but it depends on dissolved organic carbon (DOC) in the mixed layer depths (Ramaiah, Raghukumar, & Gaunsm, 1996). DOC is present in the form of concentrated carbon for heterotrophic bacterial growth and respiration (Raymond & Bauer, 2000,2001). Moreover, total dissolved organic matter (DOM) also has a high ratio of carbon and nitrogen for DOC fluxes which could facilitate bacteria to compete with phytoplankton for inorganic N and P (Steelink, 1985). In oligotrophic waters, a combination of picophytoplankton and heterotrophic bacteria biomass, could signify a considerable part of the total particulate organic carbon (Li, Dickie, Irwin, & Wood, 1992). In the surface layer, MZP play a major role in remineralization of the particulate organic materials (Sanders & Wickham, 1993) and act as a trophic intermediate between pico-/nanoplankton and mesozooplankton (Gifford, 1991).

5 | CONCLUSION

During spring intermonsoon, the average abundance of THB community was higher in the southern and northern regions compared to the central region of the Arabian Sea, and it correlated well with MZP density and picophytoplankton abundance. These results indicate a spatial variability in MZP community composition, food chain relationship, and functioning of microbial loop in the Arabian Sea. Further, the interactions within the members of the microbial loop need to be studied for better understanding their spatial scale role in biochemical fluxes.

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Asiame 2

South Asia Thematic School in Aquatic Microbial Ecology

Andaman and Nicobar Centre for Ocean Science and Technology
National Institute of Ocean Technology
Ministry of Earth Sciences, Government of India, Port Blair-744103
Andaman and Nicobar Islands

Certificate of Participation

This is to certify that **AMARA BEGUM MULLA** *of* **BIOLOGICAL OCEANOGRAPHY DIVISION, CSIR-NATIONAL INSTITUTE OF OCEANOGRAPHY, GOA** *has participated in the Second South Asia Thematic School in Aquatic Microbial Ecology held under the Indo - French academic collaborations at Andaman and Nicobar Centre for Ocean Science and Technology, National Institute of Ocean Technology, Port Blair during 6th to 18th November 2017.*

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This is to certify that Prof./Dr./Ms./Mr. Amara Begum Mulla *has attended and presented poster/~~oral~~ titled* "Detection of denitrification marker genes in axenic bacterial cultures isolated from OMZ's of Arabian Sea" *at the Seminar "New Perspectives in Biosciences", held on 7th December, 2017 at Department of Microbiology, Goa University.*

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