PHYLOGENETIC AND FUNCTIONAL DIVERSITY ANALYSIS OF BACTERIA IN THE OXYGEN MINIMUM ZONE SEDIMENTS

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By

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Dedicated to my dear parents and teachers

Certificate

*This is to certify that the thesis entitled, "**PHYLOGENETIC AND FUNCTIONAL DIVERSITY ANALYSIS OF BACTERIA IN THE OXYGEN MINIMUM ZONE SEDIMENTS**", submitted by Ms. BABY DIVYA for the award of the degree of Doctor of Philosophy in Marine Sciences is based on her original studies carried out by her under my supervision for the partial fulfillment for the award of the Doctor of Philosophy, Department of Marine Sciences during the academic session 2010 - 2011.*

Place: Dona Paula
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All the corrections suggested by the referees are incorporated.

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DECLARATION

As required under the university ordinance 0.19.8 (iv), I state that the present thesis entitled "**PHYLOGENETIC AND FUNCTIONAL DIVERSITY ANALYSIS OF BACTERIA IN THE OXYGEN MINIMUM ZONE SEDIMENTS**" is an original research work and carried out by me at National Institute of Oceanography, Dona Paula, Goa and that no part thereof has been published or submitted in part or in full, for any other degree or diploma in any university or institute. To the best of my knowledge the present study is the first comprehensive work of its kind from Arabian Sea.

The literature related to the problem investigated has been cited. Due acknowledgements have been made wherever facilities and suggestions have been availed of.

Divya

BABY DIVYA

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CONTENTS

'Whatever else it is or whatever impact it may have, the study of bacterial evolutionary relationships is central to the historical account of life on this planet. We may lay no claim to a comprehensive understanding of biology until we know this history, at least in its outline.'

Woese, 1987 (Bacterial Evolution)

CHAPTER 1

INTRODUCTION

Ocean realm is an abode of unique and interesting biotopes which includes continental margins, coral reefs, hydrothermal vents, oxygen minimum zones, methane seeps, deep sea sediments, sea ice and the surfaces of animals, plants, marine snow and inanimate objects. These biotopes gain more importance because the oceans cover more than 70% of earth's surface. Oxygen Minimum Zones (OMZs) are large volumes of oxygen-deprived waters at intermediate depths in the eastern boundary of tropical oceans where dissolved oxygen concentration is as low as 0.5 mlL^{-1} (Stramma *et al.*, 2008) (Fig.1).

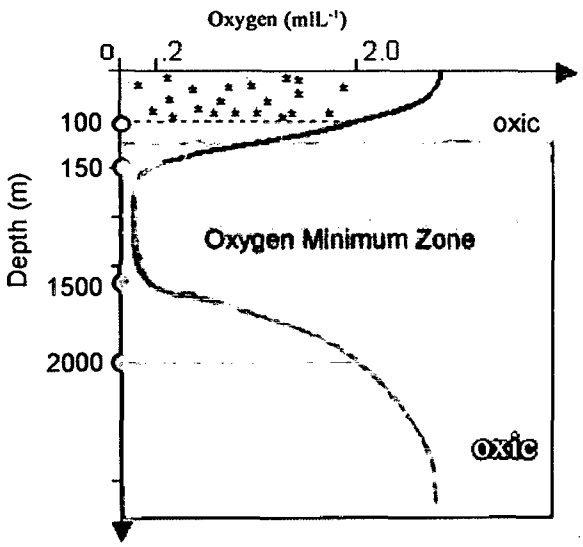


Fig. 1. Hypothetical diagram of an Oxygen Minimum Zone

Oxygen depletion is widespread in the world oceans occurring as permanent, seasonal and episodic features (Kamykowski and Zentara, 1990) (Fig. 2). The OMZs' area and volume have been estimated as ca 8% of the global ocean (Paulmier and Ruiz-Pino, 2009). Of the total OMZ area, 59% occurs in Indian Ocean (Arabian Sea and Bay of Bengal), 31% in the eastern Pacific Ocean and 10% in the southeastern Atlantic Ocean (Helly and Levin, 2004).

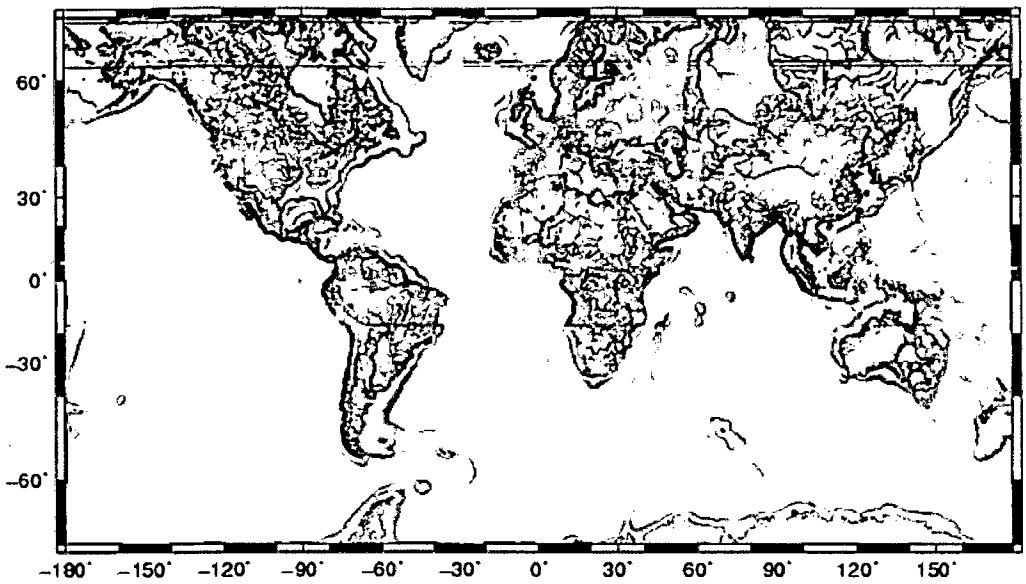


Fig. 2. Distribution of permanent (black) and seasonal (red) hypoxic regions

In modern ocean, OMZs are potential traces of Precambrian ocean where reduced chemical anomalies were prevalent and was the abode of archaea. All OMZs exhibit a similar oxygen profile, but the oxygen levels, thickness of the zone and depth of occurrence vary regionally. Most of the studies on the OMZs have been focused on the geochemistry and paleo-climatology due to their occurrence in or near the vicinity of hydrocarbon-rich regions. Molecular oxygen, due to its positive redox potential, is one of the most important reactants in the biogeochemical cycles. The first global study on denitrification of OMZ was provided by Kamykowski and Zentara (1990). However, recently, an unknown process - the anaerobic oxidation of ammonia (anammox) using nitrate in the ocean has been observed first in the sediment and then in the water column of OMZ by Kuypers *et al.*, (2005). These are also regions of ocean acidification that are marked by the presence of reduced chemical species. This region is the key to understand the present unbalanced nitrogen cycle and oceans' role on atmospheric green house gas control. Also, their expansion as the global climate changes and consequently the drastic impact this may have on the global biogeochemical cycles make these zones ecologically significant (Stramma *et al.*, 2008).

Recently, there is an increased interest both in biological and ecosystem studies of OMZ. Since they are not broad continuous habitats OMZ taxa are not global in distribution, but function as isolated habitats with a high degree of endemism. Two taxa viz. ampeliscid amphipods and lucinid bivalves are widespread within the eastern Pacific and in the Arabian Sea (AS). In most of the OMZ regions studied, though reduced macro-faunal species richness is seen, extraordinarily high dominance has been reported (Levin *et al.,* 2001). Thus, OMZs support many characteristics species which remain undescribed and need to be further explored. Hence, detailed diversity studies have to be conducted for metazoans, meiofauna, megafuana, fishes and microbes within OMZs.

One of the earliest microbiological studies in the OMZ was initiated by the discovery of large, free-living sulphur oxidizing bacteria reported as *Thioploca* by Gallardo (1977) in the eastern south Pacific sediments. This was followed by studies on the diversity, structure and behaviour of *Thioploca* in response to varying concentrations of sulphide and nitrate (Maier and Gallardo, 1984; Fossing *et al.,* 1995; Otte *et al.,* 1999). Studies on bacterial sulphate reduction regulation, hydrogen sulphide fluxes and ammonium cycling rates have been carried out in the OMZ water column (Bailey, 1991; Bruchert *et al.,* 2003; Kuypers *et al.*, 2005; Molina *et al.,* 2007). In the OMZs, eubacteria participate in various biogeochemical cycles such as the nitrogen, sulphur and carbon cycles.

The Arabian Sea Oxygen Minimum Zone (ASOMZ) was discovered during the John Murray Expedition (1933-34) aboard *RV Mabahiss* (Sewell, 1935). However, the results of this study were never published and a thorough understanding of the ASOMZ did not occur until the International Indian Ocean Expedition (IIOE) during 1959-65 (Gage *et al.,* 2000). The open ocean deep water OMZ in the AS occurs permanently between 150-1500 m (Wyrtki, 1971; von Stackelberg, 1972). The zone is formed of a column of water depleted of oxygen that is >750 m thick and extends to an area of 25,00,000 km^2 (Paulmier and Ruiz-Pino, 2009). This oxygen minimum layer impinges on the continental slope, subjecting sea floor to

permanent hypoxia that persists over thousands of years in the oceans (Wyrtki, 1962; Kamykowski and Zentara, 1990; Helly and Levin, 2004). The rain of detritus from the euphotic layers gets accumulated on the western continental margin of India and as a consequence the sediment gets enriched in organic carbon (>4%) (Paropkari *et al.*, 1992, 1993). This region where oxygen minimum zone impinges on the sea floor creates a strong gradient in dissolved oxygen concentrations and serves as a specialized habitat for the organisms.

Studies on the ASOMZ were conducted during IIOE (Gage *et al.*, 2000). Later, the sub-oxic conditions as well as biological and chemical consequences of this phenomenon in northern AS were documented (Paropakari *et al.*, 1992; van der Weijden *et al.*, 1999; Morrison *et al.*, 1999; Naqvi *et al.*, 2000).

The availability of oxygen has a tremendous impact not only on the redox potential of the environment, but also on the energetics of the organisms (Brune *et al.*, 2000). Most of the biomass and biogeochemical activity occurring therein have been attributed to the marine prokaryotes, which are being considered as the major primary producers and heterotrophic consumers in these systems (Fenchel, 1988; Fuhrman *et al.*, 1993). However, microbiological studies in this region have been sporadic. In the ASOMZ, bacterioplankton have been identified by molecular methods and the sequences have been grouped into clades SAR 11 and SAR 406, followed by sulphate reducing bacteria (*Desulphosarcina, Desulphofrigus*) and sulphide oxidizing bacteria (Fuchs *et al.*, 2005). Jayakumar *et al.*, (2004) observed the highest diversity of nitrite reductase genes (*nir*S) in sites of active denitrification compared to regions with undetectable nitrite concentrations, thus linking functional diversity and ecosystem chemistry. The oxygen depleted bottom water significantly affects the characteristics of the sediment pore water due to their close contact (Hermelin, 1992).The bacterial diversity studies pertaining to the ASOMZ sediments are sparse except the report on the presence of bacterium *Thioploca* in the ASOMZ sediments of the northeastern off Pakistan (Schmaljohann *et al.*, 2001).

Recently, the diversity of culturable and non culturable fungi from ASOMZ water and sediment have been reported (Jebaraj and Raghukumar, 2009; Jebaraj *et al.*, 2010).

Study of bacterial diversity in marine environment is important for understanding their distribution, community structure and thereby the functioning of ecosystem. The ability to measure bacterial diversity is a prerequisite for the systematic study of bacterial biogeography and community assembly. Though culture-dependant methods have been employed to study the diversity earlier, it is now well established that the fraction of bacterial community in any environment that can be detected by these methods is only less than 0.1 to 5%. Therefore, cultivation-independent approaches such as PCR based 16S rRNA clone library analysis and denaturing gradient gel electrophoresis (DGGE) are being used to study the diversity and phylogenetic prediction of bacteria. Despite the important role of OMZs in understanding the primitive marine life and chemistry as well as carbon and nitrogen cycles, very little knowledge is gained on the benthic bacterial diversity of the ASOMZ, except for the recent report by Divya *et al.*, (2010) on the diversity and activity of the culturable heterotrophic bacteria of the ASOMZ sediments.

The examination of benthic bacterial diversity will shed light on the life of bacteria thriving in this region and their role in the functioning of this unique ecosystem. Chandler *et al.*, (1997) have compared the diversity obtained from cultivation-dependant and independent approaches in deep subsurface sediments and concluded that culture based methods cannot account for all organisms in given samples and suggested that a combination of both methods would give a comprehensive assessment of diversity. Therefore, the main focus of this study, which is first of its kind in the AS, is to understand the taxonomical and functional bacterial diversity of the OMZ sediments which have been assessed using cultivation-dependant and independent methods and to elucidate the driving forces in the bacterial community structure in this unique ecosystem.

CHAPTER 2

REVIEW OF LITERATURE

2.1. INTRODUCTION

Discovery of Oxygen Minimum Zone

Marine environment is noted for the widespread occurrence of hypoxic and anoxic conditions down the geological time, especially in the Cretaceous (Rogers, 2000).

The existence of permanent hypoxic regions in marine waters, which are now recognized as OMZs was documented during Challenger Expedition (1872-76) (Dittmar, 1884). Later, the Meteor Expedition in 1925-27 reported high sulphur content from shelf near Walvis Bay and this was attributed to the decomposing wastes of whale carcass (Spiess, 1928), thus describing anoxia. This was followed by the discovery of the ASOMZ during the Murray Expedition (1933-34). Three decades later, Wyrtki published the details of the anoxia of world ocean and Eastern Pacific (Wyrtki, 1962; 1966). The term 'Oxygen Minimum Zone' was coined by Cline and Richards (1972). OMZs may be permanent, seasonal or episodic features (Kamykowski and Zentara, 1990) in the most productive regions of the eastern tropical oceans (Stramma *et al.,* 2008) where dissolved oxygen concentrations are as low as 0.5 mlL^{-1} (<22 µM) and are present at different water depths, ranging from shelf to upper bathyal zones (10-1300 m). About 1.15 million km^2 or 2% of the continental margin is intercepted by the OMZ and confer hypoxia (Helly and Levin, 2004). Of the total seafloor OMZ area, approximately 59% occurs in Indian Ocean (Arabian Sea and Bay of Bengal), 31% in Eastern Pacific Ocean and 10% in the South East Atlantic (Helly and Levin, 2004). OMZ is formed due to sinking of material from the productive zones which gets decomposed in mid-water, consuming dissolved oxygen and leading to the development of a mid-water oxygen minimum (Helly and Levin, 2004; Wyrtki, 1962; Kamkowski and Zentara, 1990). Other causes for the persistence of the oxygen minimum layer are stagnant circulation, long residence time and influx of oxygen depleted source waters (Sarmiento *et al.,* 1988). Oxygen minimum systems of the world can be divided into coastal and open ocean OMZs. The coastal/seasonal hypoxia is encountered in estuaries, embayments, enclosed seas and open continental shelves. Three of the

known major coastal hypoxic areas are the Gulf of Mexico (upto 20000 km^2, Baltic Sea (<84000 km^2) and parts of the Black Sea (<20000 km^2) (Rabalais and Turner, 2001; Mee, 2001). The coastal hypoxic areas of the world are summarized in the Table 1.

However, the open ocean deep water OMZs are permanent, extending from 150-1500 m on an average and cover 8% or 30.4 million km^2 of the ocean (Paulmier and Ruiz-Pino, 2009). Figure 1 shows the global distribution of oceanic OMZ regions.

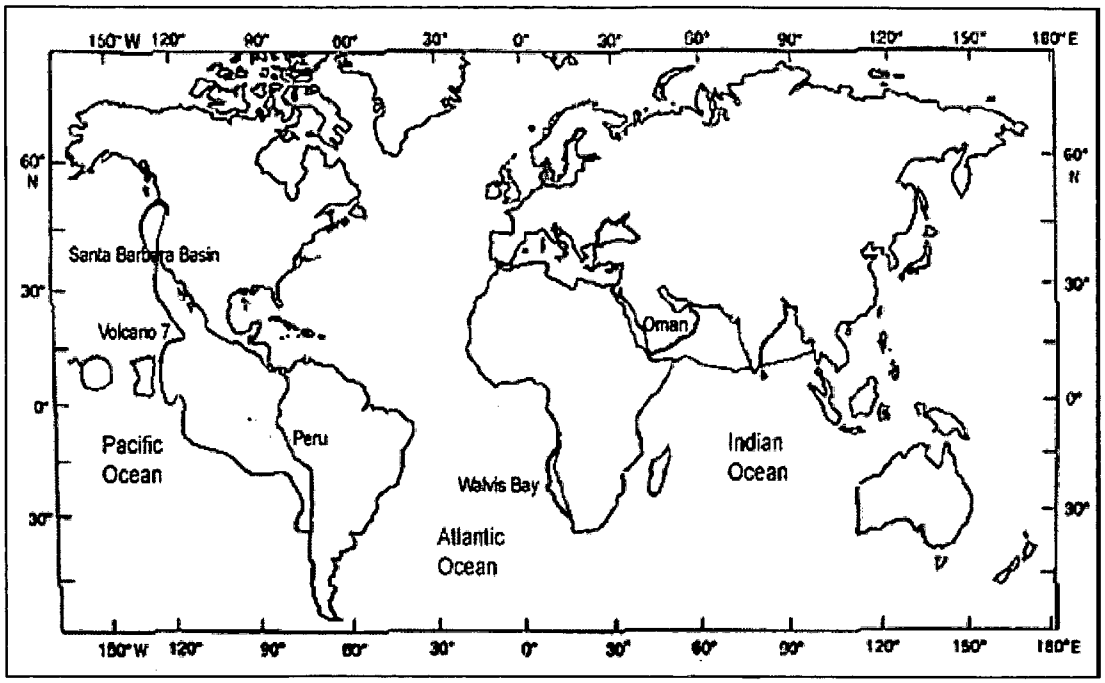


Fig. 1. Global distribution of oceanic Oxygen Minimum Zones

2.2. INTERNATIONAL SCENARIO

Since the discovery of OMZs, various studies have been carried out to understand the physical, chemical and biological characteristics of this unique region. However, most of the studies within these zones have been sporadic and have focused either on pelagic or/and benthic regions of OMZs. But scientists are beginning to understand the importance of this ecosystem for nutrient cycling, fisheries production and even as incubators of evolutionary novelty.

Hypoxia Type	Region			
	Atlantic Ocean	**Pacific Ocean**	**Indian Ocean**	**Enclosed Seas**
Seasonal	Long Island Sound, New York	Seto Inland Sea, Japan	West Indian Shelf, Arabian Sea	Black Sea NW Shelf
	Main Chesapeake Bay, Maryland	Saanich Inlet, British Columbia	Pakistan Shelf, Arabian Sea	
	Pamlico River, North Carolina	Port Hacking, Australia		
	Mobile Bay, Alabama	Tolo Harbor, Hong Kong		
	Hillsborough Bay, Florida	Japan, all major harbors, Japan		
	Louisiana Shelf	Tome Cove, Japan		
	Bornholm Basin, S. Baltic			
	Oslofjord, Norway			
	Kattegat, Sweden–Denmark			
	German Bight, North Sea			
	Laholm Bay, Sweden			
	Gullmars fjord, Sweden			
	Swedish west coast fjords, Sweden			
	Limfjord, Denmark			
	Kiel Bay, Germany			
	Lough Ine, Scotland			
	Gulf of Trieste, Adriatic			
	Elefsis Bay, Aegean Sea			
	Arhus Bay, Denmark			
Persistant	Loch Creran, Scotland			Black Sea (except NW shelf)
	Byfjord, Sweden			Caspian Sea
	Idefjord, Sweden–Norway			
	Baltic Sea, Central			
	Fosa de Cariaco, Venezuela			
	Gulf of Finland			
Aperiodic	New York Bight, New Jersey			
	Shallow Texas Shelf			
	Deep Texas Shelf			
	German Bight, North Sea			
	Sommone Bay, France			
	North Sea, W. Denmark			
Periodic	York River, Virginia			
	Rappahannock River, Virginia			

Table 1. Coastal hypoxic systems of the world

Pelagic Studies

Studies pertaining to pelagic realm have been restricted to biogeochemical processes and on the chemistry of redox sensitive elements in the suboxic regions (Bruland, 2006). The chemistry of OMZ is supported by biological processes eg. nitrate ions are used for the oxidation of organic matter and, in the process they are reduced to molecular nitrogen with nitrite as an intermediate (Codispoti and Christensen, 1985). In the suboxic regions of Pacific Ocean, particulate manganese undergoes nearly complete reductive dissolution (Rue *et al.,* 1997).

Numerous work have been carried out by various researchers on the biology of the organisms in the OMZ. Extensive work on the abundance and activity of zooplankton in the lower interface of the OMZ and feeding ecology of copepods have been carried out in the eastern tropical Pacific Ocean OMZ (Wishner *et al.,* 1995; Saltzman and Wishner, 1997, Gowing and Wishner, 1998). Escribano and Hidalgo (2000) studied the spatial distribution of copepods north of Humboldt Current System (HCS) off Chile and Allen *et al.,* (2000) reported the lipid profiles of the deep sea shrimp. Escribano *et al.,* (2007) studied the seasonal and interannual variation of mesozooplankton in the coastal upwelling zone off Chile. Distribution, abundance and biology of mesopelagic fishes in the OMZ have also been investigated (Hunter *et al.,* 1990; Vetter *et al.,* 1994; Yang *et al.,* 1992; Jacobson and Vetter, 1996; Barry and Maher, 2000, Butler *et al.,* 2001). Fernandez *et al.,* (2009) studied the primary production and nitrogen regeneration process in the surface waters of Peruvian upwelling system using tracer techniques to evaluate the potential role of regenerated nitrogen in sustaining biological productivity in surface waters. They showed that there was active regeneration of nitrogen and ammonia in the euphotic layer which were made available for primary producers.

Prokaryotic microorganisms are universally distributed in marine plankton (Suzuki and Delong, 2002). Of all the prokaryotes, eubacteria being ubiquitous and diverse, make up significant components of the ecosystem.

They are the key players in various biogeochemical cycles and thus in the functioning of the ecosystem. It has been reported that the coastal upwelling system off central Namibia has high rates of primary production and bacterial sulphate reduction (Bailey, 1991). Studies on the regulation of bacterial sulphate reduction (Bruchert *et al.,* 2003) and the massive loss of fixed nitrogen by anaerobic ammonia oxidation by the anammox bacteria in this region (Kuypers *et al.,* 2005) have been examined. Molina *et al.,* (2005) estimated ammonium cycling rates under different dissolved oxygen conditions in the OMZ water column off Chile and found that the oxycline prokaryotes were responsible for ammonium cycling. Francis *et al.,* (2005) reported the molecular evidence for the widespread presence of ammonia-oxidizing archaea in OMZ water column. Farias *et al.,* (2009a, b) studied the chemolithoautotrophic production that mediates the cycling of green house gases like nitrous oxide and methane in upwelling region off Chile and found that chemically driven chemolithotrophy could be more important than previously thought in upwelling ecosystems. Gonzalez and Quinones (2009) characterized the potential enzyme activities involved in aerobic and anaerobic energy production pathways of microplanktonic biomass in OMZ of HCS water column. Among the catabolic enzymatic activities assayed, malate dehydrogenase had the highest oxidizing activity for Nicotinamide Adenine Dinucleotide (NAD), suggesting it to be an appropriate indicator of microplankton catabolism in the OMZ. Pantoja *et al.,* (2009) examined the microbial degradation rates of two important DOM components such as peptides and amino acids to show that microbial activity is not noticeably reduced by the presence of a low oxygen layer and found similar degradation rates in both conditions. Water column distribution of phospholipids derived fatty acids of microbes in the OMZ off Peru was studied by Espinosa *et al.,* (2009).

Junier *et al.,* (2010) thoroughly reviewed the phylogenetic and functional marker genes to study ammonia-oxidizing microorganisms in the environment. Quinones *et al.,* (2009) described the relative abundance and vertical distribution of planktonic archaea off northern and central south Chile using quantitative dot blot 16S rRNA hybridization and indicated that

archaea constituted an important fraction of prokaryotic assemblages of OMZ waters of HCS. Total archaea in the central south Chile made up 6-87% while in the northern Chile it was 10-50% of prokaryotic rRNA in the water column. Crenarchaea was the most abundant archaeal group. Planctomycetes closely related to known anammox bacteria like Candidatus *Scalindua sorokini* and Candidatus *Scalindua brodae* obtained from the waters of the Benguela upwelling system by the construction of 16S rRNA library using Planctomycetes specific primers suggested the presence of anammox as a major contributing factor for the loss of nitrogen (Kuypers *et al.,* 2005). The community structure of beta ammonia oxidizing bacteria associated with the OMZ off northern Chile was estimated using 16S rRNA and *amo*A genes. Sequences affiliated to Nitrospira-like cluster I dominated the *amo*A libraries of both oxic and suboxic waters. Thus it was shown that Nitrospira-like beta ammonium oxidizing bacteria were present in both oxic and suboxic waters and clear community shift was observed at the functional level along the strong oxygen gradient (Molina *et al.,* 2005). The structure of denitrifying communities capable of water column denitrification in Eastern South Pacific (ESP) OMZ were explored by Castro-Gonzalez *et al.,* (2005) using *nir*S library and the sequences showed close similarity to sequences of cultivated *Paracoccus* sp., *Roseobacter*, *Pseudomonas*, *Marinobacter*, *Halomonas* sp.. Stevens and Ulloa (2008) constructed clone libraries in OMZ and non-OMZ waters of Eastern Tropical South Pacific (ETSP) and compared it with other pelagic marine environments such as Arctic Ocean, Aegean Sea, Sargasso Sea etc. In the non-OMZ waters, microbial community was predominately characterized by SAR 86, *Loktanella*, *Flavobacterium*, *Sulfitobacterium*, Alteromonadaceae. In the OMZ, the major groups were SAR11 and thiotropic gamma symbionts and groups such as *Chloroflexi*, AGG47 and SAR202 clades, Deltaproteobacteria, Acidobacteria and Planctomycetes. Phylogenetic diversity of heterotrophic bacteria in the sea water collected from upwelling region of Oregon coast was studied using DGGE (Longnecker *et al.,* 2005).

Benthic Studies

The largest OMZs reside at bathyal depths of the eastern Pacific Ocean, AS, Bay of Bengal (BoB) and off southwest Africa (Kamykowski and Zentara, 1990). Most of the studies were on the geochemistry and paleo-climatology due to the possible location of OMZs in the hydrocarbon-rich regions. One of the earliest studies in geology dates back to late 1980, wherein sedimentation in the OMZ off central California was described by Vercoutere *et al.*, (1987). This was followed by the works of Wakeham in 1987 on the steroid geochemistry in OMZ off Pacific Ocean. Padcard *et al.*, (1988) studied the formation of the Alboran OMZ.

Distribution of recent benthic foraminifera which are generally abundant in the anoxic regions has been an interesting area of research and foremost study was conducted by Hermelin and Shimmield (1990). The abundance and distribution of these organisms have been well documented (Perezcruz and Machaincastillo, 1990; Benhard 1992; Sengupta and Machaincastillo, 1993; Schmiedl *et al.,* 1997; Kurbjeweit *et al.,* 2000; Hogslund *et al.,* 2008; Tapia *et al.,* 2008; Pucci *et al.,* 2009). The meiofaunal distribution and bioturbation in OMZ sediments have been thoroughly investigated (Neira *et al.,* 2001a) and numerous novel benthic species have been identified (Oliver, 2001; Neira *et al.,* 2001b; Oliver and Holmes, 2006; Oliver and Levin, 2006). In addition, studies have been carried out for their use in paleoenvironmental interpretations (Kaiho, 1994), and their associations with the bacterial mats (Erbacher and Nelskamp, 2006). About 69-89% of the benthic organisms encountered within the OMZ were soft bodied with high density though of lesser diversity (Quiroga *et al.,* 2005). Extensive research on the benthic macroorganisms of the OMZ and how the dissolved oxygen concentration, sediment geochemistry and organic matter gradient affect the macroorganisms in the eastern Pacific Ocean have been studied by Lisa Levin (Levin, 2003). Levin and Dayton (2009) reviewed on the benthic ecology of continental margins with respect to expanding hypoxia. Though macrobial life within OMZs has been well documented

(Wishner *et al.,* 1995; Rogers 2000; Levin *et al.,* 2003), the systematic information on the microbiology of these regions is lacking.

Sulphur, which is one of the most abundant elements on earth is of great significance in biogeochemical processes occurring in these regions. The sulphur cycle is complex and is attributed to the wide range of oxidation states of sulphur. Sulphur is represented as pyrite (Fe_2S) and gypsum ($CaSO_4$) in rocks and sediments and as sulphate in sea water. One of the earliest microbiological studies in the OMZ sediments off Pacific Ocean was initiated by the discovery of large, free-living sulphur oxidizing bacteria reported as *Thioploca* by Gallardo (1977) in the eastern South Pacific followed by its occurrences off the coast of Chile (Schulz *et al.,* 1996) and in the coastal sediments of Namibian shelf (Gallardo *et al.,* 1998). Dense populations of conspicuous chains of pearl like *Thiomargarita namibiensis* that oxidize hydrogen sulphide to elemental sulphur, in their vacuoles has been reported off Namibia shelf (Schulz *et al.,* 1999). This was followed by studies on its diversity, structure and behaviour in response to varying concentrations of sulphide and nitrate (Maier and Gallardo, 1984; Maier *et al.,* 1990; Huettal *et al.,* 1996). It was the studies of Fossing *et al.,* (1995) which first reported the peculiar type of metabolism in these bacteria in which the cells were able to concentrate nitrate up to 500 mM. Nitrate is transported to the sediments through the gliding filaments and then reduced by the oxidation of hydrogen sulphide. Ferdelman *et al.,* (1997) postulated the role of these bacteria in the Nitrogen, Carbon and Sulphur cycles, especially in the upwelling regions. Further, Nitrogen, Carbon and Sulphur metabolisms in these organisms were determined using radiolabelled and unlabelled substrates and found to be facultative chemolithoautotrophs with mixotrophic potential (Otte *et al.,* 1999).

Recently, diversity of physiological groups like sulphate reducing bacteria has been reported. Molecular diversity of *dsr*A in sediments of continental shelf-slope transition zone of eastern Pacific belonged to *Proteobacteria* (*Desulfobulbous propionics, Desulfosarcina variabilis* and *Bacillus-Clostridium* (*Desulfotomaculum putei*) (Liu *et al.,* 2003a).

Simultaneously a group of researchers looked into the nitrogen cycling. The diversity of *nir* S and *nir* K genes in the off Mexican sediments of OMZ has been well documented (Liu *et al.,* 2003b), using 16S rDNA clone library analysis. Francis *et al.,* (2005) reported the molecular evidence for the widespread occurrence of ammonia-oxidizing archaea in the sediments. Jaeschke *et al.,* (2010) investigated the presence and abundance of anaerobic ammonia oxidizing (anammox) bacteria in the continental shelf and slope sediments of north west Africa in a combined approach applying quantitative PCR analysis of anammox specific 16S rRNA genes and anammox specific ladderane biomarker lipids. Ammonia oxidizing bacterial diversity was compared in water column and sediment-water interface and new clusters of Ammonia Oxidizing Bacteria (AOB) within *Nitrosomonas/Nitrospira* was obtained (Kim *et al.,* 2008). Diversity of sulphate reducing, iron-reducing and manganese-reducing bacteria has been quantified. These studies also revealed that bacteria dominated in the near surface and deeply buried sediments compared to archaea (Schippers and Neretin, 2006). Population structure and phylogenetic characterization of marine benthic archaea in deep sea sediments of north west Atlantic Ocean was investigated by Vetriani *et al.,* (1999) which revealed the presence of Crenarchaeota and Euryarchaeota. Schafer *et al.,* (2007) used clone library along with DGGE to investigate archaeal component of microbial community of the Benguela upwelling system and the dominant archaea detected were Euryarchaea, Marine Benthic group D (MBGD) and a Crenarchael lineage Marine Benthic group C (MBG-C). Hamdan *et al.,* (2008) studied the bacterial diversity of Sulphate-Methane Transition Zone (SMTZ) of gas hydrate bearing sediments along the mid-Chilean margin. Sequences closely affiliated to *Desulfosarcina variabilis* was found in the clone libraries. Hamersley *et al.,* (2007) investigated the phylogenetic analysis of Planctomycete-specific 16S rRNA clones and detected sequences with 98% sequence identitiy to known anammox bacteria, Candidatus *Scalindua sorokinni* in the waters of Peruvian OMZ suggesting the prevalence of anaerobic ammonium oxidation. An interesting study on the microdiversity analysis of anammox bacteria in the different OMZ waters

(Black Sea, Off Namibia, Peru and AS), revealed the presence of novel Candidatus *Scalindua* phylotype (Woebken *et al.,* 2008). Woebken *et al.,* (2007) investigated the distribution and particle association of anammox bacteria in Namibian OMZ using comparative 16S rRNA gene analysis. It was revealed that Candidatus *Scalindua* sp. colonized the particles followed by Gammaproteobacteria, Alphaproteobacteria and Bacteriodetes. The microbial diversity of sulphate reducing and methanogenic sub-surface sediments of Benguela upwelling system was analysed using DGGE and showed the presence of complex microbial communities (Schafer *et al.,* 2007). A major breakthrough was the report by Walsh *et al.,* (2009), on the metagenomic analyses of an ubiquitous and abundant but uncultivated OMZ microbe (SUP05). This microbe showed relation to the chemoautotrophic gill symbionts of deep sea clams and mussels, and harboured a versatile repertoire of genes mediating autotrophic carbon assimilation, sulfur oxidation and nitrate respiration responsive to a wide range of water-column redox states. This information may prove useful in the development of monitoring tools that assess the microbial community responses to OMZ expansion and intensification.

2.3. NATIONAL SCENARIO

Arabian Sea Oxygen Minimum Zone

The Arabian Sea OMZ (ASOMZ) was discovered during the John Murray Expedition (1933-34) aboard *RV Mabahiss* which reported the depletion of fauna in this region (Sewell, 1935). However, the results of the above study were never published and a thorough understanding of the Arabian Sea OMZ did not occur until the International Indian Ocean Expedition (IIOE) in 1959-65 (Gage *et al.,* 2000). Of the total OMZ area, 59% occurs in Indian Ocean ie. Arabian Sea (AS) and Bay of Bengal (BoB). The zone is formed of an oxygen depleted water column that is > 750 m thick and extends upto an area of 2500000 km^2 (Paulmier and Ruiz-Pino, 2009). The upper boundary of the ASOMZ is oxygenated and is approximately 150 m thick. The OMZ core is formed of the oxygen depleted waters from the

Persian Gulf. The bottom of the zone is formed of waters with the characteristic density of Red Sea water which mixes with the Indian Central water and North Indian Intermediate water (Morrison *et al.,* 1999). The open ocean deep water OMZ in the AS occurs permanently between 150-1500 m (Wyrtki, 1971; von Stackelberg, 1972) which is formed as a result of high subsurface oxygen demand arising from high surface productivity coupled with low oxygen content of water flowing into the AS from south (Naqvi, 1987).

Salient features of Arabian Sea Oxygen Minimum Zone:

1. OMZ is present in the most productive regions at intermediate depths, ie. 150-1500 m (average).
2. It is formed as a result of the decomposition of detrital matter falling from the productive waters, besides low ventilation and oxygen poor source waters.
3. The influx of detritus from the overlying productive waters to the ocean floor leads to the preservation and consequent enrichment of organic carbon in sediments.

Pelagic Studies

ASOMZ is one of the thickest and the most intense oxygen minima observed in the open ocean where the oxygen concentrations are as low as 0.5 mlL^{-1}. At the national level, the research work has mainly focused on the water column of ASOMZ, pertaining to processes in nitrogen, carbon and redox element cycling. About half of the oceanic nitrogen production occurs in the AS (Devol *et al.,* 2006). Though large volumes of chemical oceanographic data had been collected during the IIOE (1960-65) (McGill, 1973) and subsequent studies, most of these were related to the distributive aspects of different chemical constitutents (Rochford, 1966; Fraga, 1966; Reddy and Sankaranarayanan, 1968; Wooster *et al.,* 1967; Anand and Jayaraman, 1972). Post-IIOE research on the chemistry of OMZ and the Indian Ocean, examined the quantitative relationships between nutrients and

oxygen (Sengupta *et al.,* 1975; 1976 a). The AS is one of the few areas of the open ocean where the concentration of dissolved oxygen falls to vanishingly low levels, triggering extensive reduction of the oxidized nitrogen compounds (denitrification). Studies revealed that about one third of the dissolved nitrate is lost during denitrification (Sengupta *et al.,* 1976b 1980; Deuser *et al.,* 1978; Naqvi *et al.,* 1978, 1982). Numerous methods for the study of denitrification have been evaluated (Naqvi *et al.,* 1981; Naqvi *et al.,* 1982; Naqvi and Qasim, 1983). This finally led to the development of a consistent method for estimating nitrate deficits (Naqvi and Sengupta, 1985). Since then numerous attempts have been made to quantitatively evaluate the extent and other aspects of denitrification in the ASOMZ. Geographical limits of the AS denitrification zone was delineated by Naqvi (1991) from the analysis of nitrite distribution using historical datasets. Qasim (1982) suggested that poor renewal of the subsurface waters due to semi-landlocked nature of the AS is crucial in the development of oxygen deficient conditions. However, later studies revealed that oxygen depletion could be primarily due to high consumption rates as a result of rapid renewal of the intermediate waters (Naqvi, 1987; Somasunder and Naqvi, 1988). Naqvi (1991) suggested that subsurface denitrification is uncoupled from primary production in the overlying waters. Naqvi and Shailaja (1993) estimated the rate of denitrification from the activity of electron transport system (ETS) and it coincided with the Secondary Nitrite Maxima (SNM). Study by Morrison *et al.,* (1999) in US JGOFS (Joint Global Ocean Flux Study) Arabian Sea Process Study gave an internally consistent, high quality dataset for understanding the conditions of the ASOMZ over a complete monsoonal cycle of 1995 and this was consistent with the results of other investigators. Considerable efforts have been put in to study the fate and flux of nitrous oxide (Codispoti *et al.,* 1992) and Naqvi and Noronha (1991) suggested that the AS serves as a significant source of this green house gas to the atmosphere. Further studies of Naqvi *et al.,* (2000) suggested that increased production of nitrous oxide in the AS could lead to intensification of anoxia due to anthropogenic activities and subsequently to denitrifying conditions in the subsurface shelf waters. This environment is of considerable significance

to global nitrogen cycle due to the potential sensitivity of coastal denitrification to environmental conditions and global change. Microbially-mediated reduction of nitrate to nitrogen (denitrification) in the OMZ appears to greatly affect the natural isotopic abundances. It is now realized that the N_2O isotope data cannot be explained by production through either nitrification or denitrification alone, but by a possible link between the two processes as an important mechanism of N_2O production (Naqvi, 1999; Naqvi *et al.,* 2000; Patra 1999). Ward *et al.,* (2009) and Bulow *et al.,* (2010) found that it was dentrification rather than the anammox that dominated in the Arabian Sea by isotope incubation methods. Jayakumar *et al.,* (2004) have observed the highest diversity of nitrite reductase genes (*nir*S) in sites of active denitrification compared to regions with undetectable nitrite concentrations, thus linking functional diversity and ecosystem chemistry. Jayakumar *et al.,* (2009) studied the spatial dynamics of denitrifying bacterial diversity in the ASOMZ and found that they vary in space and time and exhibit striking changes in diversity associated with the progression of denitrification from initial anoxia through nitrate depletion. Anammox bacterial assemblages in AS are less diverse than denitrifier assemblages and represented by one or two phylotypes (Woebken *et al.,* 2008).

The lowered redox potential conditions in the ASOMZ affect the cycling of redox elements. The depth profiles of dissolved manganese and iron in the AS revealed the occurrence of broad maxima in their concentrations in the OMZ, attributing to *in situ* dissolution from particulate matter and lateral inputs within reducing conditions (Saager *et al.,* 1989; Lewis and Luther, 2000). Farrenkopf and Luther (2002) reported that iodate is reduced to iodide in waters of ASOMZ. Limited studies are available on the biology of OMZ region carried out by Indian researchers. The US JGOFS study in the AS found a strong relation between organisms and the oxygen concentrations in the ASOMZ (Morrison *et al.,* 1999). Studies on vertical structure of calanoid copepods in the AS (Bottger-Schnack, 1996) showed that the copepods were lesser in number in the mesopelagic zone which is the OMZ, compared to the oxygenated waters. However, Koppelmann and Weikert (1997) reported a mesozooplankton maximum at the lower boundary

layer of the OMZ in the AS during the intermonsoon. The microzooplankton biomass increased in the lower part of OMZ suggesting the possibility of active biological modification of sinking flux due to the presence of potential food levels and shorter food chains. These organisms act as a filter for carbon sinking to the sea floor and also modify it by various processes (Wishner *et al.,* 1998; Gowing and Wishner, 1998). Organisms thriving in these regions exhibit unusual adaptations in order to survive the hypoxia. Young and Vazquez (1997) reported two new species of ascidians *Agnezia monnitoi* and *Styela gagetyleri* which had specialized adaptations to live within and below the OMZ in the AS. Vetter and Lynn (1997) demonstrated that the lactate dehydrogenase activity, an indicator of anaerobic respiration, did not increase in response to hypoxia in the deep living scorpaenid fishes, *Sebastes* and *Sebastolobus*. The presence of large biomass of mesopelagic fishes such as myctophids was reported mostly living in the core of the ASOMZ, which are excellent migrators feeding on the zooplankton, forming a major component of the deep scattering layer (Nair *et al.,* 1999). Distribution and abundance of mesopelagic fishes in the ASOMZ have been thoroughly investigated (Butler *et al.,* 2001; Karuppasamy *et al.,* 2010).

Although information on the general abundance of bacterioplankton in the AS have been reported (Ducklow *et al.,* 2001; Koppelmann *et al.,* 2005), those on the bacteria associated with the OMZ is very meager. Reports on the molecular identification of baterioplankton in the ASOMZ showed the dominance of sequences falling in clades SAR 11 and SAR 406, followed by sulphate reducing bacteria (*Desulphosarcina, Desulphofrigus*) and sulphide oxidizing bacteria (Fuchs *et al.,* 2005). Jayakumar *et al.,* (2004, 2009) studied the diversity and dynamics of nirS and nirK gene in OMZ waters of AS and most of the sequences were closely related to cultivated denitrifier *Pseudomonas aeruginosa*. Horizontal and vertical variations in the bacterial community was examined in the water samples of two consecutive north east monsoon periods collected during the JGOFS AS cruise. The community was dominated by SAR 11 of Alphaproteobacteria and Cyanobacteria. Magnetotactic bacteria were found for the first time in pelagic oceanic waters (Riemann *et al.,* 1999) of ASOMZ and indicated a distinct and diverse

bacteria community. Majority of the sequences were affiliated to Gammaproteobacteria, Alphaproteobacteria and Bacteriodetes. Damste *et al.,* (2002) studied the distribution of membrane lipids in the planktonic Crenarcheota in the AS and found that the highest concentration of membrane lipids occurred at 500 m, suggesting that the planktonic Crenarcheota are facultative anaerobes.

Benthic Studies

The OMZ impinges on the western continental margin of India resulting in the deposition and consequent enrichment of organic carbon in the sediments (>4%) (Paropkari *et al.,*1992; 1993). Though the coastal hypoxia is also prevalent in the AS coast, the inner and mid-shelf hypoxia is distinct from the deeper offshore suboxic zone due to the presence of oxygenated West India Undercurrent (WIUC) which flows along the continental margin between the two systems. The rain of detritus from the euphotic waters gets accumulated on the ocean floor making the sediments high in organic carbon (Paropkari *et al.,* 1992), subjecting the sea floor to permanent hypoxia that persists over thousands of years in the oceans (Wyrtki 1962; Kamykowski and Zentara 1990; Helly and Levin, 2004). The oxygen depleted bottom water affects the characteristics of the sediment pore water as they are in close association (Hermelin, 1992). Figure 2 shows the vertical section of OMZ when it impinges on the ocean floor.

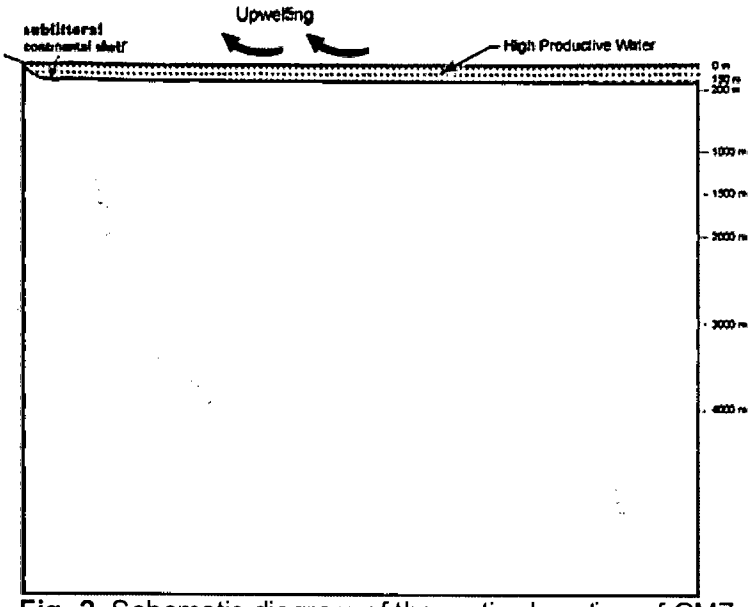


Fig. 2. Schematic diagram of the vertical section of OMZ

Molecular oxygen, due to its positive redox potential, is one of the most important reactants in the biogeochemical cycles. In the continental margins rich in organic matter, the carbon cycling is coupled to the reduction of a variety of electron acceptors including oxygen, nitrate, sulphate, manganese and iron (Canfield *et al.,* 1993; Thumdrup *et al.,* 1996). Alagarsamy *et al.,* (2005) investigated the partitioning and solid state speciation of manganese and iron diagenesis in the sediment cores of Oman margin. The availability of oxygen has a tremendous impact not only on the redox potential of the environment, but also on the energetics of the organisms (Brune *et al.,* 2000). Thus, the region where OMZ impinges on the sea floor creates a strong gradient in dissolved oxygen concentrations thereby serving as a peculiar habitat for the organisms. Levin and Edesa (1997) observed the presence of dense aggregations of cirratulid mudballs in the OMZ off Oman margin in northwest AS. Kurbjeweit *et al.,* (2000) studied the distribution, biomass and diversity of benthic foraminifera in the sediments of AS and found that the highest abundance was found in the western AS, mainly influenced by sand fraction, dissolved oxygen, calcium carbonate and organic carbon content of sediment. Panchang *et al.,* (2006) studied the effect of oxygen manipulations on benthic foraminifera from the ASOMZ. Also studies had been conducted on the distribution of foraminifera in hypoxic regions of the west coast of India and their use as paleooceanographic proxies (Mazumder *et al.,* 2003; Linshy *et al.,* 2007;

Nigam, 2007). OMZ plays an important role in generating biodiversity and certain organisms are endemic to these regions with peculiar adaptations (Rogers, 2000; Levin, 2003). Novel species of a gromiid protist, *Gromia pyriformis* seen attached to large arborescent foraminiferan *Pelosina* sp. confined to narrow bathymetric zone in the lower portion of OMZ and a new species of mollusc namely, *Amygdalum anoxicolum* was reported by Gooday and Bowser (2005) and Oliver (2001), respectively in ASOMZ. Polychetes were the dominant group in macrofauna with lesser species diversity in the OMZ region compared to the non-OMZ (Ingole *et al.,* 2010).

Total microbial biomass in the sediments of the AS was estimated by Boetius and Lotche (2000) using lipid biomarkers. Bacteria play a significant role in the redox transformations of sulphur. *Thioploca* sp. has been encountered in sediments of northern AS (Gallardo *et al.,* 1998). Also the sediments of northeastern AS off Pakistan form a good habitat for the bacterium *Thioploca* (Schmaljohann *et al.,* 2001). Recently, the diversity of culturable and non-culturable fungi was reported by Jebaraj and Raghukumar, (2009); Jebaraj *et al.,* (2010). Thus, little or no systematic studies on culturable and non-culturable bacterial diversity in this ecosystem have been attempted. Although the review of the literature reveals considerable gaps in our knowledge of OMZ, recent observations suggest that OMZ is an important frontier for discovery of new adaptations and process at all levels.

Studies on the sediments of OMZ have been hampered largely by limited access to the samples. The OMZ in the AS and BoB remains largely unexplored. Overall the knowledge of OMZ is truly in its infancy and an integrated approach using biochemical, microbiological, molecular and ecological tools is imperative in advancing our understanding of sediments of OMZ especially in the AS. Bacteria play a key role in the biogeochemical cycles and thus information on who these players are, what function they are carrying out and how they help in the biogeochemical processes in this unique habitat is essential.

With this as the backdrop the following queries arise:

Are bacteria the key players in supporting the macrofauna of the richest sediments? What are the species and how do they adapt their physiology, enzymatic and molecular function?

To answer the above questions the following objectives were identified:

1. To assess the diversity of culturable and uncultured bacteria in different areas of OMZ of AS using cultivation-dependant and cultivation-independent techniques.
2. To characterize the spatial abundance and diversity of the components of bacterial community to environmental parameters.
3. To elucidate the metabolic/functional diversity of the bacterial community.

CHAPTER 3

MATERIALS AND METHODS

3.1. STUDY AREA

3.1.1. Description and Location

Arabian Sea (AS) occupies an area of 6.2 x 10^6 km^2 in the north-western Indian Ocean. It is a semi-enclosed sea on the western side of the Indian peninsula, bounded by African and Asian landmasses to the west and north, respectively. The continental shelf is generally wide all along the Indian west coast with the maximum off the Gulf of Cambay (350 km). AS also receives about 350 km^3y^{-1} river runoff. The rainfall over the AS occurs during the southwest monsoon (June-September) which may often exceeds 300 cmy^{-1}. The surface waters are less saline in the south east and more saline in the north west AS. The large rainfall and land runoff result in a positive water balance (excess of precipitation and runoff over evaporation) in the northern part of AS and vice versa in the southern region. However, the net water balance is negative for AS as a whole. It is also subjected to extreme seasonal changes in the atmospheric forcing that are divided into northeast and southwest monsoons and two inter-monsoonal periods, producing dramatic physical, chemical and biological changes in the upper layers of water column. Consequently, the seasonal reversal of monsoon winds, during the southwest monsoon, leads to the upwelling phenomenon which increases the productivity of the region, thereby making AS one of the highly productive system. This high biological productivity of 1.03 to1.64 $Cm^{-2}d^{-1}$ (US JGOFS) has led to increased flux of organic matter to the ocean floor. This resulted in the formation of a permanent oxygen minimum layer at the mid-depths. Accordingly, the sampling stations covered a spatial gradient along 3 transects, Karwar (K), Goa (G), Ratnagiri (R), of the continental margin, viz 50, 200, 500 and 1000 m (Fig. 1).

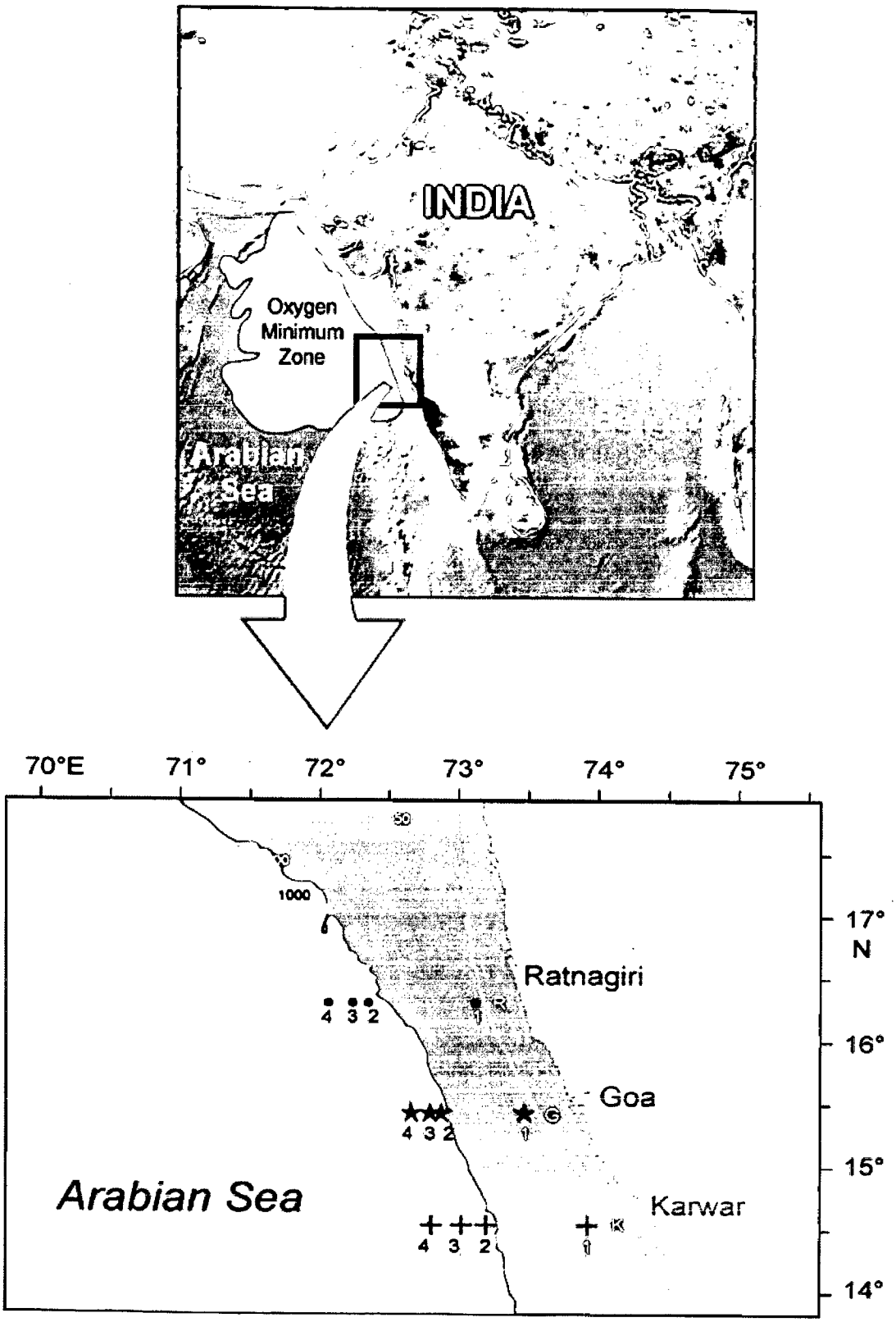


Fig. 1. Location of the sampling stations at 50 m, 200 m, 500 m and 1000 m (1, 2, 3 and 4 respectively) along transects Karwar (K), Goa (G) and Ratnagiri (R).

The site at 50 m is located in the continental shelf and is oxic while the sites at 200, 500 and 1000 m are suboxic situated in the continental slope which falls in the OMZ. The coordinates of the stations are listed in Table 1.

Transects	Stations	Depth (m)	Latitude	Longitude
Karwar	K1	50	14°32'267" N	73°54'477" E
	K2	200	14°32'145" N	73°11'283" E
	K3	500	14°32'148" N	73°07" E
	K4	1000	14°32'223" N	73°03'042" E
Goa	G1	50	15°26'088" N	73°29'131" E
	G2	200	15°25'988" N	72°52'702" E
	G3	500	15°25'465" N	72°47'215" E
	G4	1000	15 °25'529" N	72°40'877" E
Ratnagiri	R1	50	16°17'491" N	73°07'436" E
	R2	200	16°18'133" N	72°21'941" E
	R3	500	16°18'201" N	72°19'140" E
	R4	1000	16°16'25" N	72°16'64" E

Table 1. Coordinates of the sampling stations

3.1.2. Sample Collection

Water and sediment samples were collected onboard FORV *Sagar Sampada* (Cruise No. 254) from transects off Karwar, Goa and Ratnagiri during May 2007. Water samples were collected using a Seabird Conductivity-Temperature-Depth (CTD) rosette system fitted with 1.7 L Niskin samplers from a mandate depth close to the seafloor, ie., 10 m above the seafloor at all stations. Water samples for determining physico-chemical parameters were sub-sampled in polypropylene bottles and preserved at -20°C for analysis.

Sediment samples were collected using Smith McIntyre grab, having an area of 0.1 m^2 and undisturbed surface sediments were collected from all the 12 stations. Multiple subsamples from each of the stations were pooled together and analyzed for better homogeneity. Samples for molecular analysis were preserved in Liquid Nitrogen (-196°C), whereas those for

bacteriological analysis were sub-sampled using sterile syringes and stored at 4-5°C till analysis. For biochemical analysis the sediment samples were stored at -20°C.

3.2. HYDROGRAPHICAL PARAMETERS

Water parameters were measured, processed and analyzed on board using standard methods. All analyses except depth and temperature were carried out in triplicates.

3.2.1. Depth

The station depths were recorded using an inbuilt echo sounder of the vessel.

3.2.2. Temperature

Temperature was determined using the Seabird CTD (rosette system which possess a temperature sensor and determines temperature in real time.

3.2.3. Salinity

Water salinity was determined using Autosal.

3.2.4. pH

A digital pH meter (Thermoelectron Corporation, USA) was used for determining pH of water samples after calibrating it with the standard buffers of pH 4, 7 and 9.2 (Fluka), respectively.

3.2.5. Dissolved oxygen

Dissolved oxygen concentration was estimated using Winkler's titrimetric method (Carpenter, 1965). Water samples were collected by siphoning in 125 ml acid washed (10% HCl) glass stoppered bottles without formation of air bubbles. Winkler A (1 ml of 3 M manganous chloride) and

Winkler B (1 ml of 8 M alkaline iodide) were added to the sample. The precipitate formed was dissolved using 1 ml of 10 N H_2SO_4 and was titrated with 0.01 N sodium thiosulphate using starch as indicator. Concentration of oxygen is expressed as mlL^{-1}.

3.2.6. Nitrite

Nitrite in the sample was allowed to react with sulphanilamide in an acid solution. The resulting diazo compound reacted with N-(1-naphthyl)-ethylenediamine, forming a highly coloured azo dye. The extinction was read in a spectrophotometer (Shimadzu, Japan) at 543 nm (Grasshoff *et al.,* 1983). Standards were run with analytical reagent quality sodium nitrite (Standard curve in Appendix section-A4). Values are expressed in μM.

3.2.7. Nitrate

Water sample was passed through cadmium-copper column, where the nitrate chemically gets reduced to nitrite. Nitrite estimation was carried out as in Section 3.2.6. Potassium nitrate was used for standardization (Standard curve in Appendix section-A4). Values are expressed in μM. (Grasshoff *et al.,* 1983).

3.2.8. Phosphate

Phosphate and ammonium molybdate were allowed to react in acid solution to give phosphomolybdic acid, which was reduced by ascorbic acid. After 10 minutes of incubation, the optical density was measured 882 nm using a spectrophotometer (Shimadzu, Japan). Potassium dihydrogen orthophospate was used as standard (Standard curve in Appendix section-A4) and the concentration is expressed in μM (Grasshoff *et al.,* 1983).

3.2.9. Silicate

Sample was allowed to react with ammonium molybdate resulting in the formation of silicomolybdate complexes and oxalic acid was added to reduce the above to silicomolybdous acid and the absorbance of blue colour

was measured at 810 nm (Grasshoff *et al.,* 1983) The concentration is expressed in µM. (Standard curve in Appendix section-A4).

3.3. GEOCHEMICAL PARAMETERS

3.3.1 Sediment Temperature

Sediment temperature was determined using a thermometer.

3.3.2. Redox Potential (Eh)

Eh was estimated using ORP meter (Thermo Electron Corporation, USA). The probe was placed in the sediment directly and the value was noted after attaining a steady reading and expressed in mV.

3.3.3. Water content

The weight difference between wet and dried sediment (in triplicates) was recorded. This difference in weight was multiplied by 100 to give the percentage of water content.

3.3.4. Sediment Texture

Grain size was analyzed using pipette analysis in triplicates (Krumbein and Petti John, 1938).

3.3.5. Elemental CHNS

The total concentration of C, H, N and S was estimated in triplicates of sediment samples directly using the CHNS analyzer (Perkin Elmer).

3.3.6. Total organic carbon (TOC)

Sediment samples (in triplicates) were decarbonated by Sulphurous acid and total organic carbon (TOC) was analysed using Elemental Analyser (Thermo Finnigan, Italy).

3.3.7. Labile Organic Matter (LOM)

Sediment samples (in triplicates) were dried and powdered for estimating labile organic matter.

3.3.7.1. Estimation of Carbohydrates

Sediment samples of 0.5 g (in triplicates) were treated with 2 ml of 5% Tricarboxylic Acid (TCA) and heated in a boiling water bath for 3 hours at 80-90°C. The mixture was centrifuged at 5000 rpm for 5 minutes to obtain a clear supernatant. To 0.5 ml of this supernatant, equal amount of distilled water, 1 ml phenol reagent and 5 ml concentrated H_2SO_4 were added and mixed immediately and incubated for 30 minutes. The absorbance of the reagent blank and the samples were measured at 490 nm in a spectrophotometer (Shimadzu, Japan) (Kochert, 1978) (Standard curve in Appendix section-A4).

3.3.7.2. Estimation of Proteins

To 500 mg of sediment (in triplicates), 2 ml of 1 N NaOH was added and boiled at 100°C for 5 minutes. The tubes were cooled and centrifuged at 5000 rpm for 5 minutes. 0.5 ml of supernatant was mixed with equal amount of distilled water. Further 5 ml of Reagent C was added, mixed and kept for 10 minutes in dark. 0.5 ml of Folin Ciocalteau reagent was added, mixed and kept at 20 minutes in dark. The absorbance of the samples and reagent blank was measured at 730 nm in a spectrophotometer (Shimadzu, Japan). (Lowry, 1951) (Standard curve in Appendix section-A4).

3.3.7.3. Estimation of Lipids

To 3.8 ml of organic solvent, 0.5 g of sediment sample (in triplicates) was added. The mixture was homogenized at 5000 rpm for 5 minutes, followed by centrifugation at 2500 rpm for 5 minutes. The supernatant was mixed in 1.9 ml organic solvent (chloroform: methanol: distilled water in the ratio 5:10:4), 1.5 ml chloroform and 1.5 ml distilled water and collected in an evaporating flask which was further evaporated to dryness. 2 ml of 0.15%

acid dichromate was added to the dried lipid sample and heated in a boiling water bath for 15 minutes, cooled and made up to 6.5 ml by adding 4.5 ml of distilled water. The absorbance was measured at 440 nm in a spectrophotometer (Shimadzu, Japan) against acid dichromate solution as the reagent blank (Bligh and Dyer, 1959) (Standard curve in Appendix section-A4).

3.4. BACTERIAL ABUNDANCE

3.4.1. Estimation of Total Microbial Biomass –Adenosine Triphosphate (ATP)

Fresh wet sediment in triplicates (1 g) was mixed thoroughly with 5 ml of Tris buffer (pH 7.7) and boiled at 100°C for 5 minutes in a water bath. The tubes (after cooling) were centrifuged at 4500 rpm for 5 minutes. The supernatant was collected and kept frozen until analysis. For analysis 100 µl of the supernatant was mixed with equal volume of enzyme extract (a mixture of luciferin and luciferase from firefly lanterns) and the light emitted was measured using a luminometer. The concentration of ATP in the sample was calculated using standard ATP solution (Holm-Hansen and Booth, 1966) and expressed as biomass in $\mu Cg^{-1}dw$ (Standard curve in Appendix section-A4).

3.4.2. Direct Total Counts (DTC)

Bacterial abundance was determined by DAPI (4,6-Diamidino-2-Phenylindole) method (Porter and Feig, 1980). Approximately one gram (in triplicates) of wet sediment was suspended in 99 ml of sterile sea water and vortexed for three minutes. One ml each of this sample were fixed immediately with buffered formalin on board and stored at 4°C. These samples were stained with DAPI (Fluka) (final concentration 0.01% w/v) for five minutes before filtering it through 0.22 µm polycarbonate Nuclepore filter (Whatman, USA). Samples were enumerated at 100X magnification under a

Nikon epifluorescence microscope, and at least 10 fields of >30 bacteria per field were counted. Bacterial abundance is expressed as numbers g^{-1}DW.

3.4.3. Total Viable Counts (Aerobic and Anaerobic)

Total Viable Counts (TVC) was obtained using the method of Kogure *et al.,* (1979). Samples (in triplicates) were prepared as in section 3.4.2. and incubated at 20°C for 6 hours with nalidixic acid (0.02% w/v), piromidic acid (0.001% w/v), pipemidic acid (0.01% w/v) (Sigma, USA) and yeast extract (0.01%). In case of anaerobic TVC, sodium sulphide (0.125%) was added. Incubation was terminated by the addition of buffered formalin (final concentration 2%) after 6 hours. Samples were stained with DAPI (as mentioned for DTC) and observed under epifluorescence microscope. Only swollen and elongated cells were enumerated as viable bacterial cells and their counts are expressed as numbers g^{-1}DW.

3.4.4. Retrievable Counts

3.4.4.1 Colony Forming Counts of Aerobic Bacteria

About 1 cubic centimetre of sediment was added to 99 ml of sterile sea water and thoroughly blended for 60 seconds. Ten fold dilutions were prepared from this homogenate. About 0.1 ml of the sample was spread on to agar plates with varying concentrations of nutrients (100%, 25%, 10% and 0%). The plates were incubated for seven days at 25°C and monitored at 24 hours interval and the colony forming units (CFU) were enumerated. The abundance is expressed as $CFUg^{-1}$DW.

3.4.4.2. Colony Forming Counts of Anaerobic Bacteria

Anaerobic bacteria were cultured using the shake agar method, in which nutrient agar (Hi Media, India) with varying concentrations (0% and 100%) was used. The tubes were incubated at 23-24°C and monitored for the growth of colonies. A layer of paraffin mix (paraffin oil and paraffin wax in

the ratio 2:1) was added on the surface to prevent oxidation. The numbers are expressed as $CFUg^{-1}DW$.

3.4.4.3. Sulphate Reducing Bacteria (SRB)

Lactate and acetate utilizing SRBs were enumerated in shake agar using the modified Hatchikian's method (1972). A layer of paraffin mix was added on the surface to prevent oxidation. Black colonies were counted and the numbers are expressed as $CFUg^{-1}DW$.

3.4.4.4. Thiobacillus Denitrificans Like Organisms (TDLO)

TDLOs were enumerated in shake agar using modified Lieske medium (Rodina, 1972). A layer of sterile paraffin mix was added on the surface to prevent oxidation. The colony forming units were counted and expressed as $CFUg^{-1}DW$.

3.5. TAXONOMIC DIVERSITY

Benthic bacterial diversity was estimated using cultivation-dependant and cultivation-independent methods. A detailed study was carried out on samples collected off Goa transect.

3.5.1. Cultivation Dependant

Different morphotypes (CFUs) were isolated from the enumeration plates. These isolates were purified by quadrant streaking. Well-isolated colonies that appeared after 24-48 hours of incubation at 25°C were purified and transferred to nutrient agar slants. A total of 105 (50 m-31; 200 m-19; 500 m-23; 1000 m-32) of these isolates were identified using polyphasic approach ie., identification by both biochemical and molecular methods.

3.5.1.1. Biochemical Method

3.5.1.1.1. Morphological Characteristics

i. Size: Approximate size of the colonies was measured.

ii. Pigmentation: Colony colour was noted visually.

iii. **Form:** The colony forms such as punctiform, circular, filamentous, irregular, rhizoid, or spindle were recorded.

iv. **Elevation:** Elevation of the colony was noted as flat, raised, convex, pulvinate, or umbonate.

v. **Margin:** Margin of the colony entire, undulate, lobate, erose filamentous, or curled was observed.

vi. **Texture:** This was recorded as butyrous, viscous or dry.

3.5.1.1.2. Biochemical Characteristics

The isolated bacterial colonies were sub cultured on nutrient agar plates and the actively growing bacteria were subjected to various biochemical tests (Gerhardt *et al.,* 1981). All the tests were carried out with cultures in logarithmic phase. Composition of the media and reagents used are given in Appendix A1&A2.

i. Nature of cell wall

Two tests were carried out to determine the nature of bacterial cell wall.

a. Gram stain

It is a differential staining process, used to differentiate gram positive and gram negative bacteria depending on their cell wall composition. On a clean, dry glass slide, a smear of bacterial culture was made with distilled water. Then the smear was air dried and heat fixed. Primary stain crystal violet was flooded on the smear and allowed to stand for 1 minute, and then excess stain was washed off. This was followed by flooding with Gram's iodine solution and was kept for 1 minute (This acts as a mordant that fixes the crystal violet-iodine complex). After 1 minute Gram's iodine was washed off and slide was decolourized with 70% alcohol for less than 15 seconds. Then counter stain saffranin was added, kept for 1 minute and the excess stain was washed off. The slide was allowed to air dry and viewed under oil immersion microscope (100X, Olympus)

b. String test

This test is used to differentiate gram positive and gram negative bacteria. On a clean glass slide a loopful of 18 hours old bacterial culture was mixed with a drop of 3% KOH solution with a sterile toothpick. If the mixture became viscous within 15 seconds with the formation of a string (KOH positive), culture was considered as gram negative (Halebian *et al.*, 1981).

ii. Oxidase test

Small amount of bacterial culture was placed on a filter paper moistened with oxidase reagent N, N, N' N'-tetramethyl-p-phenylene diamine dihydrochloride. If a purple colour due to formation of indophenol develops within 10 seconds, the isolate was considered as oxidase positive. This test demonstrated the presence of cytochrome oxidase in bacterial cell.

iii. Catalase test

This test was determined by the addition of a drop of 3% hydrogen peroxide solution on clean glass slide, to a loopful of bacterial culture was suspended. The evolution of gas bubbles demonstrated the production of catalase or peroxidase enzyme.

iv. Motility test

Bacterial cultures were stabbed onto the mannitol motility agar medium in a test tube and incubated for 24 hours. Motile bacteria showed a spreading growth from the stabbed line.

3.5.1.2. Molecular Characterization

i. Deoxyribonucleic Acid (DNA) Isolation

Genomic DNA was extracted from the 105 bacterial cultures using standard protocol (Ausbel *et al.*, 2003). The steps followed were:

1. Bacterial strain was grown in 5 ml Luria Bertani (LB) broth at optimum conditions.
2. About 1.5 ml of the culture was spinned in a microcentrifuge for 2 minutes until a compact pellet was formed. The supernatant was discarded.
3. The pellet was resuspended in 567 µl Tris EDTA (TE) buffer and 30 µl of 10% sodium dodecyl sulfate (SDS) and 3 µl of 20 mg/ml proteinase K were added, mixed thoroughly and incubated for 1 hour at 37°C. This was done to lyse the bacterial cell walls.
4. About 100 µl of 5 M NaCl was added and mixed thoroughly. Salt concentration was not allowed to drop below 0.5 M at room temperature as a CTAB–nucleic acid precipitate would form on the addition of CTAB in the subsequent step. The aim here was to remove cell wall debris, denatured protein, and polysaccharides complexed to CTAB, while retaining the nucleic acids in solution.
5. CTAB/NaCl solution (80 µl) was added, mixed thoroughly and incubated for 10 minutes at 65°C.
6. An approximately equal volume (0.7 to 0.8 ml) of chloroform/isoamyl alcohol (24:1) was added to the above, mixed thoroughly, and spinned for 4 to 5 minutes in a microcentrifuge. A white interface was visible after centrifugation.
7. The aqueous phase was collected in a fresh microcentrifuge tube, leaving the interface behind.
8. To the above, isopropanol (0.6 ml) was added to precipitate the nucleic acids (salt was not added since the NaCl concentration was already high). The tube was shaken back and forth until a stringy white DNA precipitate became clearly visible. The precipitate was pelleted by spinning briefly at room temperature.
9. The DNA was washed with 70% ethanol for the removal of residual CTAB and re-spinned for 5 minutes at room temperature. The

supernatant was removed carefully and the pellet was briefly dried in a lyophilizer.

10. The pellet was re-dissolved in 50 µl TE buffer.

ii. Agarose gel electrophoresis

To 100 ml of electrophoresis buffer (1X Tris Acetate EDTA buffer (TAE)) 0.8 g of electrophoresis grade agarose was added. The agarose was melted in a microwave oven (IFB, USA) and was swirled in between to ensure even mixing. The molten agarose was allowed to cool down to 50-60°C and poured into a gel tray. The gel was allowed to solidify for 20-30 minutes at room temperature. After removing the comb, gel casting tray was placed in the electrophoresis tank and sufficient 1X TAE buffer was added to completely immerse the gel. DNA samples were mixed with 6X loading dye in the ratio 1:5 and 5µl loaded into the gel wells with a micropipette at the negative electrode. 3 µl of *Hae* III digest λ DNA ladder DNA molecular weight marker was loaded as reference. The leads were connected and voltage applied so that DNA could migrate toward the anode. The gel was electrophoresed at 1-10 volt/cm of gel length until the dye front had migrated to about three-fourth of the gel. The DNA fragments were visualized using the gel documentation system Gel Logic 1500 (Eastman Kodak Company, USA) and the image was captured with cooled CCD camera.

iii. Spectrophotometric estimation of DNA

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

iv. Amplification and Purification of 16S rRNA genes

The purified DNA was subjected to Polymerase Chain Reaction (PCR) for the amplification of 16S rRNA genes using the primer pairs: 27F- AGA GTT TGA TCC TGG CTC AG and 1492R- TAC GGY TAC CTT GTT ACG ACTT (Lane, 1991) in a thermal cycler (Eppendorf, Germany). Amplification was carried out in a 50 µl reaction mixture containing 50 ng of purified DNA, 3 mM concentration of $MgCl_2$, 2.5 mM of each deoxynucleotide triphosphate (dNTP), 10 pM of each primer and 3 U/µl of taq polymerase in 1X taq buffer (Bangalore Genei, India). The PCR conditions for the first set of primers were: initial denaturation at 94°C for 5 minutes, 30 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 90 seconds and final extension at 72°C for 5 minutes. Two reactions, a positive control with DNA template known to be successfully amplified and a negative control lacking template DNA was set up in each PCR run. Agarose gel electrophoresis was carried out as in Section 3.5.1.2.-ii in 1.5% gel and using 500bp DNA ladder as reference.

v. Amplified Ribosomal DNA restriction analysis (ARDRA)

Amplified Ribosomal DNA restriction analysis (ARDRA) was performed to group the isolates into different phylotypes. The 16S rRNA gene was digested using four base-cutting restriction enzyme, *Hae* III (1U) at 37°C for one hour. The restricted products were electrophoresed on a 1.2% agarose gel and viewed on Gel Imaging System (Kodak Gel Logic). The patterns in the gels were compared using Bionumerics Software Version 4.6 (Applied Maths, Belgium). Representative phylotypes were sequenced (Bioserve Biotechnologies Pvt. Ltd., Hyderabad).

vi. Sequencing of PCR products

PCR amplification was carried out in a 50 µl reaction mixture containing 50 ng of purified DNA, 3 mM concentration of $MgCl_2$, 2.5 mM of each dNTP, 10 pM of each primer and 3 U/µl of taq polymerase in 1X of taq

buffer (Bangalore Genei, India). The PCR conditions used were: initial denaturation at 95°C for 5 minutes, 30 cycles consisting of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 1 minute and final extension at 72°C for 5 minutes. Two reactions, a positive control with DNA template known to be successfully amplified and a negative control lacking template DNA was set up in each PCR run. Sequencing of the PCR products were carried out by a commercial company (Bioserve Biotechnologies Pvt. Ltd., Hyderabad).

vii. Sequence editing, BLAST analysis and GenBank submission

The sequences obtained were edited using DNA Baser and compared with sequences in GenBank using Basic Local Alignment Search Tool (BLAST) (www.ncbi.nih.nlm.gov). The partial 16S rRNA gene sequences of the bacterial cultures obtained were deposited in the GenBank database.

3.5.2. Cultivation-Independent Method

3.5.2.1. DNA Extraction from Sediments

Various DNA extraction protocols under both cell extraction (Steffan *et al.,* 1988) and direct lysis (Purohit *et al.,* 2003 ; Gray and Herwig, 1996; Lovell and Piceno 1994; Yeates *et al.,* 1997; Zhou *et al.,* 1996) methods were tried in order to standardize the best method for humic acid containing, organic rich OMZ sediments. All the methods were carried out with a positive control (MTCC-434, *Stenotrophomonas maltophila*) and a negative control (Reagent blank). Higher DNA yield was only obtained from the Zhou *et al.,* (1996) method. Also none of the methods gave PCR-amplifiable DNA due to the presence of PCR inhibitors such as humic acids. The details of all the methods tried are given in the Appendix-A5.

3.5.2.2. Modified Zhou Method

As DNA was obtained using Zhou *et al.,* (1996) method, further modification steps were attempted for removing the contaminating humic acids, which would get co-extracted with DNA and resulting in the inhibition

of PCR. The main aim was to extract PCR amplifiable DNA with a single protocol.

Additional steps adopted in the modified protocol were the initial wash steps, lysis and incubation, protein removal, DNA precipitation and purification. It was hypothesized that washing the sediment prior to lysis helps in removing the potential PCR inhibiting materials. Also chemicals like PEG and potassium acetate were used for the efficient removal of humic acids.

The modified protocol carried out was as follows:

1. Sediment samples (200 mg) were taken in fresh microcentrifuge tubes and were subjected to four washing steps as described below.
2. First two washes were made with 850 µl of wash buffer, 100 µl of 0.5 M NaOH, 50 µl of guanidine thiocyanate (GTC) and 100% Triton-X, 50 µl of NaCl. Vortexed the mixture thoroughly and spinned at 2000 rpm for 2 minutes. Discarded the supernatant.
3. In the third wash 850 µl of wash buffer, 50 µl of guanidine thiocyanate (GTC), 50 µl of 100%Triton-X, and 50 µl 10% CTAB/NaCl were added to the pellet. Vortexed the mixture thoroughly and spinned at 2000 rpm for 2 minutes and the supernatant was discarded.
4. The final wash done using 1000 µl extraction buffer.
5. In the lysis step 500 µl of extraction buffer, 50 µl of 10% CTAB, 5 M Guanidine thiocyanate (GTC) and 10 µl of proteinase K were added to the pellet. Mixed by vortexing.
6. The mixture was incubated at 55°C for 2 hours.
7. 50 µl of 20% SDS was added after incubation, and kept for further incubation at 65°C for 1 hour.
8. Tubes were centrifuged at 10000 rpm for 10 minutes, and supernatants recovered.

9. To the supernatant 75 µl of 5 M potassium acetate and 100 µl of 30% polyethylene glycol in 1.6 M NaCl were added and kept for incubation at -20°C for 1 hour. This was followed by centrifugation at 13000 rpm for 20 minutes and pellets were taken.
10. Pellets were dissolved in 100 µl 2% CTAB, 140 µl of 1.4 M NaCl , 260 µl 10 mM Tris and 0.1 M EDTA (wash buffer) and incubated at 68°C for 15 minutes.
11. To the suspension chloroform-isoamyl alchohol (24:1) was added, mixed by inversion for 15 minutes and spinned at 10000 rpm for 15 minutes.
12. Aqueous layer was taken in a fresh tube and added 0.6 volumes of isopropanol followed by 12 hours incubation. Spinned at 13,000 rpm for 15 minutes.
13. The pellet was washed with 70% ethanol and the supernatant was removed after centrifugation at 10000 rpm for 10 minutes.
14. The pellet was air dried and resuspended in 30 µl of 10 mM TE buffer (pH 8).

3.5.2.3. Spectrophotometric Estimation Of DNA

DNA was quantified by measuring absorbance at 260 nm as mentioned in Section- 3.5.1.2-iii.

3.5.2.4. Electrophoretic Analysis of DNA

DNA was electrophoresed on agarose gel as given in Section-3.5.1.2-ii.

3.5.2.5. Amplification and Purification of 16S rRNA Genes

The purified DNA was subjected to PCR amplification and was carried out in a 50 µl reaction mixture containing 50 ng of purified DNA (ie. 5 µl from a 1:5 diluted genomic DNA) and the concentration of the reagents was as in Section 3.5.1.2-iv. A nested PCR approach was used prior to DGGE to

increase the sensitivity of amplification. The oligonucleotides used in the study are summarized in the Table 2.

S.I No.	Primers	Sequence	Annealing Temperature	Reference
1	27F	F:AGAGTTTGTCCTGGCTCA	55°C	Amann *et al.*, 1995
	1090R	R:GCTCGTTGCGGGACTTAACC		
2	27F	F:AGAGTTTGATCCTGGCTCAG	60°C	Lane *et al.*, 1991
	1492R	R:TACGGYTACCTTGTTACGACTT		
3	341F*	F:CGCCCGCCGCGCGCGGCGGGC GGGGCGGGGGCACGGGGGGCC TACGGGAGGCAGCAG	55°C	Muyzer *et al.*, 1993
	907R	R:ATTACCGCGGCTGCTGG		
4	M13F	F:GTAAAACGACGGCCAG	55°C	Messing *et al.*, 1993
	M13R	R:CAGGAAACAGCTATGAC		

*These primers were used in a 'touchdown' protocol from 65°C to 55°C.

Table 2. Oligonucleotides used in the diversity analysis of 16S rRNA gene.

The PCR products were purified using Qiagen kit (Qiagen, USA) following manufacturer's instructions.

3.5.2.6. DGGE analysis

Sediment DNA was extracted, purified, estimated, electrophoresed and amplified as given in Sections 3.5.2.1-3.5.2.5. and DGGE analysis was carried out by method described by Muyzer *et al.,* (1993).

i. Preparation of acrylamide gel

a. Perpendicular gel

A perpendicular gel was casted and run for the unknown samples to define the range of denaturant concentration that allows the best separation possible. Perpendicular gel of 1 mm thickness was prepared with 10% polyacrylamide gel (ratio of 40% acrylamide/bisacrylamide 37.5:1), using gel sandwich sizes of 16x16 cm. A denaturant gradient was made ranging from 0% to 100% (100% denaturant is a mixture of 7 M urea and 40% (vol/vol) formamide). The perpendicular gel was casted as given in the manufacturer's instructions. The gel was allowed to set for one hour at room

temperature. The electrophoresis tank filled with 7 L of 1X TAE buffer was preheated at a temperature of 60°C. The samples were loaded (1-3 µg) after mixing with equal volume of 2X loading dye. Electrophoresis was carried out at a temperature of 60°C and voltage of 100 V for 17 hours. (Gel picture in Appendix section-A10).

b. Preparation of Parallel gel

Optimization of runtime in parallel gel

A parallel gel was prepared as above with slight differences as given in the Biorad Dcode instruction manual. In order to optimize the run time, a 'time travel' experiment was carried out with samples loaded at hourly intervals and electrophoresed at 100 V at a buffer temperature of 60°C. (Gel picture in Appendix section-A10).

Sample analysis

A denaturant gradient ranging from 20-80% were made for 16S rRNA genes as determined from perpendicular gel electrophoresis. The electrophoresis was carried out at buffer temperature of 60°C at 100 V for 17 hours as optimized by the time travel experiment (Gel pictures in Appendix section-A10).

ii. Viewing the gel

After electrophoresis run was completed, the core sandwich assembly was removed from the buffer tank. The gel sandwiches were carefully detached and the gel removed and stained with 0.01% SybrGold (Tuma, 1999) in 1X TAE buffer for 15 minutes. The gel was viewed in a gel documentation system (Kodak) and photographed.

iii. Excision of bands and re-amplification

The documentation of the DGGE gel was followed by printing of the gel picture and careful marking and labeling of bands. The bands were excised with a sterile surgical blade. Exposure of the bands to UV radiation

was kept at a minimum in order to minimize the damage to the DNA in the gel. The gel was transferred to the labeled tube with 10 µl of deionized water. The tubes were incubated at 4°C for 12 hours followed by a spin for about 15 seconds. The supernatant was aspirated. About 0.5 µl of the supernatant was used as a template for reamplification using the primers 341F and 907R (Section 3.5.2.5.). The PCR products were run on 1.5% agarose gel. Further they were electrophoresed on a parallel gel along with the environmental sample in order to make sure of its position and distinction. The eluted products were also amplified using PCR primer without GC clamp. The resulting PCR products were run on 1.5% agarose gel for assessment of quality and quantity.

iv. Cloning of the PCR product

The cloning reaction was performed using TOPO TA Cloning kit (Invitrogen, USA). To 3 µl of fresh PCR product, 1 µl salt solution and 1 µl TOPO vector was added and made up to a final volume of 6 µl with the water provided in the kit. The above mixture was incubated at 22-23°C. TOPO cloning reaction mixture (2 µl) was added to a vial of chemically competent *Escherichia coli* that was previously thawed on ice and mixed gently followed by incubation on ice for 5-30 minutes.

The cells were given heat shock at 42°C for 30 seconds without shaking and immediately transferred to ice. Thawed SOC (Super Optimal Broth with catabolite repression) medium of 250 µl was added to the tubes. The tubes were capped tightly and shaken horizontally (200 rpm) at 37°C for one hour. After shaking, 10-50 µl of the transformation mixture was spread on prewarmed selective plates (LB medium amended with ampicillin) and incubated at 37°C. The colonies were counted and picked for further analysis after 8-12 hours. The bacterial colonies were streaked for single colony isolation on Luria Bertani (LB) plates containing 100 µg/ml ampicillin and incubated at 37°C.

Well-isolated colonies were inoculated into 1-2 ml of LB broth containing 100 µg/ml ampicillin and incubated overnight with shaking at 37°C

until the culture was saturated. The culture (0.85 ml) was mixed with 0.15 ml of sterile glycerol and transferred to a cryovial and stored at -80°C for long term storage.

v. Screening of clones

A few clones were selected at random and their plasmid DNA was extracted using Qiagen Miniprep Kit (Qiagen, USA), following manufacturer's instructions. The plasmids were subjected to PCR amplification using primers, M13F and M13R for screening the positive clones (clones with inserts), the characteristics of which are given in Section 3.5.2.5. The plasmids of the five positive clones were further processed for sequencing.

vi. Sequencing of PCR products

Amplification of plasmid DNA was carried out in a 50 µl reaction mixture containing 50 ng of purified DNA, 3 mM concentration of $MgCl_2$, 2.5 mM of each dNTP, 10 pM of each primer (M13F and M13R-details given in Section 3.5.2.5.) and 3 U/µl of taq polymerase in 1X of taq buffer (Bangalore Genei, India). The PCR conditions used were: initial denaturation at 95°C for 5 minutes, 30 cycles consisting of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 1 minute and final extension at 72°C for 5 min. Two reactions, a positive control with DNA template known to be successfully amplified and a negative control lacking template DNA was set up in each PCR run. Sequencing of the gene was carried out by Bioserve Biotechnologies Pvt. Ltd., Hyderabad.

3.5.2.7. 16S rRNA clone library analysis

Total genomic DNA from sediments was extracted, purified, estimated, electrophoresed and 16S rDNA was amplified as given in Sections 3.5.2.1-3.5.2.5.16S rRNA clone library analysis was carried out as below.

i. Cloning of PCR product

The PCR product was cloned using TA cloning kit as given in section (3.5.2.6-iv).

ii. Screening of clones

The plasmid DNA of the clones was extracted using Qiagen Miniprep Kit (Qiagen, USA), following manufacturer's instructions. The plasmids were subjected to PCR amplification using primers M13F and M13R, the characteristics of which are given in Section 3.5.2.5. The positive clones were further processed for sequencing.

iii. Sequencing of PCR products

Amplification of plasmid DNA was carried out as in Section 3.5.2.7-iii.

3.5.2.8. Sequence editing, BLAST analysis and GenBank Submission

The sequences obtained after DGGE and clone library analyses were edited using DNA Baser and compared with sequences in GenBank using Basic Local Alignment Search Tool (BLAST). The partial 16S rRNA gene environmental clone sequences obtained from this study were deposited in the GenBank.

3.5.2.9. Phylogenetic analysis

The 22 sequences obtained from DGGE analysis and 115 sequences obtained from the clone libray were assembled using DNA baser sequence assembly software Version 2 (Heracle Software, Germany) and analyzed for putative chimeric sequences using Chimera_Check software of Ribosomal Database Project (Cole *et al.,* 2003) and Pintail Software (Ashelford *et al.,* 2005). DGGE sequences were not chimeric. However, twelve sequences in the clone library were found to be chimeric and hence excluded from further analysis. Thus, 22 DGGE sequences and 103 clone library sequences were aligned using multiple sequence alignment tools Clustal X version 1.83

(Thompson *et al.*, 1997). A PHYLIP (Phylogeny Inference Package) tree was generated by neighbor-joining method and edited using Treeview 1.6.6 (Page, 1996). Bootstrap analysis was carried out using 1000 iterations.

3.6. FUNCTIONAL DIVERSITY

3.6.1. Cultivation-Dependant Method

The aerobic culturable isolates were grown on various substrates to study their enzymatic activity (Gerhardt *et al.*, 1981).

i. Amylase test

This test is used to determine the ability of bacteria to produce amylase enzyme that can degrade starch supplied in the media to glucose. Medium was prepared by amending nutrient agar with 0.2% soluble starch. The medium was sterilized at 115°C for 10 minutes. A single streak of the test culture was made across the center of the plate. After incubation, the plate was flooded with iodine solution and observed for the clear zone around the colony.

ii. Gelatinase test

This test determines the ability of bacteria to use animal protein, gelatin as a source of energy. Agar medium supplemented with 0.4% gelatin, was prepared, sterilized and poured into plates. A single streak of culture was made across the center of plate and incubated. After incubation the plates were flooded with gelatin precipitating reagent and observed for clear zones around the colonies.

iii. Lipase test

This is to determine the ability of bacteria to produce lipase enzyme that degrade lipids. Agar medium was supplemented with 0.01% Calcium Chloride. Tween 20 and Tween 80 were sterilized by autoclaving at 121°C for 20 minutes. Sterilized Tween was added to the molten agar medium at 45-50°C to give a final concentration of 1% (vol/vol). The medium was

shaken well until the Tween was completely mixed and was dispensed into petri plates, allowed to solidify, and inoculated the culture by streaking at the centre of plate. After the incubation for 24 hours, the plates were observed for the formation of an opaque halo containing crystals of calcium soaps around the colonies.

iv. DNase test

This method is used to test the ability of bacteria to produce enzyme DNase that can degrade DNA. DNA containing test medium was prepared and added 100 mgL^{-1} of toluidine blue to it prior to autoclaving. A single streak of culture was made across the plate and incubated. Bluish colouration of the colonies indicated a positive result.

v. Phosphatase test

Phenolphthalein phosphate agar containing sodium phenolphthalein phosphate (0.01%) was prepared, sterilized at 121°C for 15 minutes. Streaked plates were incubated for 2-5 days. After incubation the colonies were subjected to ammonia fumes. Positive test gave pink colonies within 3 seconds, as a result of the presence of free phenolphthalein.

vi. Urease test

Christensen's urease agar medium was prepared and sterilized at 115°C for 20 minutes. Filter sterilized urea (40%) was added to the sterilized media and mixed well. The plates were streaked in the center with the bacterial culture and incubated for 2-3 days. The plates were observed for positive result that will give a pink colour on medium.

vii. Indole production test

Indole production test is used to determine the ability of bacteria to produce tryptophanase enzyme that can degrade tryptophan supplied in the medium to indole. Indole is extracted from the medium in to the reagent layer by the acidified butanol component and forms a cherry-red complex with

para dimethyl amino benzaldehyde. Sterilized peptone broth was inoculated with a loopful of bacterial culture and incubated for 24 hours at room temperature (22-23°C). After incubation about 0.2 ml of Kovac's indole reagent was added through the sides of the test tube and color developed was observed within 1-2 minutes.

viii. Citrate utilization test

This test is used to determine the ability of the bacteria to utilize citrate as a sole source of carbon. Citrate supplied in the medium is acted upon by the enzyme citrase, which produces oxaloacetate and acetic acid that are enzymatically converted to pyruvic acid and carbon dioxide. Carbon dioxide reacts with sodium and water to form sodium carbonate that makes an alkaline pH which changes the colour of bromothymol blue indicator from green to Prussian blue. Simmons's citrate agar slants were streaked with a loopful of bacterial culture and incubated for 24-48 hours. After incubation, colour of the slant was observed.

ix. Nitrate reduction test

This test determines the ability of facultative, aerobic and anaerobic bacteria to reduce nitrate to nitrite in the absence of molecular oxygen. Sterile nitrate broth (5 ml) was inoculated with the culture and checked for turbidity after 24 hours of incubation. After incubation, nitrate reduction was tested by adding two nitrate reagents. Reagent 1 (sulphanilic acid) and Reagent 2 (alpha–naphthylamine), were added in equal proportion and mixed well. A cherry red colouration of the broth immediately after adding reagents indicated a positive result.

x. Arginine dihydrolase

Sterile Mollers Arginine dihydrolase broth (5 ml) was inoculated with a loopful of test culture. After inoculation the broth was over laid with 10 mm of sterile mineral oil and incubated for 4 days. A positive reaction gave purple-violet colour.

xi. Lysine decarboxylase

Sterile Mollers lysine decarboxylase broth (5 ml) was inoculated with a loopful of bacterial culture and overlaid with 10 ml sterile mineral oil and incubated for 4 days. A positive test gave purple-violet colour.

xii. Ornithine decarboxylase

Sterile Mollers ornithine decarboxylase broth (5 ml) was inoculated with a loopful of bacterial culture and overlaid with 10 mm sterile mineral oil and incubated for 4 days. A positive reaction gave purple-violet colour.

xiii. Carbohydrate fermentation tests

The following tests were done in order to study the utilization of sugars.

a. Triple Sugar Iron (TSI) test

This is used to test the ability of bacteria to ferment glucose, lactose, sucrose and produce hydrogen sulphide. TSI slant contained 1% lactose and sucrose and 0.1% glucose. Phenol red, the acid-base indicator was incorporated in the medium to detect carbohydrate fermentation. Bacterial culture was inoculated by stabbing the center of butt and streaking the slope. The tubes were incubated for 24 hours at 22-23°C. The colour of both slant and butt were observed.

b. Marine oxidation fermentation

Hugh and Leifson medium was prepared and dispersed upto $3/4^{th}$ volume in tubes and sterilized. After sterilization tubes were plunged in cool water for few minutes to remove most of the dissolved oxygen. The tubes were inoculated by stabbing and incubated for 24-48 hours. After incubation, tubes were observed for change in colour. If top of the tube was yellow, it was indicative of oxidative metabolism and if bottom of the tube was yellow, it was indicative of fermentative metabolism. If the colour of the medium changes to blue, it was indicative of alkaline reaction.

c. Voges-Proskauer test

This test is used to determine the capacity of microorganism to ferment carbohydrate with the production of non-acidic or neutral end products such as 2, 3 -butylene glycol. 5 ml of sterile Methyl Red Voges-Proskauer (MRVP) broth was inoculated with a loopful of bacterial culture and incubated for 48 hours at room temperature (22-23°C). After incubation, 0.6 ml of 5% solution of alpha – naphthol in ethanol and 0.2 ml of 40% KOH were added and vigorously shaken and observed for color change. The red colour indicated positive reaction.

d. Methyl red test

This test demonstrates production of acid during fermentation of glucose. 5 ml of MRVP broth was inoculated with loopful bacteria and incubated for 24-48 hours at room temperature. After incubation, 2-4 drops of 0.04% methyl red indicator solution was added to the broth and thoroughly mixed. Appearance of red coloration of the broth was the positive reaction.

3.6.2. Cultivation-independant method

The DNA was extracted, purified, estimated and electrophoresed as given in sections 3.5.2.1-3.5.2.5. The diversity of the functional gene dissimilatory sulphite reductase B (*dsr*B) was estimated using clone library and Denaturing Gradient Gel Electrophoresis (DGGE).

3.6.2.1. Diversity Analysis

The purified DNA was subjected to PCR for the amplification of dissimilatory sulphite reductase AB (*dsr*AB) gene. This PCR product was used as template for the amplification of *dsr*B gene. The oligonucleotides used are summarized in Table 3.

Sl. No.	Primers	Sequence	Annealing Temperature	Reference
1	DSR 1F	F:ACGCACTGGAAGCACG	60°C	Wagner *et al.*, 1998
	DSR 4R	R:GTGTAGCAGTTACCGCA		
2	DSR*p2060F	F:CGCCCGCCGCGCGCGGC GGGCGGGGCGGGGGCAC GGGGGGCCCAACATCGTC CACACACCCAGGG	55°C	Wagner *et al.*, 1998
	DSR 4R	R:GTGTAGCAGTTACCGCA		
3	M13F	F:GTAAAACGACGGCCAG	55°C	Messing *et al.*, 1993
	M13R	R:CAGGAAACAGCTATGAC		

*These primers were used in a 'touchdown' protocol from 65°C to 55°C.

Table 3. Oligonucleotides used in the diversity anlysis of *dsr* B gene.

The diversity analysis was carried out using clone library and DGGE as given in Sections 3.5.2.7.-3.5.2.9.

3.7. STATISTICAL ANALYSIS

3.7.1 Environmental parameters

Hierarchical clustering and Principal Component Analysis (PCA) was carried out using Primer 6 software.

3.7.2. Abundance

Principal Component Analysis (PCA) was carried out using Primer 6 software. In order to link abundance to environmental parameters Canonical Corresponding Analysis (CCA) was carried out using MVSP 3.13 statistical package (Kovach, 1998).

3.7.3. Diversity

i. DGGE Analysis

Analysis of the fingerprint data was performed using the Bionumerics software Version 4.6 (Applied Maths, Belgium) (Rademaker *et al.*, 1999). The digitized gel images were converted, normalized and analyzed. The DGGE bands were detected and transformed into a presence/absence

binary matrix. The binary matrix was converted into a similarity matrix by Bray-Curtis measure, using Primer 6 software (Clarke et al., 2005). Hierarchical cluster analysis of the DGGE profiles was done by the group average method. Canonical correspondence analysis (CCA) were performed with DGGE profiles and environmental variables in order to evaluate the relation of bacterial community to environmental parameters using MVSP 3.13 (Kovach, 1998).

ii. Clone library Analysis

DOTUR (Distance-based OTU and Richness) software was used to assign sequences to operational taxonomic units, perform rarefaction analysis and calculation of diversity indices (Schloss and Handelsman, 2004). Sequences with distance values of 0.03 ($\geq$ 97% sequence similarity) corresponding to the species level were grouped into a single Operational Taxonomic Unit (OTU) (Stackebrandt and Goebel, 1994). Rarefaction curves with 95% confidence intervals were constructed by plotting the number of OTUs observed against the number of sequences sampled in each of the clone libraries that were being compared. The diversity indices included the non-parametric richness estimators such as Chao1 richness estimate, the abundance-based richness estimator (ACE), the Simpson diversity index and Shannon-Wiener diversity index. The coverage (C) of the various clone libraries under comparison was calculated using the equation: C=1- (n/N), where n is the number of different unique OTU types from a clone library and N is the total number of clones sequenced (Good, 1953).

LIBSHUFF analysis was performed in order to compare the homologous $C_X(D)$ and heterologous $C_{XY}(D)$ coverage of the ASOMZ sediment 16S rRNA clone library with sequences from ASOMZ water and sediments of North Sea, South China Sea and Gulf of Mexico. The software used was weblibshuff (http://libshuff.mib.uga.edu) (Singleton *et al.*, 2001). This was carried out to estimate the similarity between clone libraries from the five different samples based on the evolutionary distance (D) of all sequences. The homologous coverage of the clone library X was calculated

using the equation C_X =1- (N_X/n), where N_X is the number of unique sequences in the sample and n is the total number of sequences. Similarly, the heterologous coverage of clone library X by a second clone library Y is defined as C_{XY} =1- (N_{XY}/n), where N_{XY} is the number of sequences in the clone library X not found in the second clone library Y and n is the number of sequences in X. If the clone libraries are similar, then the coverage curves C_X(D) and C_{XY}(D) are also expected to be similar. For DOTUR analysis and web LIBSHUFF comparison all the sequences were aligned (multiple sequence alignment) using Clustal X in PHYLIP format, followed by the generation of distance matrices in the DNADIST program PHYLIP (version 3.68) which were further used as the input files in the respective programs.

CHAPTER 4

RESULTS

4.1 HYDROGRAPHICAL PARAMETERS

4.1.1. Physical Parameters

Profiles of temperature, salinity and pH of the water column at 50, 200, 500 and 1000 m depths in the three transects are depicted in the Figures 1-3. High temperature was recorded in the surface waters of the three transects (30.8, 30.9 and 30.7°C at Karwar, Goa and Ratnagiri respectively) irrespective of station depths (Fig. 1). Temperature of the water column of 50 m stations of the 3 transects ranged between 30.8 and 28.7°C i.e. decreased with increase in depth at all stations. Minimum variation in temperature was observed at 50 m depth station. Decrease in temperature was also observed for the rest of the station depths. The temperature of the overlying water at 200, 500 and 1000 m depth stations of the three transects was below 16°C. At 1000 m station depth, it ranged from 9.0 to 9.6°C. There was no significant difference in temperature between the three transects.

Salinity of the water column was almost constant at all depths of the three transects with an average of 35.6±0.7. Salinity of the overlying water of three transects did not show any marked variation with the station depths (Fig. 2).

The pH of the surface and close to the bottom water varied between 8.4 and 8.1 at 50 m depth stations of the three transects. However, beyond 100 m depth the pH of the water column was less than 8. There was a decrease in pH of the overlying water with increase in station depths for the three transects (Fig. 3).

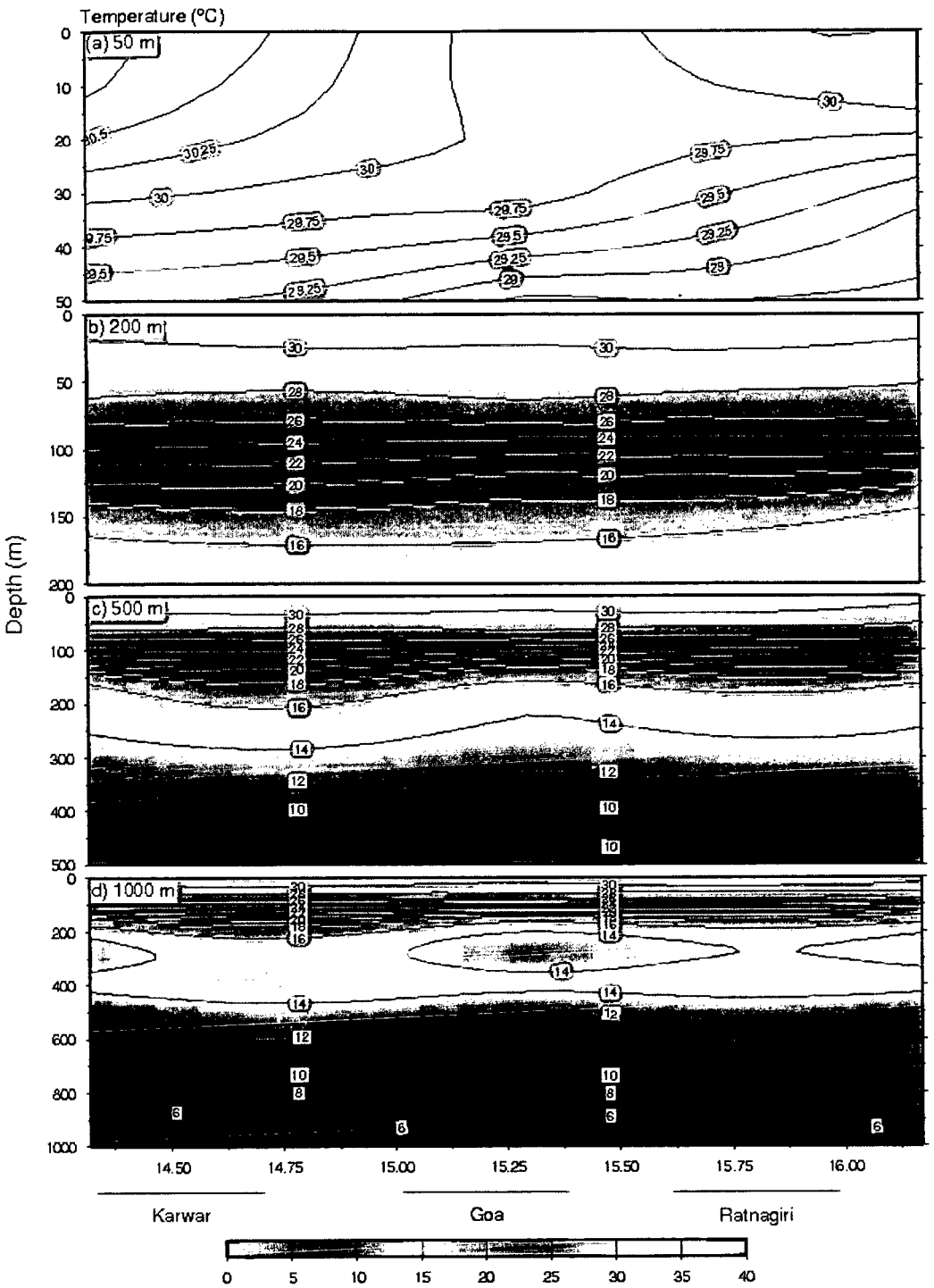


Fig.1. Distribution of temperature at Karwar, Goa and Ratnagiri.

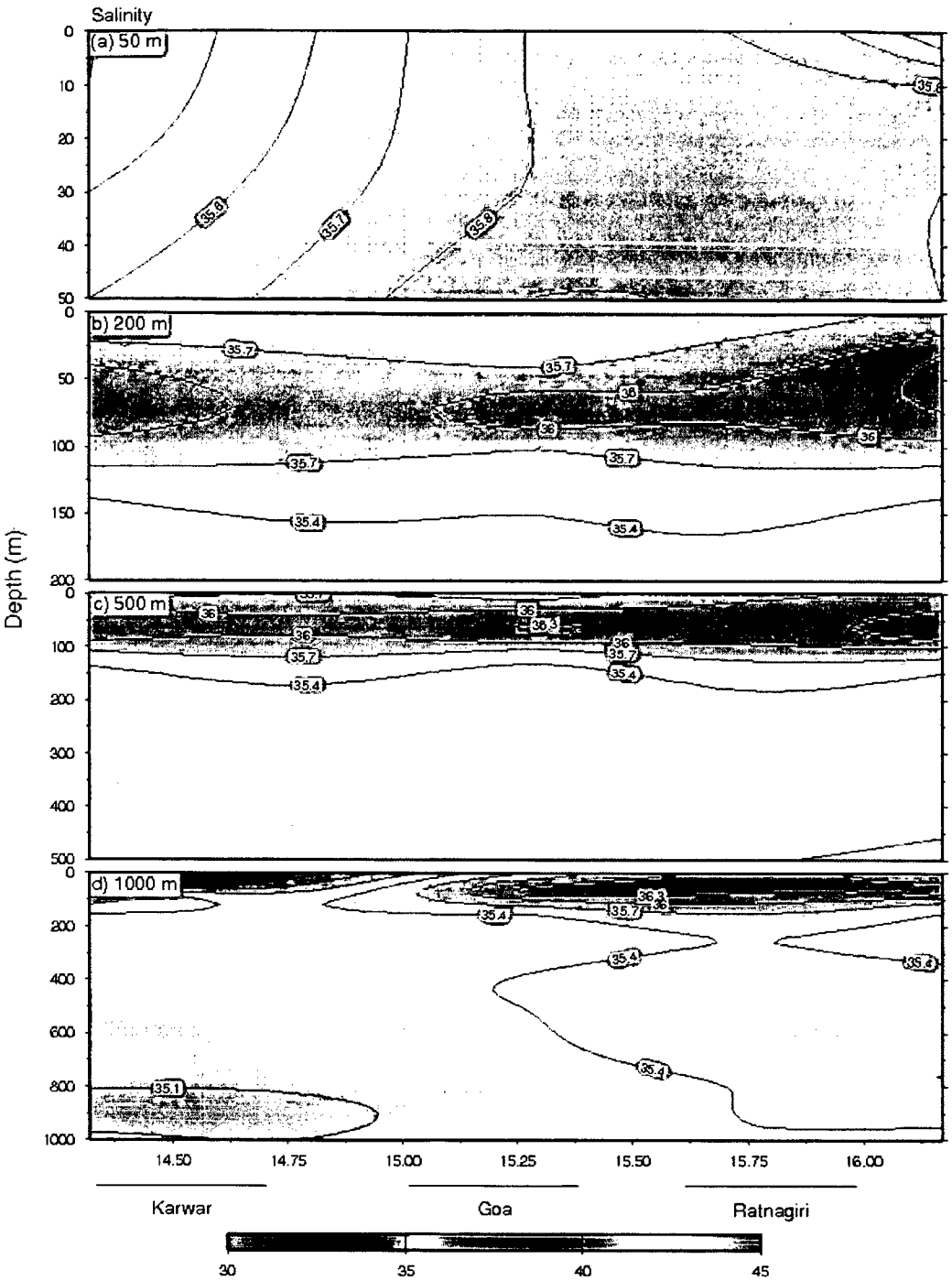


Fig.2. Distribution of salinity at Karwar, Goa and Ratnagiri.

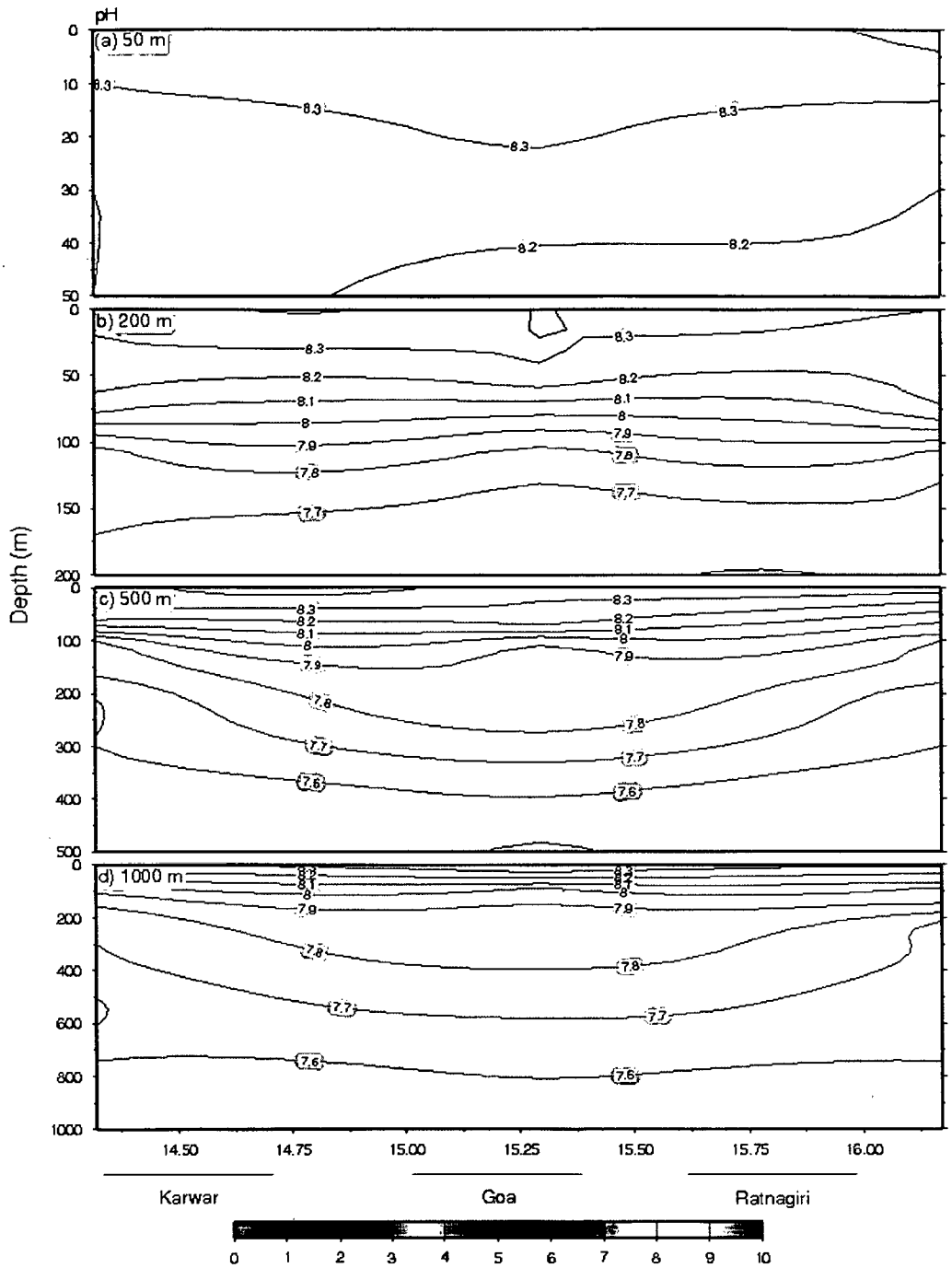


Fig.3. Distribution of pH at Karwar, Goa and Ratnagiri.

4.1.2.Chemical Parameters

4.1.2.1.Dissolved Oxygen

Dissolved oxygen (DO) concentration decreased with depth of the water column. At 50 m depth station the concentration was >3 mlL^{-1}. At the three other depth stations, there was a drop in DO concentration by ca 1 mlL^{-1} below 50 m. The overlying water of 1000 m depth stations of all transects showed an increase in oxygen concentration but was within the DO level of OMZ. OMZ showed depth variation between transects. As DO concentration was <0.5 mlL^{-1} below 100 m at Karwar and Goa transects, the OMZ of these two transect was from this depth. In the case of Ratnagiri, OMZ was beyond 150 m. This OMZ extended upto 1000 m at sediment-water interphase (Fig. 4A). The actual values are presented in Figure 4B.

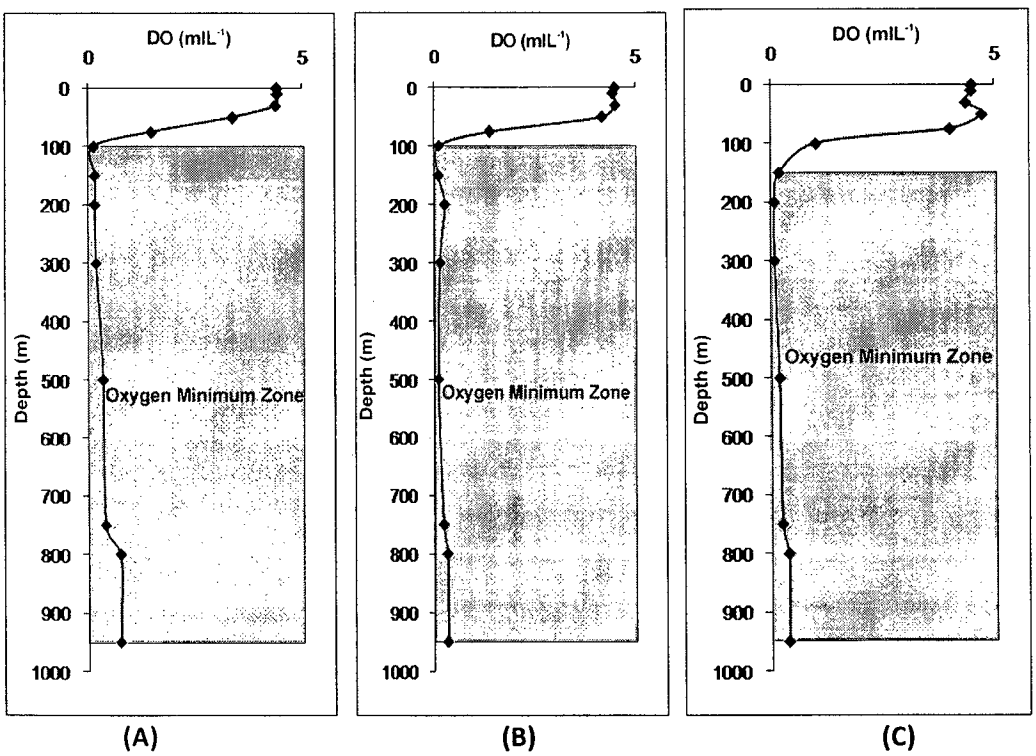


Fig. 4A. Schematic plot of DO profiles at 1000 m depth stations at three transects (A) Karwar (B) Goa (C) Ratnagiri. The shaded area depicts the OMZ.

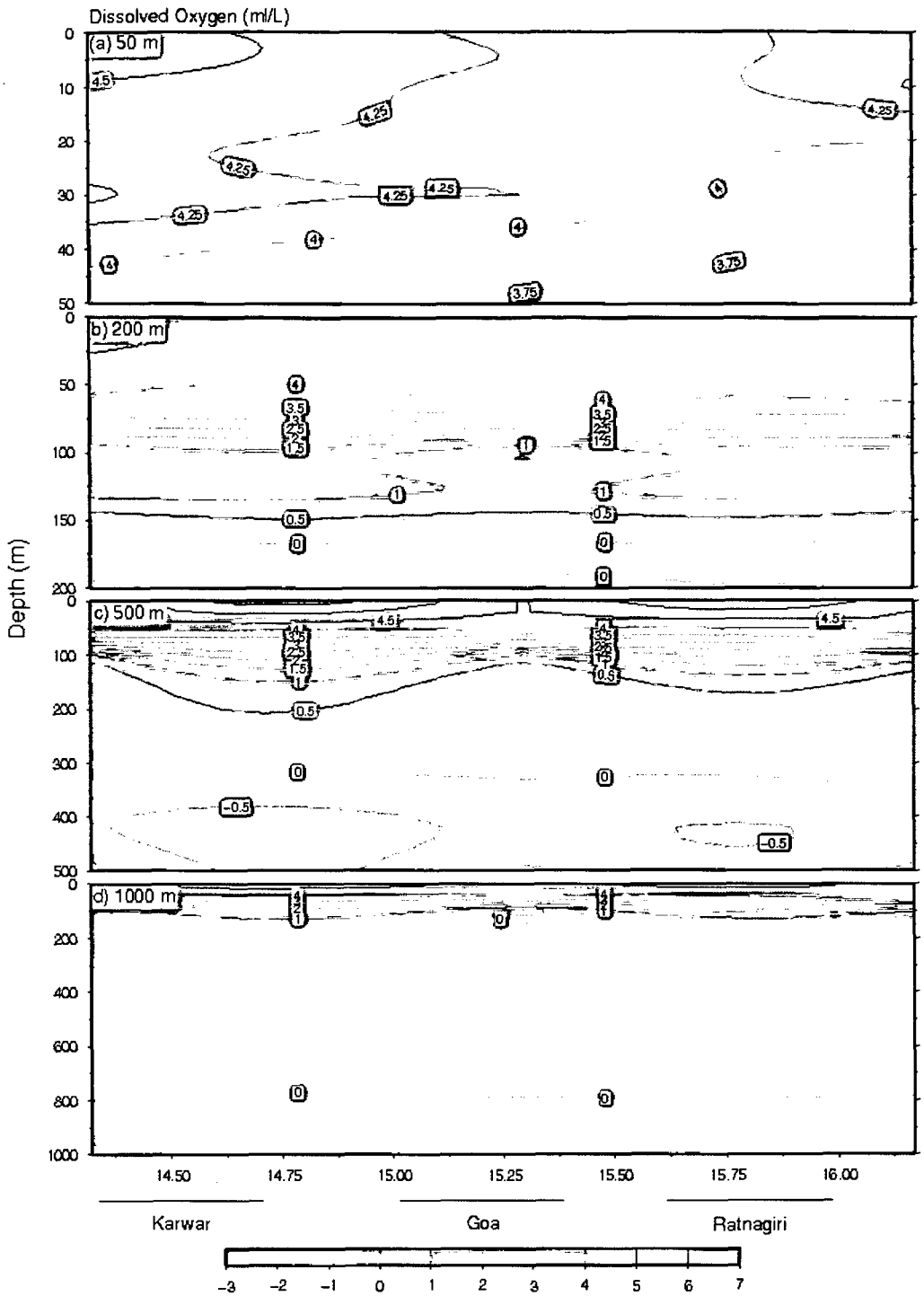


Fig.4. Distribution of dissolved oxygen at Karwar, Goa and Ratnagiri.

4.1.2.2. Nitrate

Nitrate concentration of the surface waters of the stations (50, 200, 500 and 1000 m of three transects were low and ranged from not detectable to 0.31±0.01 µM. At Karwar transect, nitrate concentrations of the surface waters were 0.05±0.01, negligible, 0.23±0.01 and 0.05±0.01 µM at 50, 200, 500 and 1000 m respectively whereas at Goa transect the concentrations were negligible, 0.17±0.01, 0.31±0.01 and 0.04±0.01 µM for 50, 200, 500 and 1000 m respectively. At Ratnagiri, the concentration of nitrate was generally low. There was no particular trend in the nitrate concentration either based on depth of the stations or transect (Fig. 5A). Nitrate concentration of the overlying waters was higher than the surface waters and ranged from 0.21±0.04 to 44±0.5 µM for all stations of the three transects. The nitrate values were negligible at 50 m station depth of all transects and ranged from 0.2±0.04 to1.36±0.01 µM, whereas for 200 m station depth the values ranged from 21.8±0.95 to 22.5±1.25 µM. For deeper stations viz. 500 and 1000 m, it was 26.8±0.6 to 28.7±0.5 µM and 34.9±0.57 to 44.4±0.5 µM respectively. At Karwar transect the concentrations of nitrate at 50 m and 1000 m were 0.21±0.04 and 34.98±0.57 µM respectively. Similar trend of increase in concentration with station depths was observed in Goa and Ratnagiri transects. Transect-wise, Goa recorded high concentration of nitrate with 1000 m depth stations recording the highest concentration of 44.4±0.5 µM. Thus the nitrate concentration of the overlying waters showed a distinct trend in its distribution (Fig. 5B).

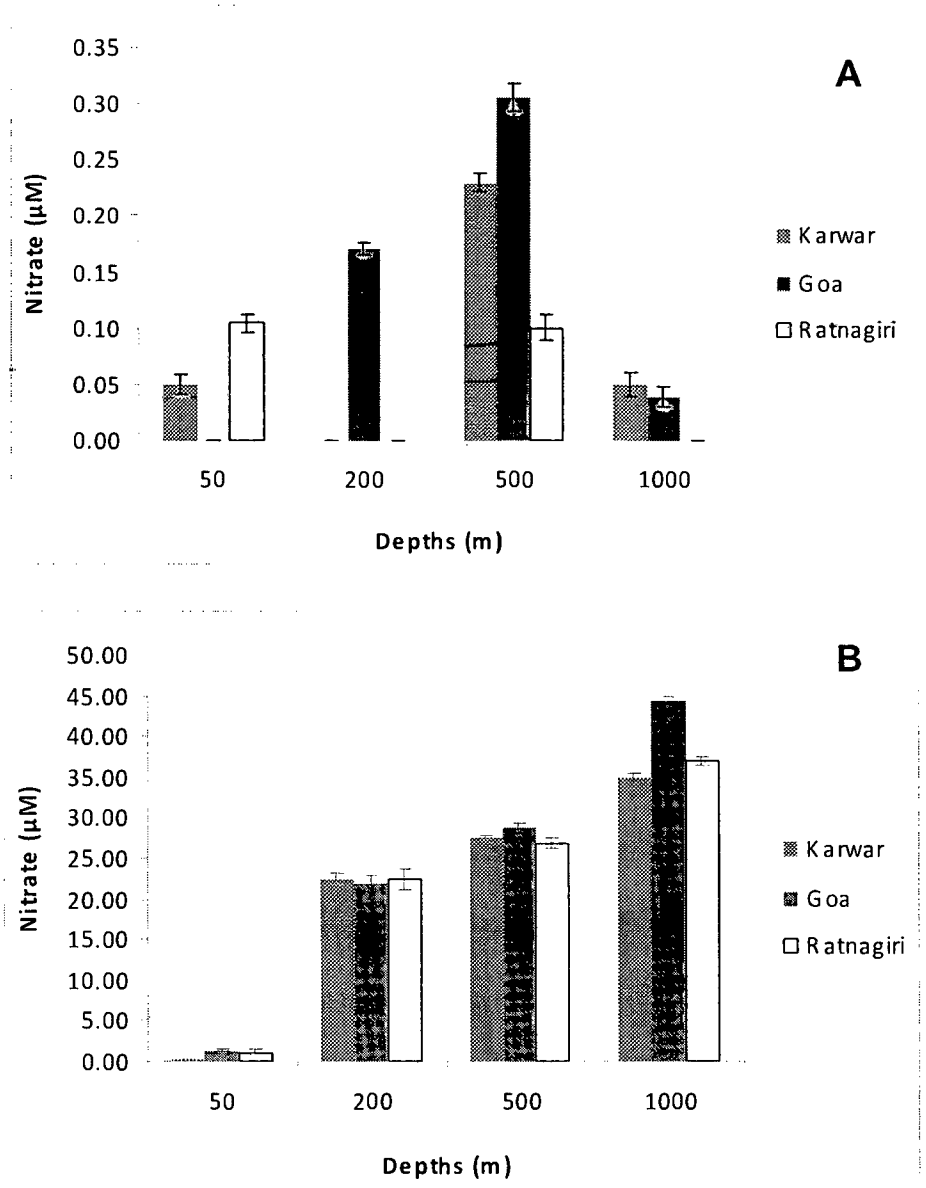


Fig. 5. Variation in nitrate concentration at (A) surface and (B) overlying waters of the three transects at four station depths (50, 200, 500, 1000 m).

4.1.2.3. Nitrite

Like nitrate, the nitrite concentration of the surface water of the three transects were low and ranged from non-detectable to 0.05±0.001 µM. There was no particular trend either transect-wise or depth of the stations. At Karwar transect, highest concentration of 0.05±0.001 µM was recorded at 200 m whereas in Goa transect the concentration was 0.04±0.002 µM at 200 and 500 m depths (Fig. 6A). In case of the overlying water, at 50 m (non-OMZ) the concentration of nitrite ranged from 0.2±0.01-0.29±0.01 µM while

in the deeper stations, 200, 500 and 1000 m (OMZ) stations the nitrite concentration ranged from 0.03±0.01-0.14±0.01 µM. Unlike nitrate concentration of the overlying water, no trend was observed for nitrite concentration with station depths (Fig. 6B).

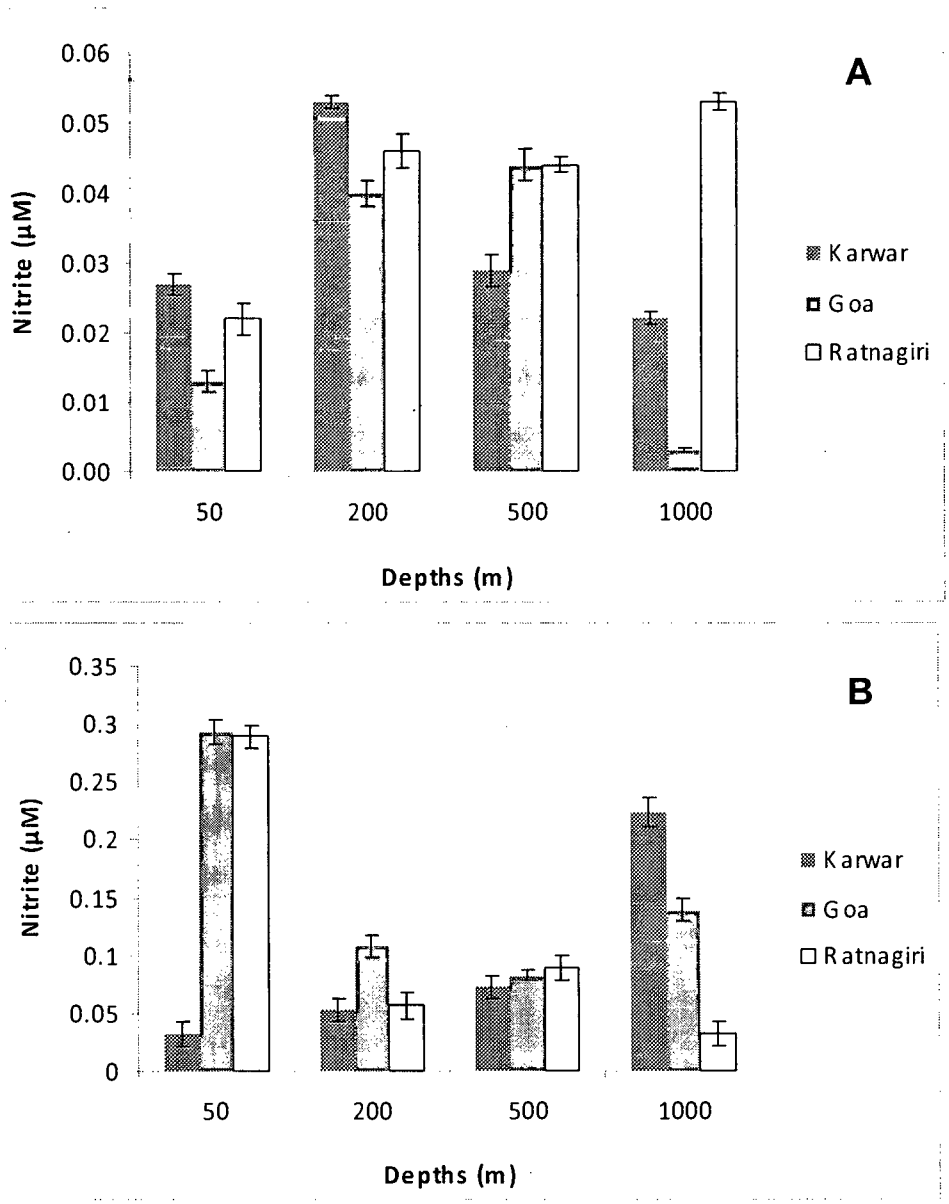


Fig. 6. Variation in nitrite concentration at (A) surface and (B) overlying waters of the three transects at four station depths (50, 200, 500, 1000 m).

4.1.2.4. Phosphate

Phosphate concentration of the surface water of the three transects were low and ranged from 0.11±0.008 to 0.37±0.01 µM. Among the three

transects, Ratnagiri recorded highest concentration of phosphate. Transect and depth-wise there were no trend in the distribution of phosphate (Fig. 7A). Along Karwar transect, the highest concentration was recorded at 1000 m depth station (0.27±0.005 µM) whereas in Goa transect it was at the 50 m depth station (0.31±0.006 µM). Phosphate concentration of the overlying water was higher than the surface waters and ranged from 0.29±0.02 to 3.47±0.1 µM. Like the surface waters, the overlying waters did not show any particular trend though 50 m depth stations (non-OMZ) recorded lower concentration than the deeper stations (OMZ). The highest concentration was recorded at different station depths for different transects. At Karwar and Goa it was at 1000 m depth whereas for Ratnagiri it was at 500 m depth station (Fig. 7B).

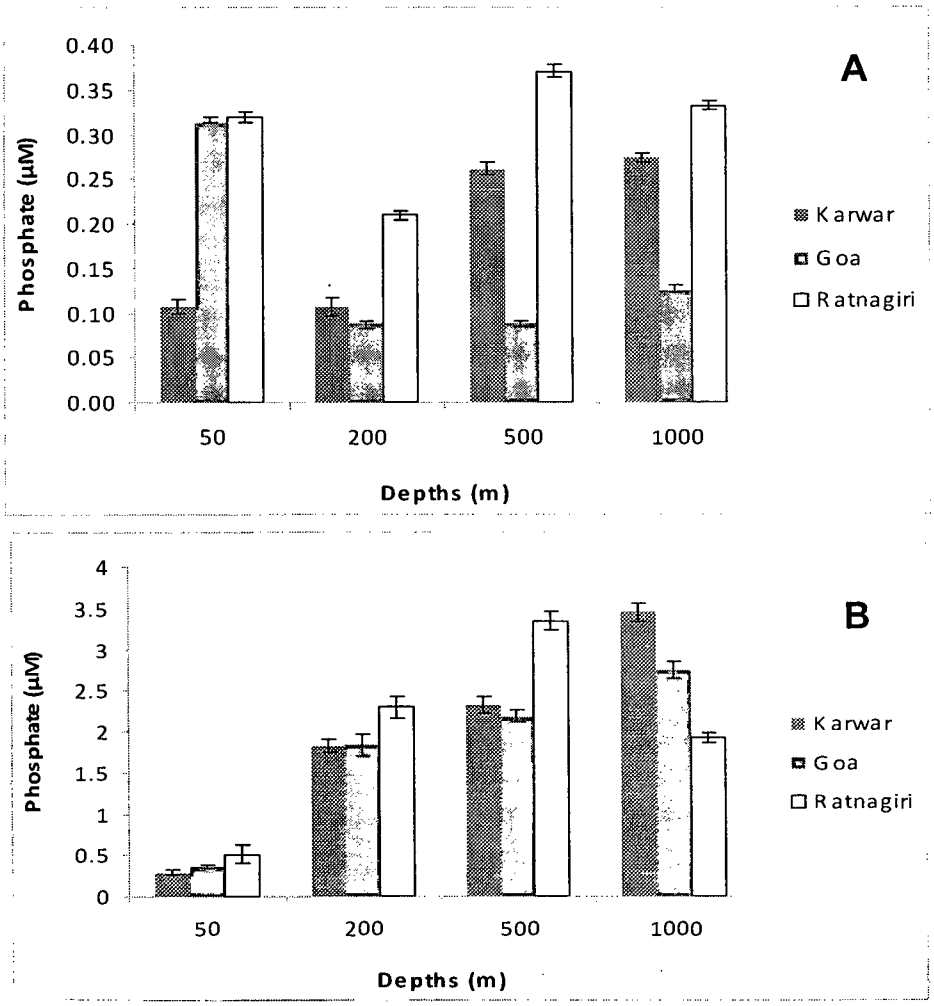
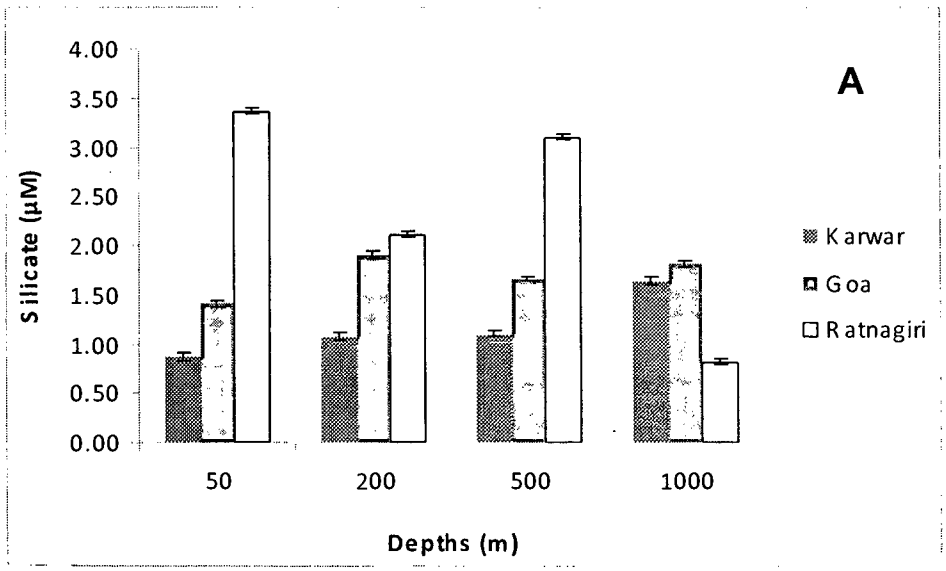


Fig. 7. Variation in phosphate concentration at (A) surface and (B) overlying waters of the three transects at four station depths (50, 200, 500, 1000 m)

4.1.2.5. Silicate

In the surface waters, the concentration of silicate ranged from 0.82±0.03 to 3.36±0.03 µM for the three transects. Transect-wise, there was variation in silicate concentration. At Karwar, it ranged from 0.87±0.04 to 1.64±0.03 µM, at Goa it ranged from 1.4±0.02 to 1.9±0.04 µM and at Ratnagiri it was from 0.82±0.85 to 3.36±0.03 µM. Among the three transects, Ratnagiri recorded the highest concentration (Fig. 8A). Depth-wise, there was no particular trend, as at Karwar highest concentration was recorded at 1000 m, at Goa it was 200 m and at Ratnagiri it was 50 m depth. The overlying waters of the three transects had higher concentration of silicate compared to the surface waters. The concentration ranged from 5.4±0.39 to 75.5±0.9 µM for all depths in the three transects. The silicate concentration increased with station depth for Karwar and Goa transects (Fig. 8B). Higher concentration was observed for all the 1000 m depth stations (>60 µM). Overall, the non-OMZ stations had lower concentration compared to the OMZ stations of the three transects.



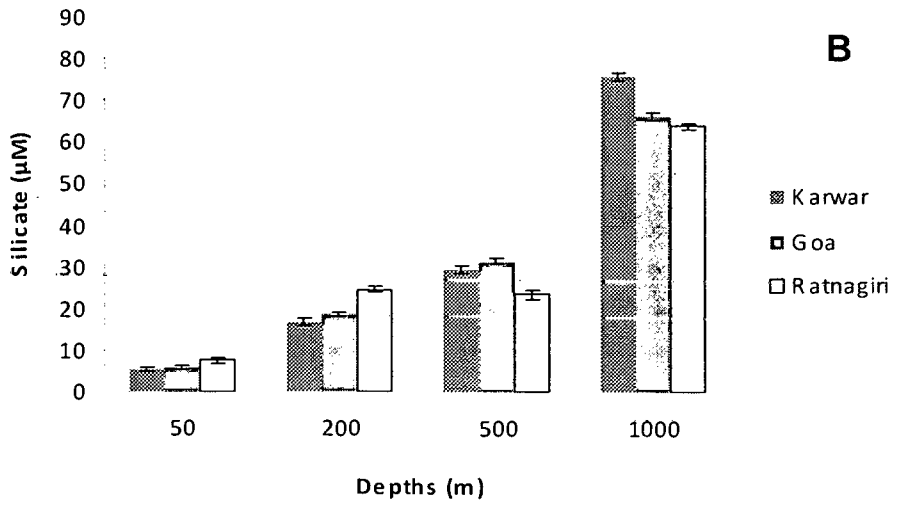


Fig. 8. Variation in silicate concentration at (A) surface and (B) overlying waters of the three transects at four station depths (50, 200, 500, 1000 m).

4.2. SEDIMENT PARAMETERS

4.2.1. Temperature

Sediment temperature of 50 m depth stations of all transects was high. Temperature decreased with depth of the stations with the lowest at 1000 m depth. The variations in temperature were significant ($p<0.001$) between different depths. There was no significant difference between transects (Fig. 9).

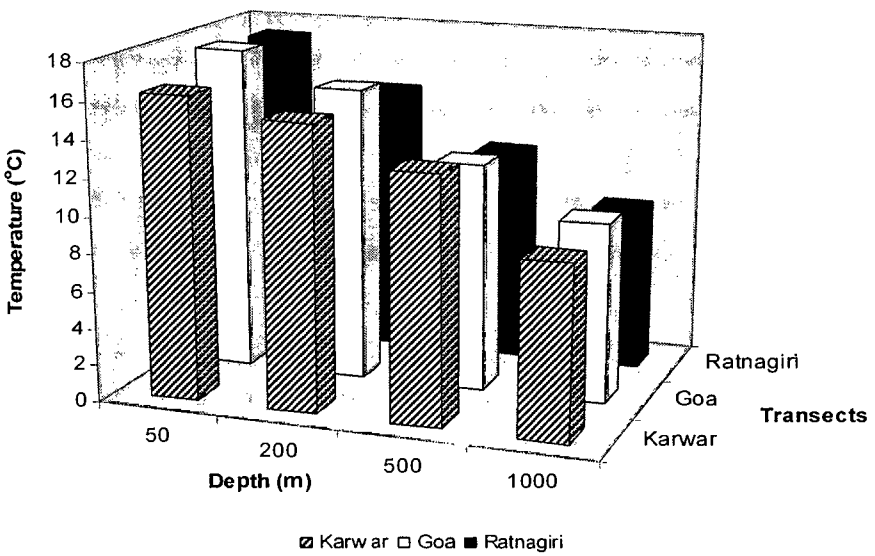


Fig. 9. Sediment temperature at different depths

4.2.2. Redox Potential (Eh)

The redox potential was negative for all the sediment samples. No particular trend was observed in the Eh values with respect to station depths or transects. Goa transect recorded high values with the highest value of -94.5 mV at 500 m station depth (Table 1). There was difference in Eh values between OMZ and non-OMZ stations except at Goa transect where the difference was not much.

Transect	Depth (m)			
	50	200	500	1000
Karwar	-37	-52.6	-62	-66.9
Goa	-78	-96.7	-94.5	-58.6
Ratnagiri	-68.1	-54.7	-56.9	-36.7

Table 1. Depth-wise variation in Eh (mV) in the three transects.

4.2.3. Sediment Colour And Texture

The sediment colour in almost all the samples was olive green except at 200 m stations of Goa and Ratnagiri where it was slightly yellowish brown. Most of the samples had sand, silt and clay in varying proportions (Fig.10). Transect-wise sand content was 8.9±11.5, 7.7±15.1 and 5.6±6.8% for Karwar, Goa and Ratnagiri, respectively. The proportion of sand was greater than 10% at 200 m depth in all the three transects. Along the Karwar transect there was a decrease in sand content with station depth. Clay and silt fractions were proportionately more in all the station depths in all transects. The percentage of silt ranged between 42.9±12.5 and 48.7±16.0% and clay between 43.7±15.7 and 51.4±11.3% (Table 2).

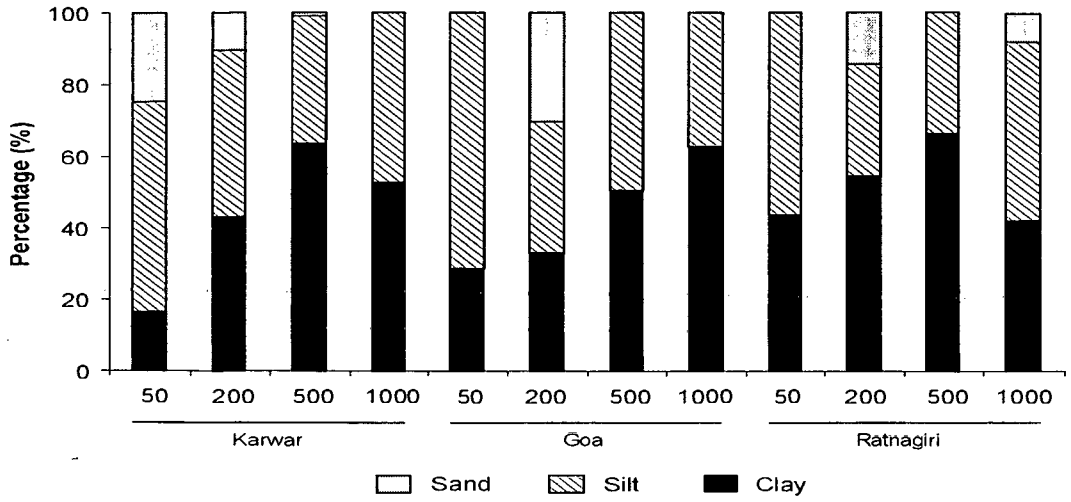


Fig. 10. Depth-wise variation in the grain size analysis at the three transects.

Transect	Sand	Silt	Clay
Karwar	8.9±11.5	47.2±9.4	43.8±20.0
Goa	7.7±15.1	48.7±16.0	43.7±15.7
Ratnagiri	5.6±6.8	42.9±12.5	51.4±11.3

Table 2. Transect-wise grain size analysis (Average±SD)

4.2.4. Water Content (WC)

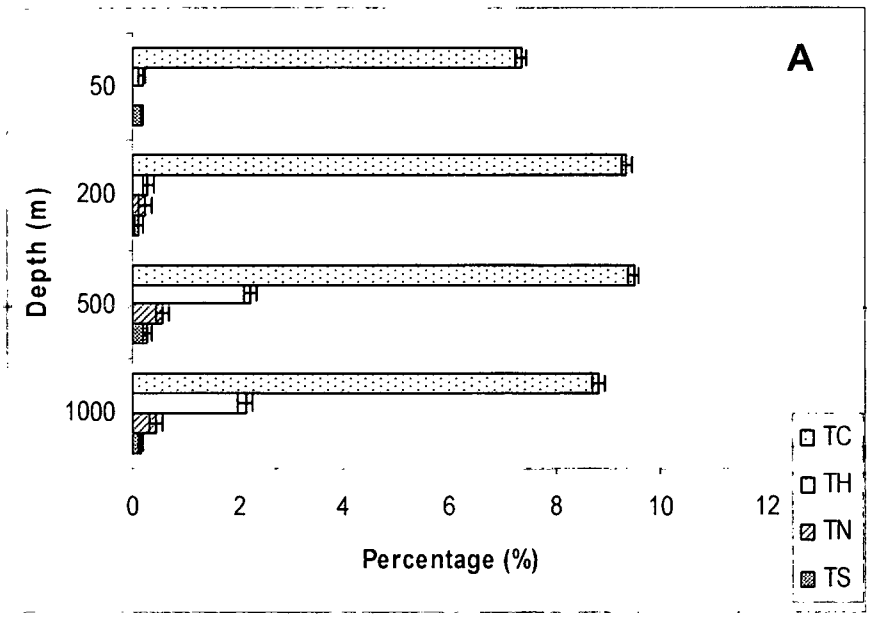
Water content of the sediments from the three transects is given in Table 3. In the Karwar transect, the water content ranged from 25±0.37 to 85±0.62% with the highest water content of 85% at 500 and 1000 m depth stations. However, in the Goa transect, water content showed a wide range from 7±0.9 to 89±0.35%. The lowest of 7±0.9% was observed in 200 m of Goa transect where sand was also recorded. In the Ratnagiri transect, the values varied from 11±1.75 to 99±0.8%. Though there was no significant variation between transects, there was a significant variation between station depths ($P<0.005$).

Transect	Depth (m)			
	50	200	500	1000
Karwar	66±0.79	25±0.37	85±0.62	85±0.87
Goa	35±1.05	7±0.9	86±1.9	89±0.35
Ratnagiri	98±1.01	11±1.75	99±0.8	79±3.18

Table 3. Depth-wise variation in water content (%) in the three transects

4.2.5. Elemental CHNS

Total elemental carbon (TC), nitrogen (TN), hydrogen (TH) and sulphur (TS) of the sediment are presented transect-wise. (Fig.11). The TC concentration was comparatively higher in all samples. In the shallow depth stations of all transects, TC ranged from 4.2±0.09 to 7.35±0.09% (Fig 11). However, in the deeper stations it ranged from 8.82±0.1 to 10.58±0.11%. The total nitrogen (TN) ranged from non-detectable to 0.69±0.01% in the surface sediment. In the Goa transect, TN ranged from non-detectable levels to 0.69±0.1% with the highest observed in the 500 m depth (Fig 11B). The total hydrogen (TH) in the Karwar transect ranged from 0.19±0.05-2.22±0.13% whereas in the Goa transect it ranged from 0.67±0.09 to 0.79±0.12%. The TS in the sediments off Karwar varied from 0.12±0.07 to 0.28±0.08 (1.05±0.78) and in Goa it ranged from 0.49±0.08-0.94±0.1 (0.71±0.23). In the Ratnagiri transect the TS varied from non-detectable to 0.49±0.1 (Fig.11C) with an average of (0.18±0.21). In the Ratnagiri transect the TH and TN values were on an average 1.2±0.8 and 0.29±0.23 respectively (Table 4).


A
Depth (m)
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200
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1000
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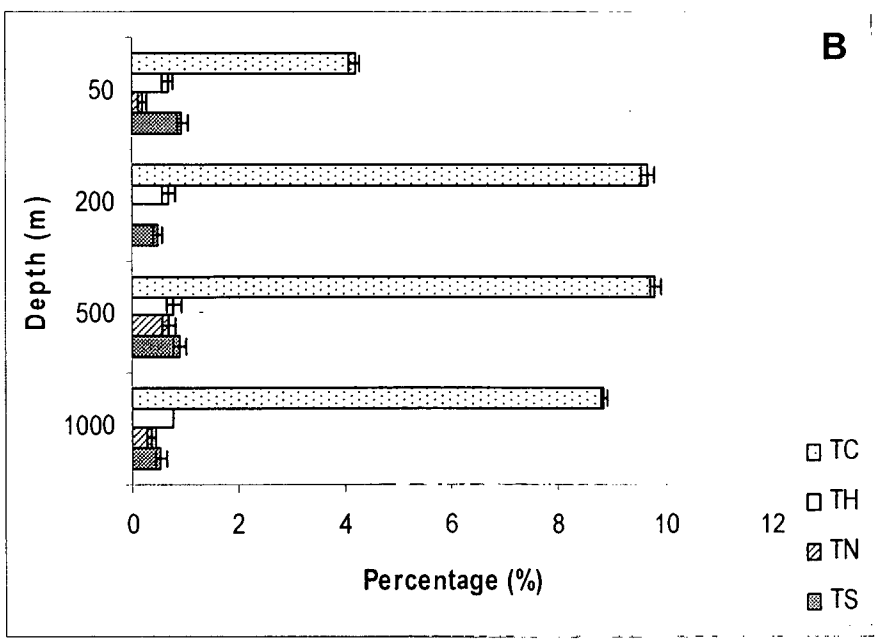
2
4
6
8
10
12
Percentage (%)
TC
TH
TN
TS


B
Depth (m)
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Percentage (%)
TC
TH
TN
TS

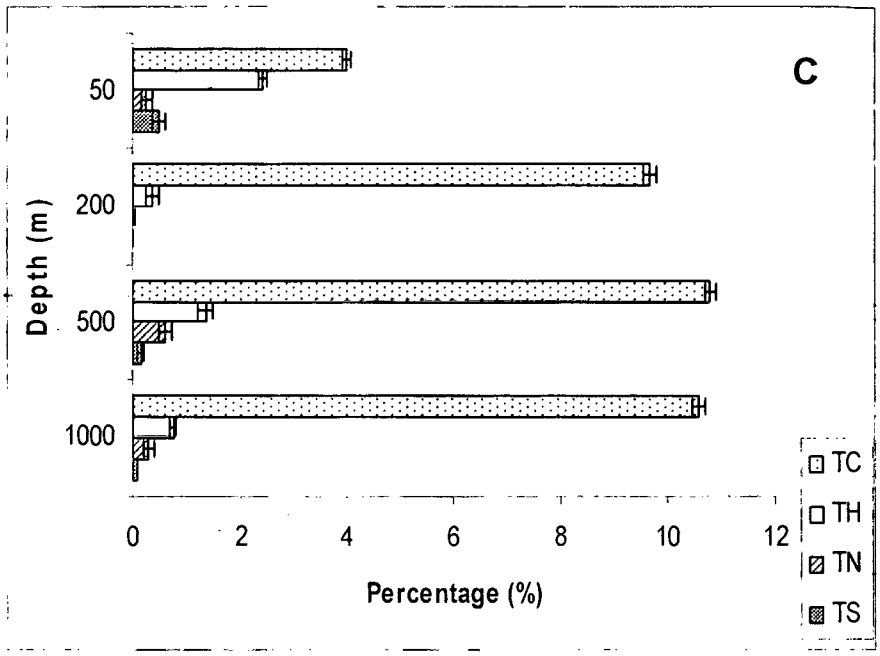


Fig. 11. Depth-wise variation in elemental CHNS in the three transects (A) Karwar, (B) Goa and (C) Ratnagiri.

Transect	TC	TH	TN	TS
Karwar	8.8±0.97	1.2±1.11	0.31±0.24	0.20±0.07
Goa	8.1±2.70	0.7±0.05	0.32±0.29	0.70±0.20
Ratnagiri	8.8±3.20	1.2±0.90	0.3±0.23	0.18±0.21

Table 4. Transect-wise variation in the percentage of total elements (Average±SD)

Total Carbon was highest in the OMZ compared to non-OMZ in all the transects with 9.2 ± 0.35, 9.4 ± 0.5 and 10.3 ± 0.6% in Karwar, Goa and Ratnagiri (Fig. 11 D, E & F). The other parameters such as Total Hydrogen, Nitrogen and Sulphur varied between transect. In the Goa transect the values of these were similar while in Karwar and Ratnagiri showed different trends.

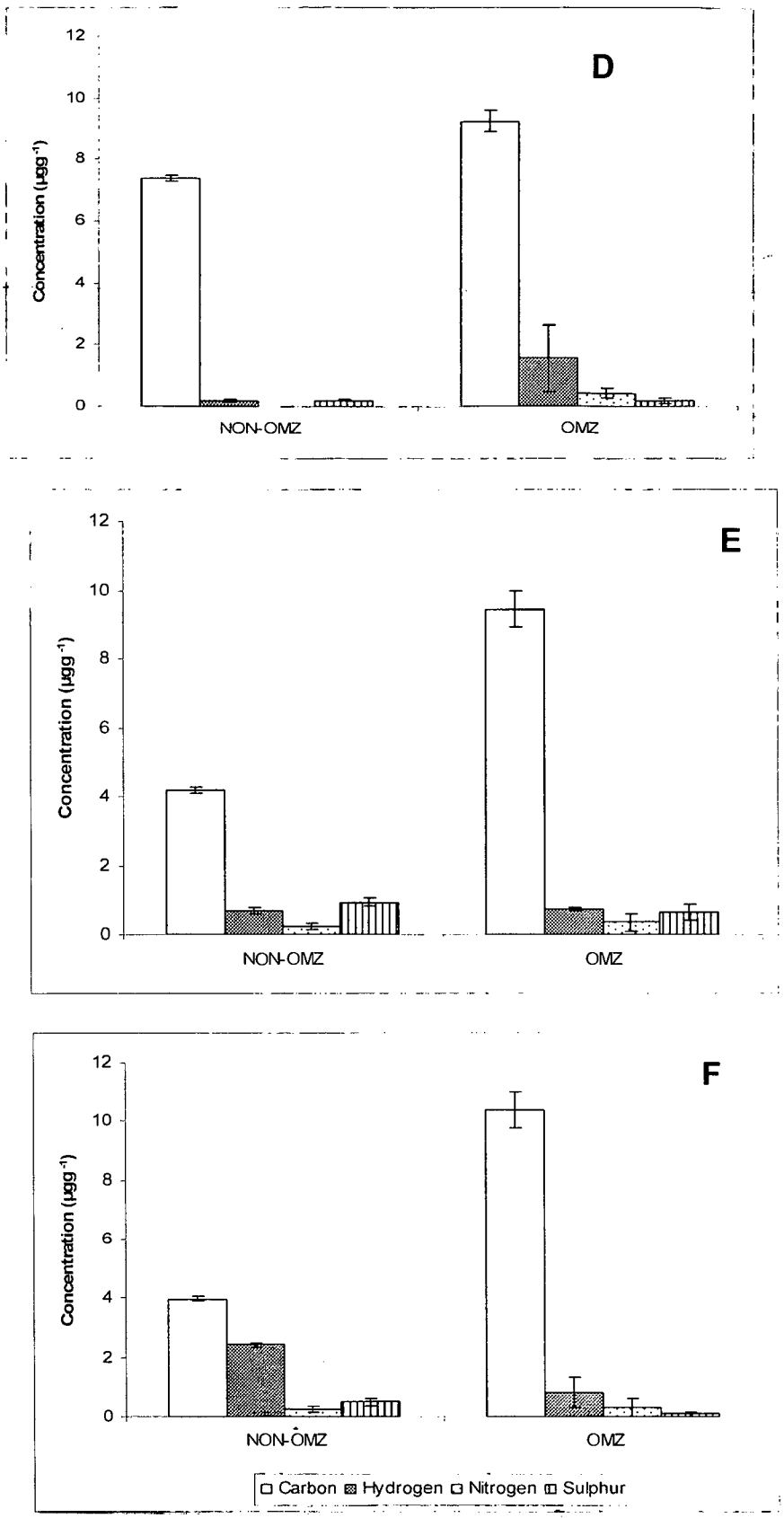


Fig.11. Comparison of Carbon, Hydrogen, Nitrogen and Sulphur in the OMZ and non-OMZ of (D)Karwar, (E)Goa and (F)Ratnagiri transects.

4.2.6. Total Organic Carbon (TOC)

The average percentage of TOC concentrations at Karwar, Goa and Ratnagiri were 8.6±0.9, 7.7±2.6 and 8.6±3.2%, respectively. At the shallower depths the TOC was 7±0.1, 3.8±0.09 and 3.7±0.2, in Karwar, Goa and Ratnagiri respectively (Fig.12A, B & C). The high percentage of TOC was detected in the sub-oxic regions off Ratnagiri ie., 200, 500, 1000 m with 9.5±0.15, 10.56±0.14 and 10.55±0.17% respectively (Fig.12C). However, in the Goa and Karwar transects the highest TOC was observed at 500 m (Fig. 12 A&B).

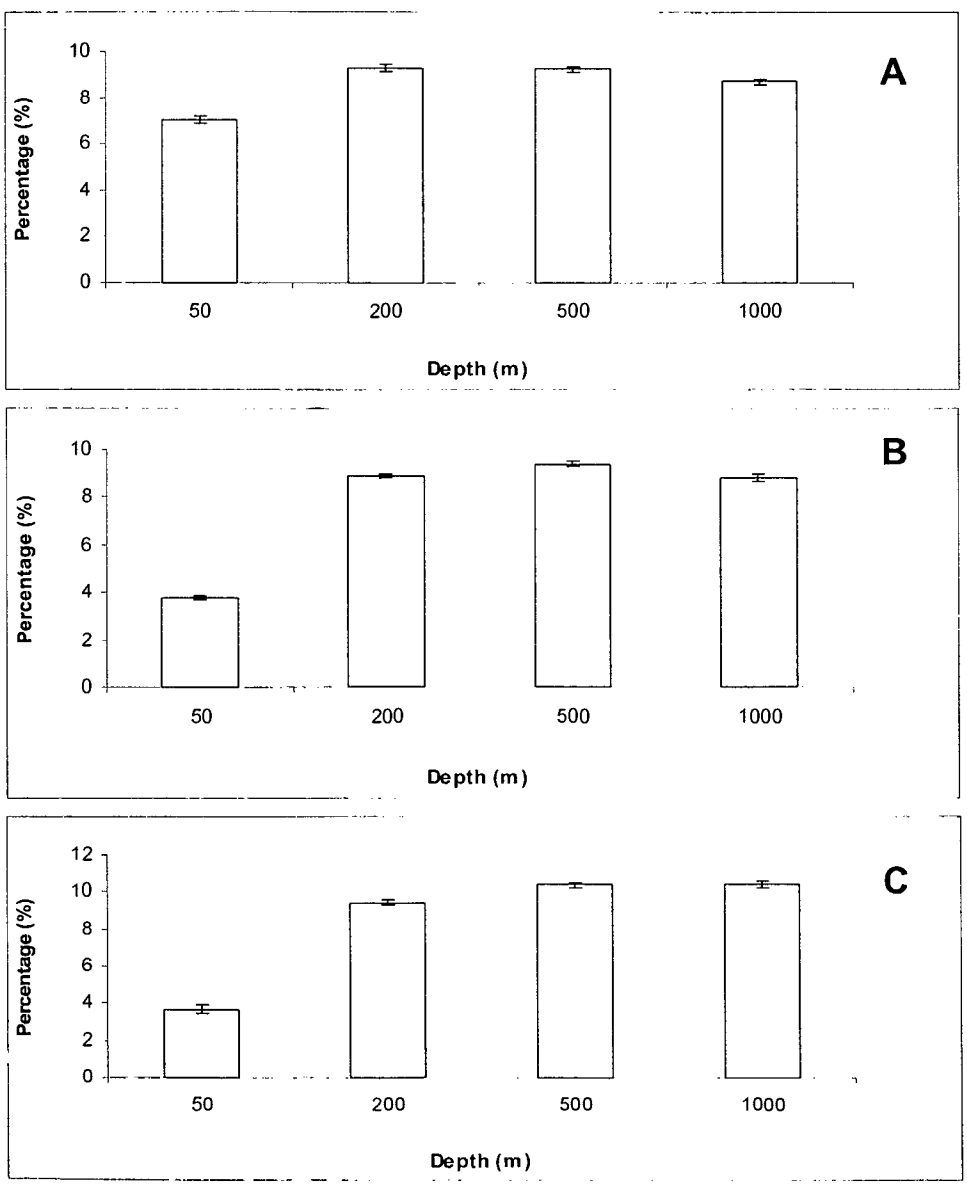
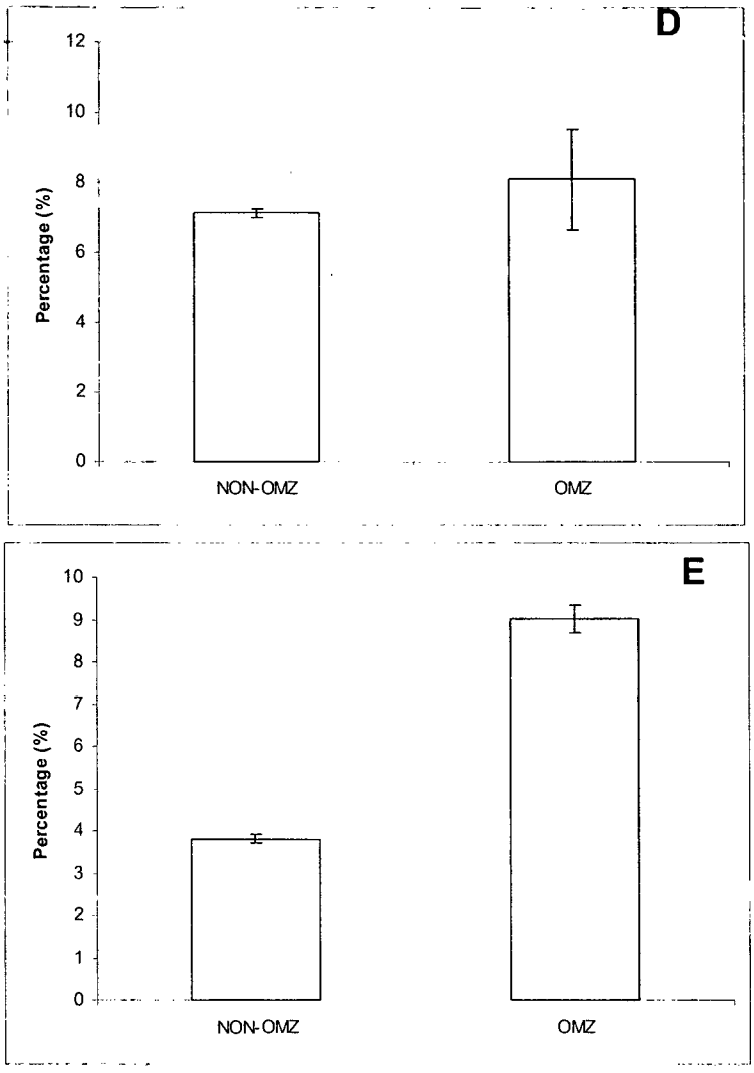


Fig.12. Depth-wise variation in TOC of (A) Karwar,(B) Goa and (C) Ratnagiri transects

The total organic carbon (TOC) was observed higher in OMZ region with 8.08 ± 1.44, 9 ± 0.32 and 9.5 ± 0.57 % in Karwar, Goa and Ratnagiri transects (Fig 12 D, E & F). The TOC in the non-OMZ region of Karwar was very close to the OMZ values with 7.1 ± 1.1%.



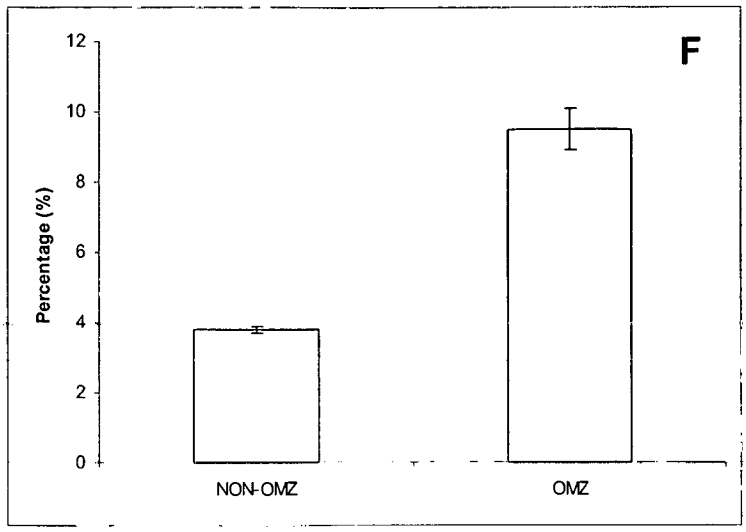
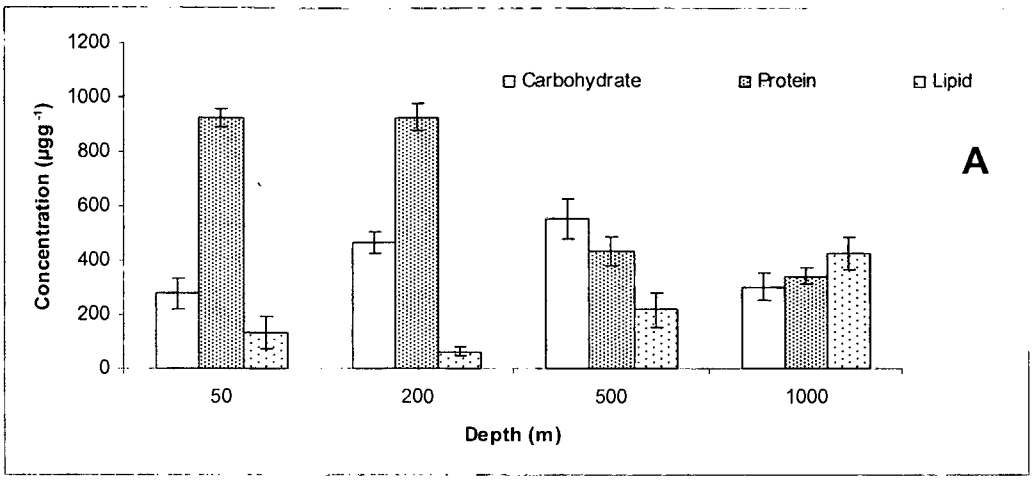


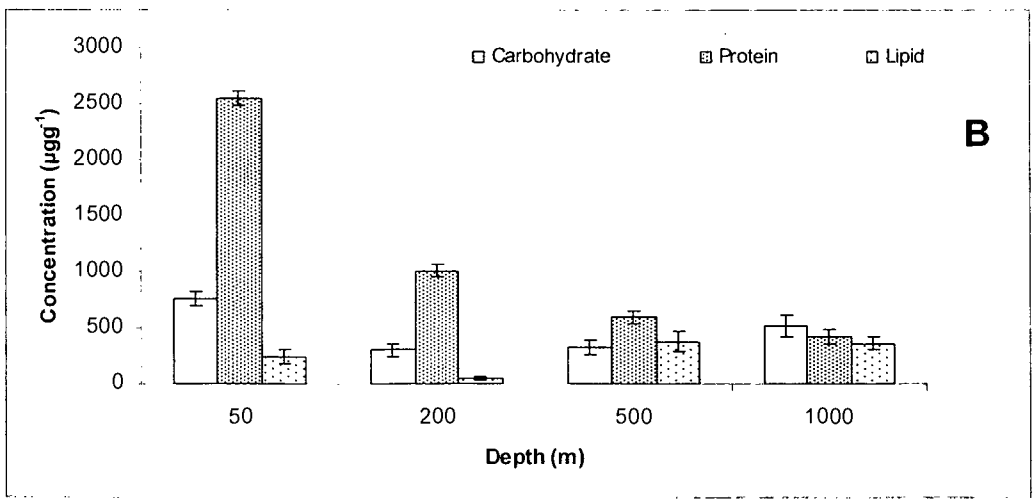
Fig.12. Comparison of TOC in the OMZ and non-OMZ of (D)Karwar, (E)Goa and (F)Ratnagiri transects

4.2.7. Labile Organic Matter (LOM)

The total labile organic matter (carbohydrates, proteins and lipids) at Karwar, Goa and Ratnagiri were 1267.6, 1874.9 and 1364.7 μgg^{-1} respectively. The average concentration of carbohydrates in the Karwar transect was 400.5 ± 114.6 μgg^{-1} and ranged from 278±58.5 to 554.98±74.4 μgg^{-1}. The concentration of proteins was 657.5±271.4 μgg^{-1} and was higher than carbohydrates (Fig. 13A). The lipid concentration ranged from 61.2±17.7 to 424±57.3 μgg^{-1}. There was no particular trend in the distribution of labile organic matter viz. carbohydrates, proteins and lipids with depth of the station for Karwar transect as the highest concentration of carbohydrates was at 500 m depth, proteins at 200 m and for lipids it was at 1000 m station depth. Analysis of variance showed that there was no significant variation with depth. In the Goa transect, the concentration of carbohydrates on an average was 474.5±212.5 μgg^{-1} with the highest observed in 50 m station depth. The protein content in the sediments off Goa was on an average 1141.8±969.3 μgg^{-1}. The sediment lipid concentration ranged from 60.6±12.7 to 379±95.5 $\mu g.g^{-1}$. There was no particular trend for distribution of carbohydrates and lipids at different station depths off Goa also (Fig.13B).

The same was also observed for the Ratnagiri transect. On an average, carbohydrates in the sediments off Ratnagiri were 387.8±181.4 μgg^{-1}. Protein and lipid concentrations in this transect were 817.6±793.9 and 159.4±155.7 μgg^{-1} respectively (Fig.13C).





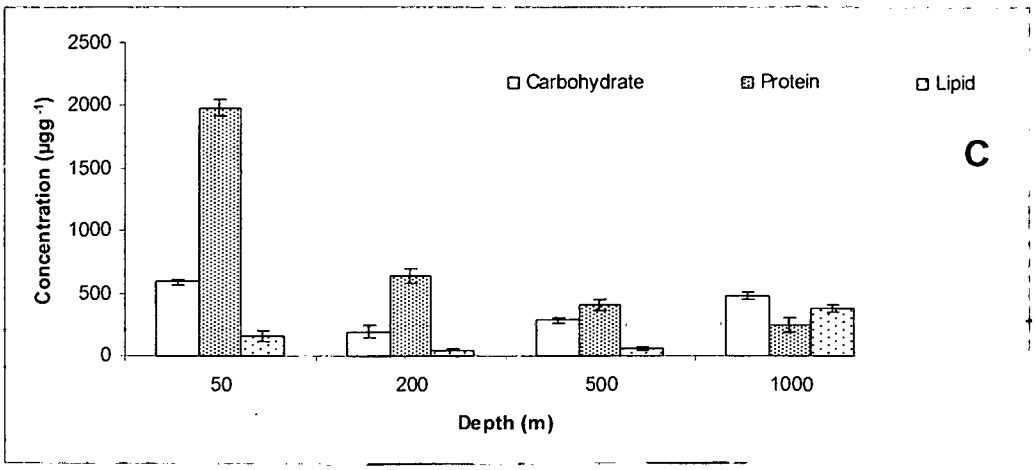
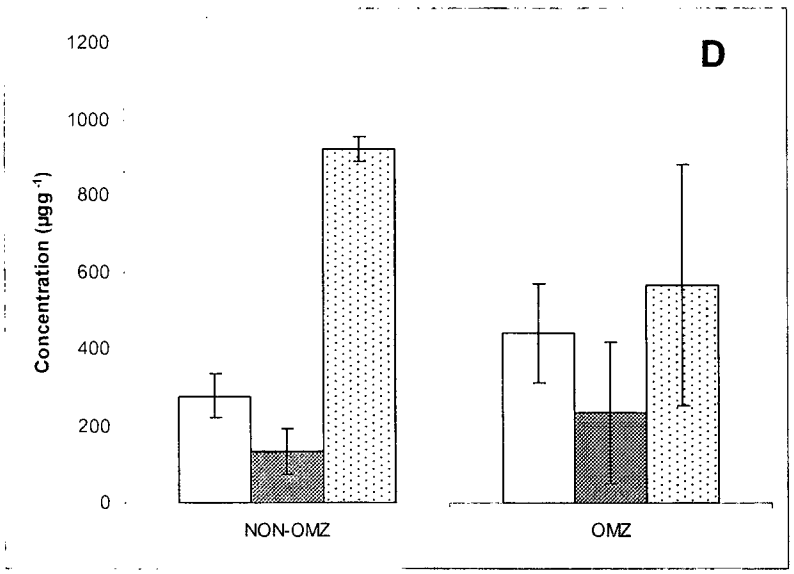


Fig.13. Depth-wise variation in Labile Organic Matter (LOM) of transects (A) Karwar, (B) Goa and (C) Ratnagiri.

Among the labile organic carbon, proteins were the highest components in the OMZ and non-OMZ regions. Protein was highest in the non-OMZ region with 924.3± 34, 2548±57.1 and 1981.2±58.9 μgg^{-1} in Karwar, Goa and Ratnagiri respectively compared to the OMZ region (Fig. 13 D, E & F). Other components did not show any particular trend.



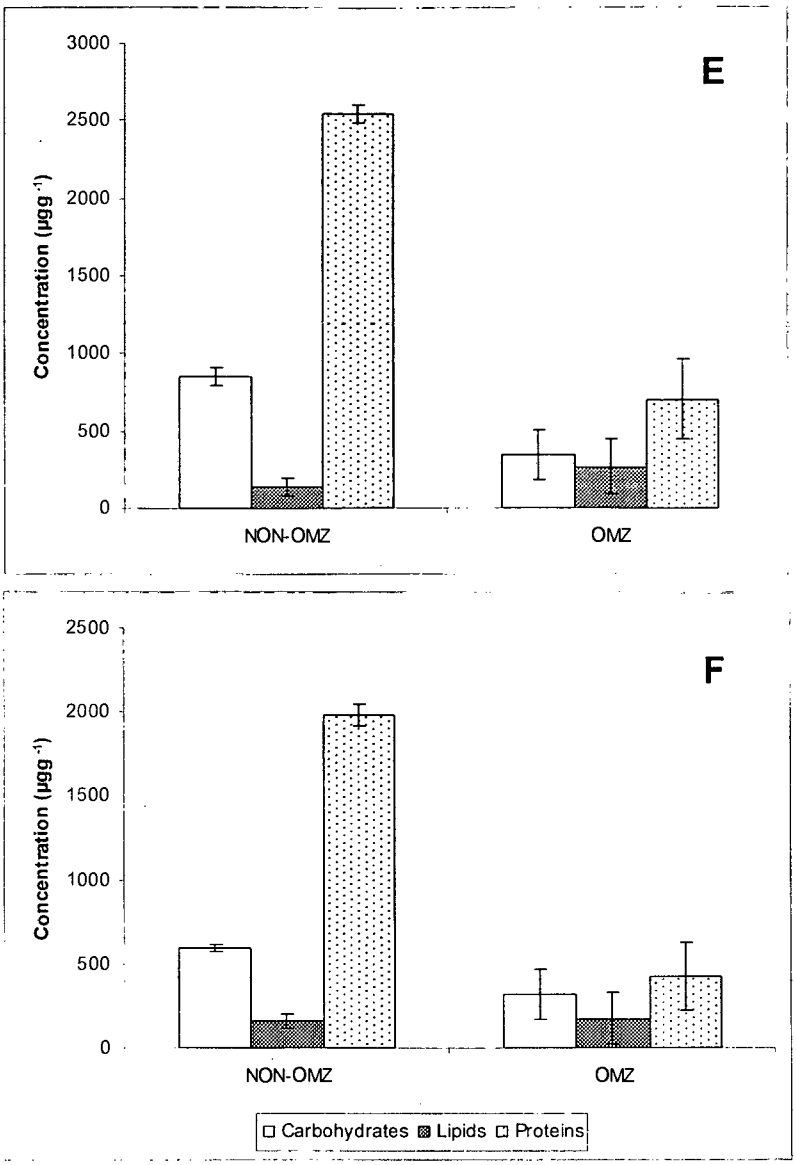


Fig.13. Comparison of carbohydrates, lipids and proteins in the OMZ and non-OMZ of (D)Karwar, (E)Goa and (F)Ratnagiri transects.

4.3. STATISTICAL ANALYSIS

Hierarchical cluster analysis of hydrographical parameters showed that the sampling stations are structured into two main zones (Fig.14) at about the Euclidean distance 8. All the non-OMZ stations at 50 m depth clustered together at a distance of 7 while the OMZ stations in 200, 500 and 1000 m depths clustered at 5.5 Euclidean distances. The OMZ cluster, further sub-grouped into two clusters differentiating into the shallower sites at 200 m and deeper stations at 500 and 1000 m.

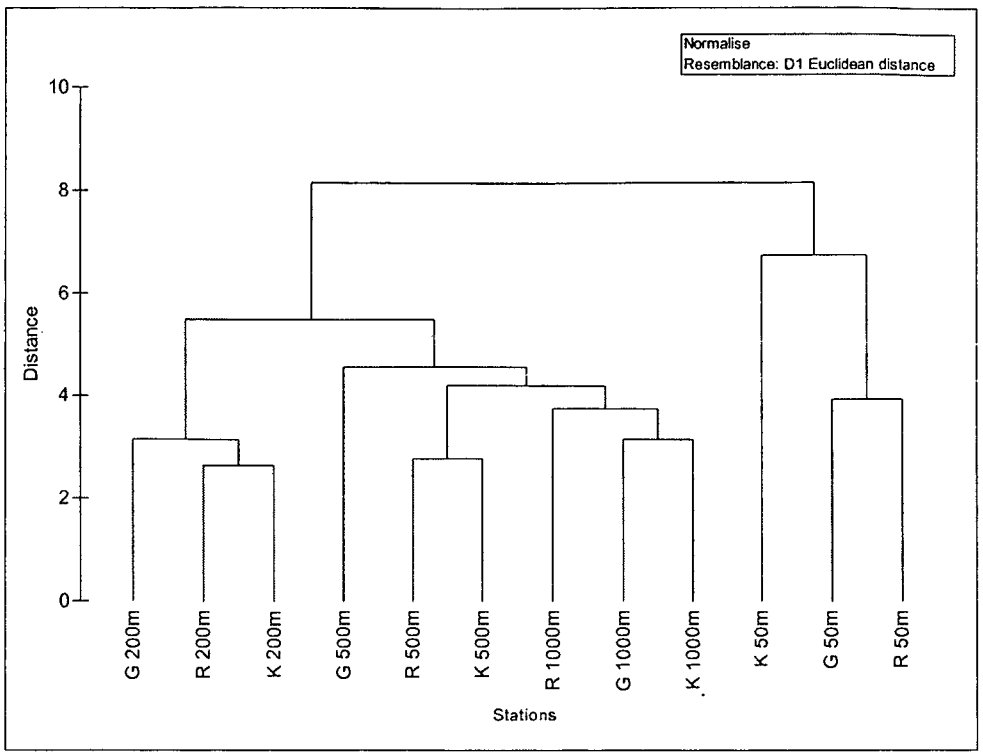


Fig.14. Hierarchical cluster analysis of sampling sites (G-Goa; K-Karwar; R-Ratnagiri)

A principal component analysis (PCA) was carried out in order to understand which environmental parameters were responsible for the variations at different sites. PCA of all the stations aggregated the variables into two main components, together explaining 72% of the variation (Table 5). The variables that were highly influencing in the first principal component (PC1) were temperature, nitrate, phosphate, total carbon, TOC, proteins, dissolved oxygen, salinity and pH, bringing about 48% of the variation. The major players in the second axis (PC2) were nitrite, total hydrogen, total nitrogen, lipids, sand and water content. The PCA ordination gives the dissimilarity measures which were seen in the Euclidean distance (Fig.15).

PCA

Eigenvalues

PC	Eigen values	%Variation	Cum. %Variation
1	10.2	48.8	48.8
2	4.87	23.2	72.0
3	1.81	8.6	80.6
4	1.53	7.3	87.9
5	0.945	4.5	92.4

Eigenvectors

(Coefficients in the linear combinations of variables making up PC's)

Variable	PC1	PC2	PC3	PC4	PC5
Temperature	**-0.26**	-0.15	**0.264**	0.113	-0.01
Nitrate	**0.29**	0.093	-0.03	-0.18	0.136
Nitrite	-0.18	**0.286**	0.092	0.006	**0.455**
Phosphate	**0.285**	0.093	0.115	0.075	0.132
Silicate	0.226	0.169	**-0.29**	-0.23	**0.313**
TC	**0.286**	-0.14	0.05	-0.05	-0.21
TN	0.147	**0.333**	0.114	0.091	**-0.43**
TH	0.014	**0.305**	0.012	**0.442**	0.236
TS	-0.15	0.227	**0.292**	**-0.41**	-0.24
TOC	**0.293**	-0.12	0.012	-0.02	-0.18
Carbohydrates	-0.16	0.244	-0.03	-0.08	0.188
Lipids	0.075	**0.313**	**-0.32**	**-0.4**	-0.02
Proteins	**-0.29**	0.059	0.162	-0.05	0.157
Sand	-0.04	**-0.41**	-0.02	-0.13	0.05
Silt	**-0.25**	0.113	**-0.26**	**-0.25**	-0.14
Clay	0.222	0.2	0.219	**0.293**	0.079
Eh	0.049	**-0.25**	**-0.52**	**0.245**	0.108
Water Content	0.063	**0.322**	**-0.29**	**0.279**	**-0.36**
DO	**-0.28**	0.033	**-0.26**	0.065	-0.07
Salinity	**-0.28**	0.113	0.018	0.163	-0.21
Ph	**-0.28**	-0.03	-0.23	0.147	-0.12

Table. 5. Eigen values and vectors of all variables in the three transects. Values in bold are >0.25

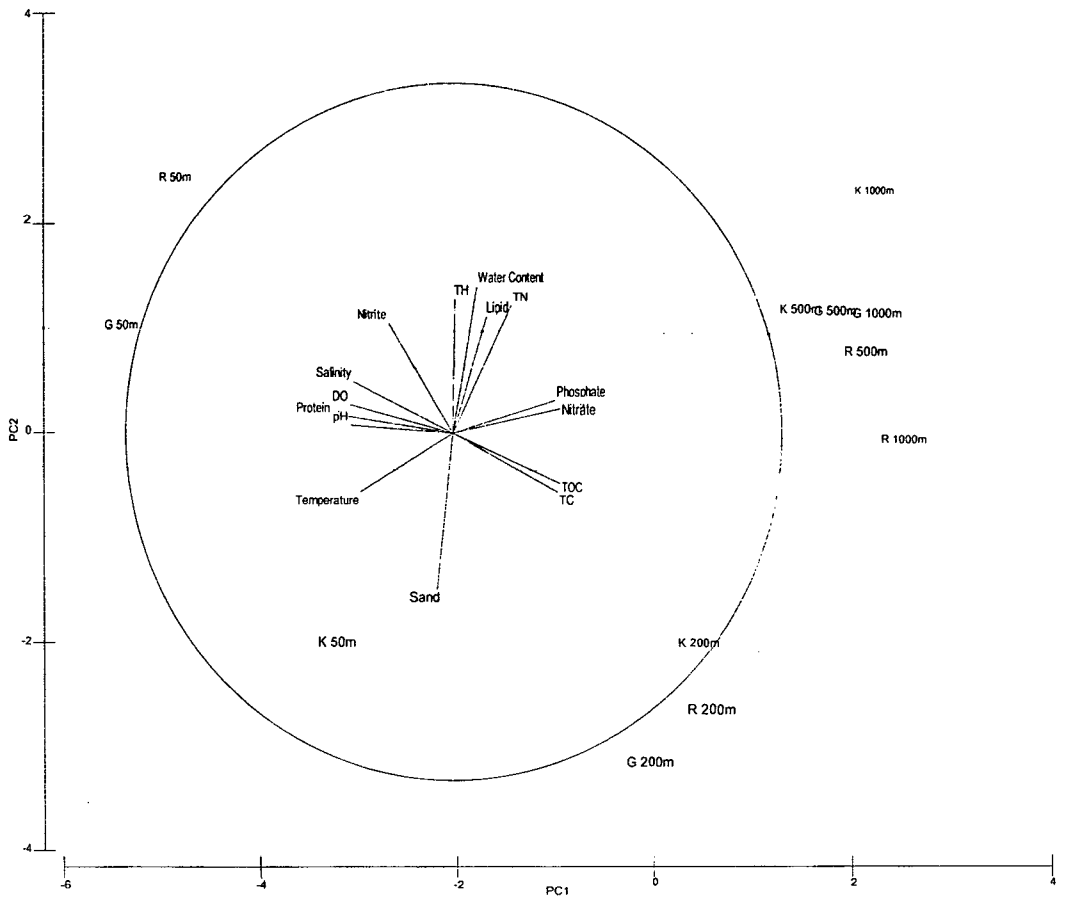


Fig.15. Principal Component Analysis of variables with Eigen vectors > 0.25 in the three transects

PCA of the OMZ sites showed that the first component (PC1) brought about 38% of the variation while in the non-OMZ sites it showed 72% of the variation (Table 6 & Fig.16 A&B). The major variables that influenced the OMZ sites were temperature, nitrate, silicate, lipids, proteins, sand, water content and dissolved oxygen, while in the non-OMZ regions the key players were temperature, nitrate, nitrite, total carbon, TOC, sand and water content. The second component (PC2) in the OMZ included nitrite, clay and salinity, explaining 57% of the variation. However, in the non-OMZ the second component which contributed to 100% of the variation included phosphate, lipids, silt, water content and dissolved oxygen. Interrelationships between environmental parameters are tabulated in the Appendix-A6 section.

PCA

Eigenvalues OMZ

PC	Eigenvalues	%Variation	Cum. % Variation
1	8.09	38.5	38.5
2	3.96	18.8	57.3

Eigenvalues non-OMZ

PC	Eigen values	%Variation	Cum. % Variation
1	15.2	72.3	72.3
2	5.81	27.7	100.0

Eigenvectors

(Coefficients in the linear combinations of variables making up PC's)

Variable	OMZ		non-OMZ	
	PC1	PC2	PC1	PC2
Temperature	**0.321**	0.013	**-0.254**	-0.062
Nitrate	**-0.291**	0.096	**-0.254**	-0.059
Nitrite	-0.195	**0.276**	**-0.256**	0.024
Phosphate	-0.235	-0.07	-0.182	**0.293**
Silicate	**-0.291**	0.201	-0.158	**0.327**
TC	0.067	**-0.393**	**0.255**	-0.052
TN	-0.243	-0.241	-0.248	0.107
TH	-0.224	-0.05	-0.157	**0.328**
TS	-0.043	0.053	-0.216	-0.223
TOC	-0.016	**-0.406**	**0.256**	-0.031
Carbohydrates	-0.122	0.004	-0.247	-0.112
Lipids	**-0.294**	0.146	-0.19	**-0.278**
Proteins	**0.299**	0.157	-0.247	-0.114
Sand	**0.288**	0.18	**0.256**	-0.029
Silt	-0.125	0.152	-0.11	**-0.375**
Clay	-0.18	**-0.266**	-0.206	0.248
Eh	0.012	-0.058	**0.253**	0.067
Water Content	**-0.316**	-0.2	0.016	**0.414**
Dissolved Oxygen	**-0.264**	0.236	0.165	**-0.317**
Salinity	0.022	**-0.465**	-0.248	-0.108
pH	0.161	-0.011	0.231	0.181

Table 6. Eigen values and vectors of all variables in the OMZ and non-OMZ sites

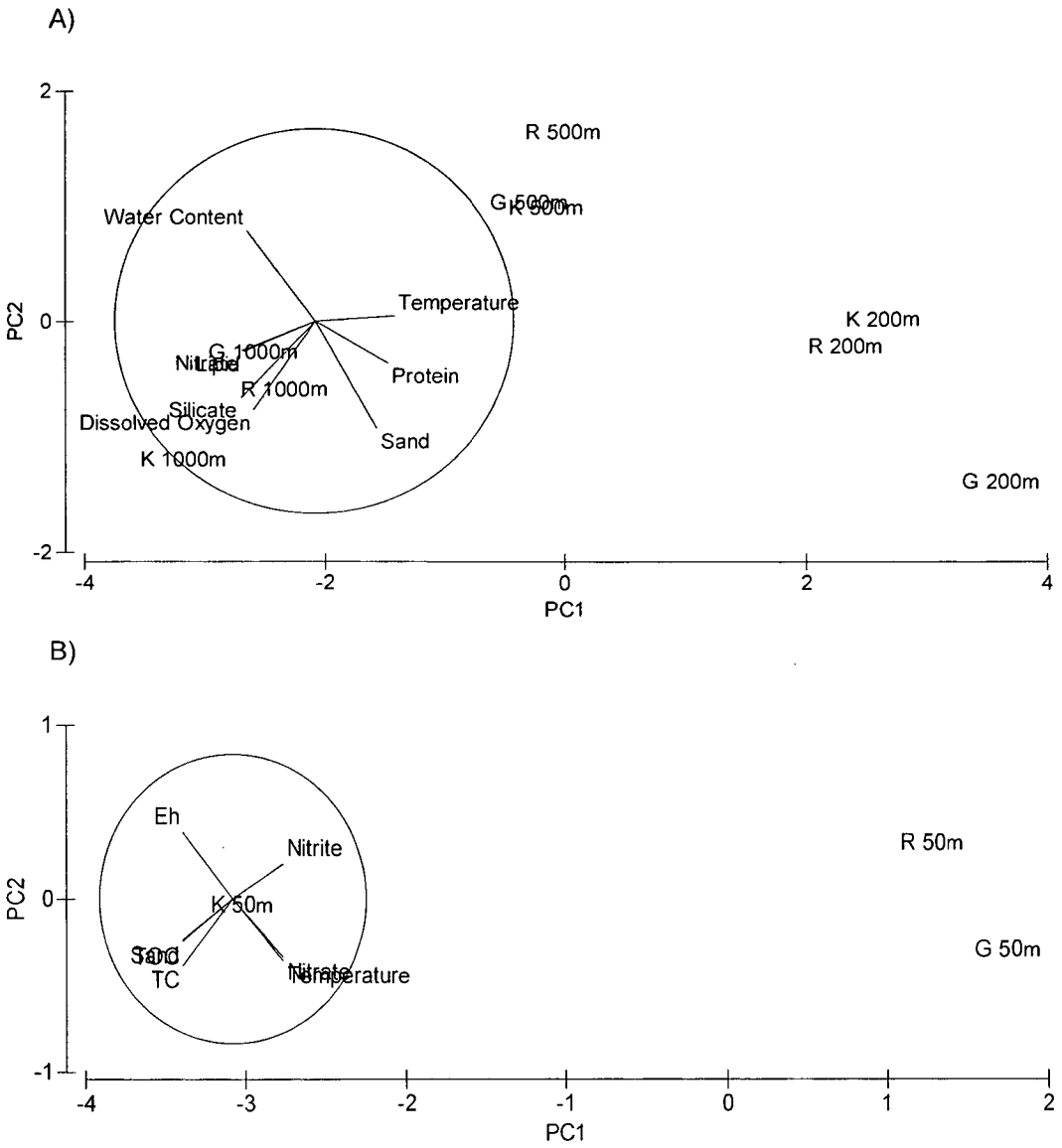


Fig. 16. Principal Component Analysis of variables with Eigen vectors > 0.25 in the A) OMZ sites B) non-OMZ sites

4.4. BACTERIAL PARAMETERS

4.4.1. Total Microbial Biomass (ATP)

Total benthic microbial biomass of Goa transect was 1601.3±561.9 μgCg^{-1} dry weight (DW). High biomass of 2184±37.02 μgCg^{-1} DW was recorded at 1000 m depth and least biomass was observed at 200 m depth (Fig.17A).

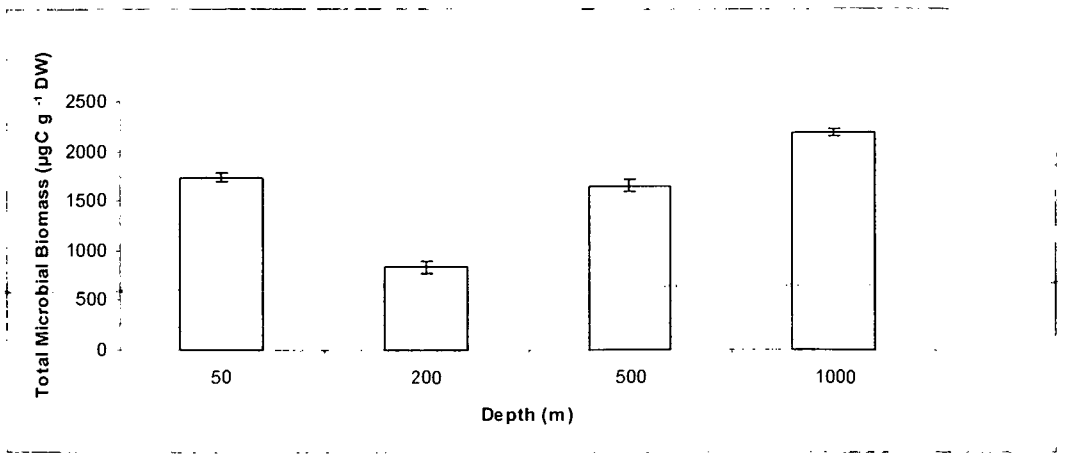


Fig. 17A. Variation in the total microbial biomass at Goa transect

Average biomass of Ratnagiri transect was 1133.7±606.2 µgC g^{-1} DW. Like Goa transect, the highest biomass (1938.4±57.15 µgC g^{-1} DW) was recorded at 1000 m depth and the least (466.3±28.4 µgC g^{-1} DW) at 200 m depth (Fig.17B).

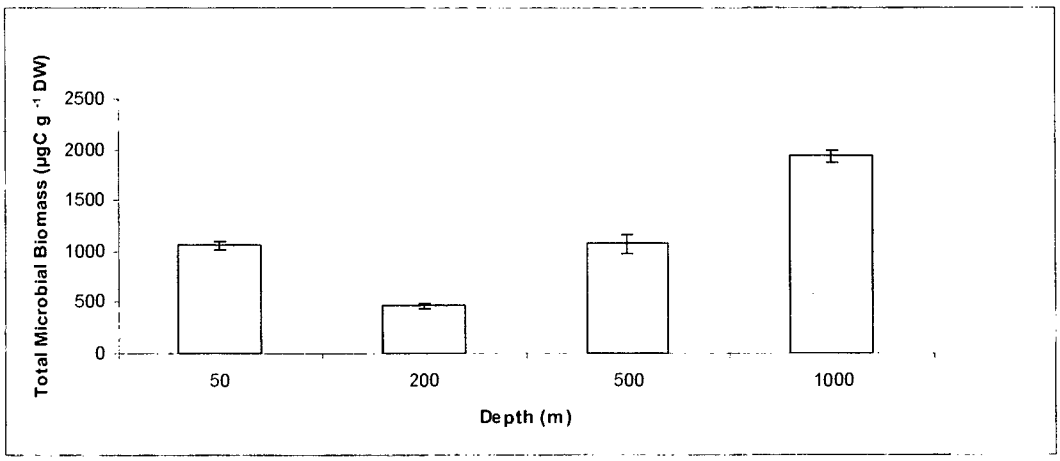


Fig. 17B. Variation in the total microbial biomass at Ratnagiri transect

The total benthic microbial biomass of Karwar transect was 1378.9± 534 µgC g^{-1} DW which was higher than Ratnagiri transect. Depth-wise distribution of the biomass showed the same trend as the other two transects with low biomass at 200 m depth (668.7±69.3 µgCg^{-1} DW) and high biomass of 1893.6±69.9 µgC g^{-1} DW at 1000 m depth (Fig.17C).

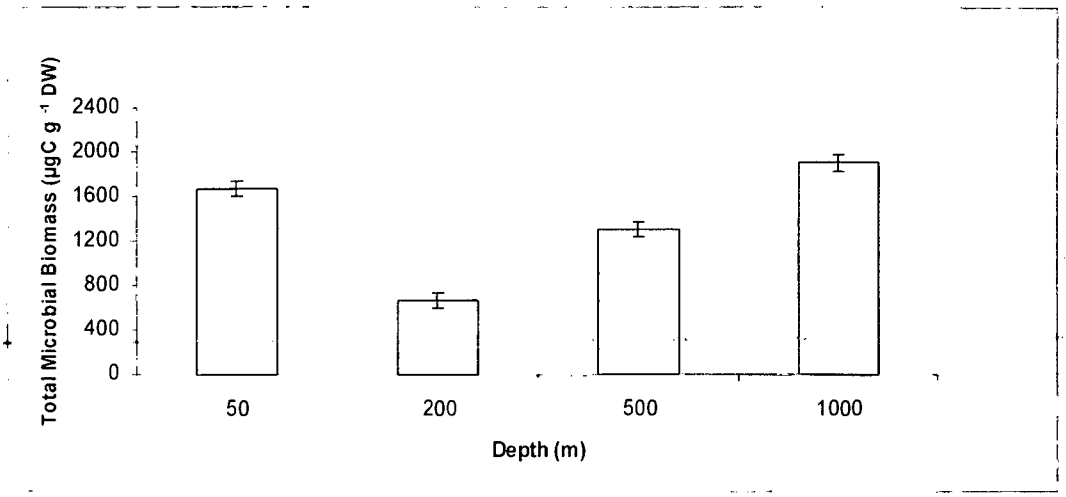


Fig. 17C. Variation in the total microbial biomass of Karwar transect

ATP concentration was highest in the non-OMZ region in Karwar (1659.8 ± 67.58 µgCg^{-1} DW) and Goa (1738.7 ± 41 µgCg^{-1} DW) transects while it was higher in the OMZ region in the Ratnagiri transect with 1159.3 ± 739 µgCg^{-1} DW (Fig. 17D).

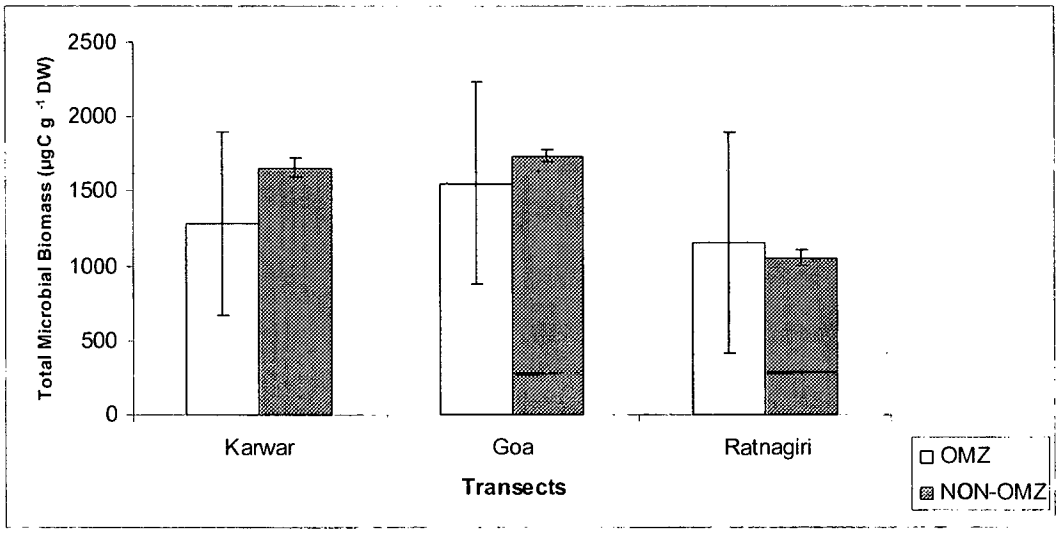


Fig.17D. Comparison of ATP in the OMZ and non-OMZ of (D)Karwar, (E)Goa and (F)Ratnagiri transects.

4.4.2. Bacterial Abundance

4.4.2.1. Direct Counts

(i)Total Direct Count (TDC)

In Goa transect, TDC was 42.85 ± 27.60 x 10^8 g^{-1}DW averaged over depths. In the deeper depths (500 and 1000 m), the total bacterial abundance was an order higher (10^9 g^{-1}DW). Maximum abundance (67±4.27 x10^8 g^{-1}DW) was recorded at 1000 m depth (Fig. 18A).

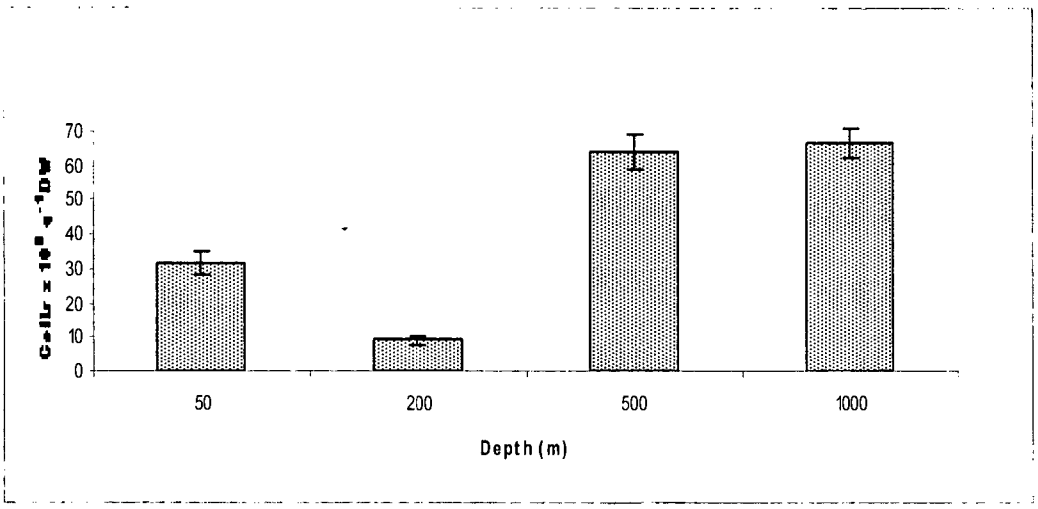


Fig. 18A. Variation in TDC at Goa transect

Total count on an average was 46.3 ± 39.4 x 10^8 g^{-1}DW in Ratnagiri transect. The highest count (98±1.45 x 10^8 g^{-1}DW) was recorded at 1000 m and the lowest (6.3±1.456 x 10^8 g^{-1}DW) at 200 m depth. At 50, 500 and 1000 m depths, the TDC were an order higher (10^9 g^{-1}DW) compared to 200 m (Fig. 18B).

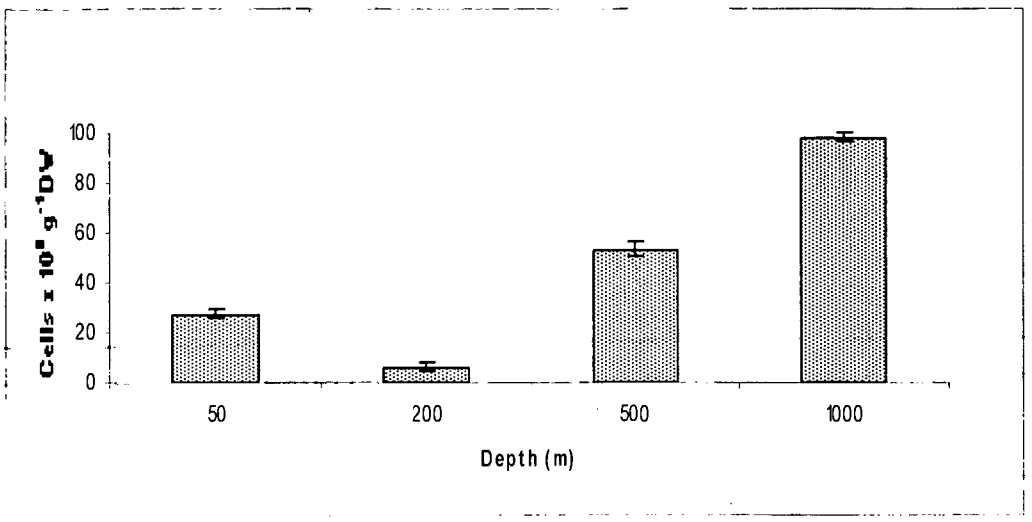


Fig. 18B. Variation in TDC at Ratnagiri transect

Average TDC was 39.5 ± 24.5 x 10^8 g^{-1}DW for Karwar. Unlike Goa and Ratnagiri, TDC of Karwar was high at 200 m (5.9±1.22x10^9 g^{-1}DW). Like the other two transects higher count was recorded at 1000 m depth (Fig.18C).

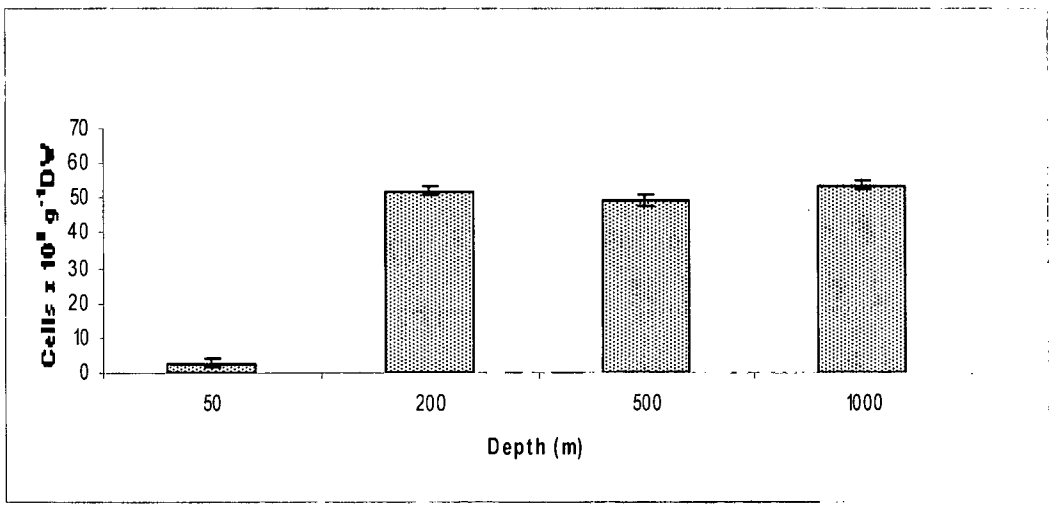


Fig. 18C. Variation in TDC at Karwar transect

TDC was higher in the OMZ compared to the non-OMZ with all the transects with almost equal proportions ie 51.73±2.3, 46.6±32.4 and 52.6±45.8 x 10^8 g^{-1}DW in Karwar, Goa and Ratnagiri respectively (Fig. 18D). Among the non-OMZ region Karwar gave the lowest TDC ie 2.8± 1.2 x 10^8 g^{-1}DW.

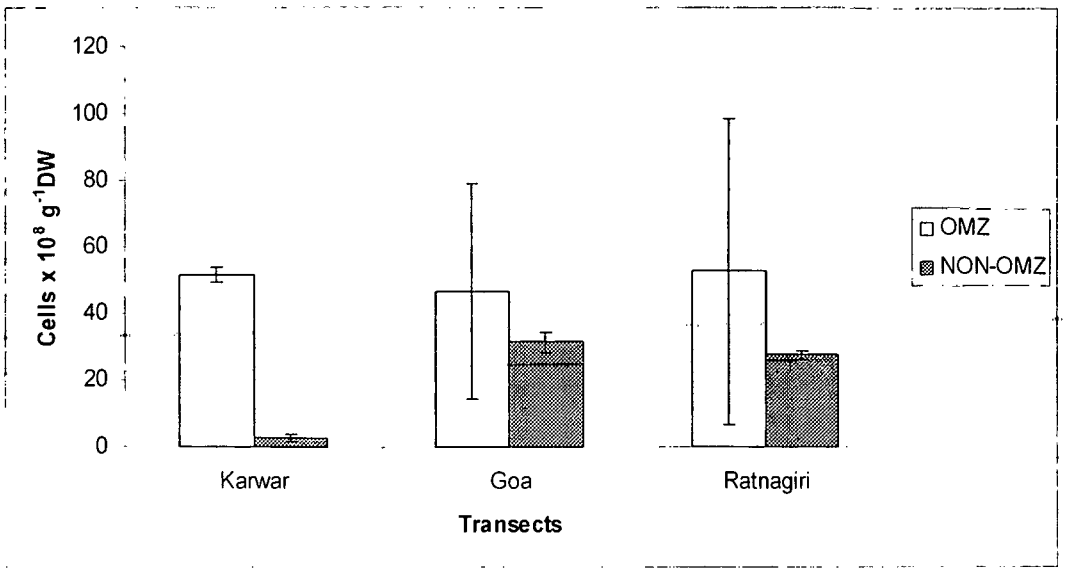


Fig.18D. Comparison of TDC in the OMZ and non-OMZ of (D)Karwar, (E)Goa and (F)Ratnagiri transects.

(ii) Total Viable Count - aerobic and anaerobic (TVCa and TVCan)

The average TVCa and TVCan at Goa transect were 4.21± 2.54 x10^8 g^{-1}DW and 1.59 ± 1.69 x10^8 g^{-1}DW, respectively with aerobic counts being three times higher than anaerobic counts. The TVCan was of the order 10^8 in 500 and 1000 m depths while it was of the order of 10^7 in 200 m. Depth-wise distribution of TVCa and TVCan is given in Figure 19A.

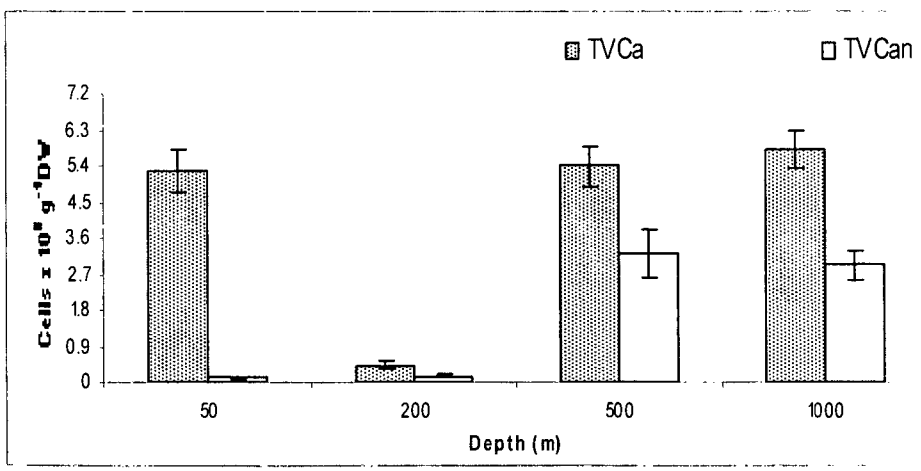


Fig.19A. Variation in TVCa and TVCan at Goa transect.

TVCa and TVCan were 3.9 ± 2.3 x 10^8 g^{-1}DW and 1.62 ± 1.5 x10^8 g^{-1}DW, respectively for Ratnagiri transect. The distribution of TVCa and TVCan at different depths is given in Figure 19B. The number and trend in

distribution of the two groups was similar to Goa transect except that it was one order less at 50 m depth.

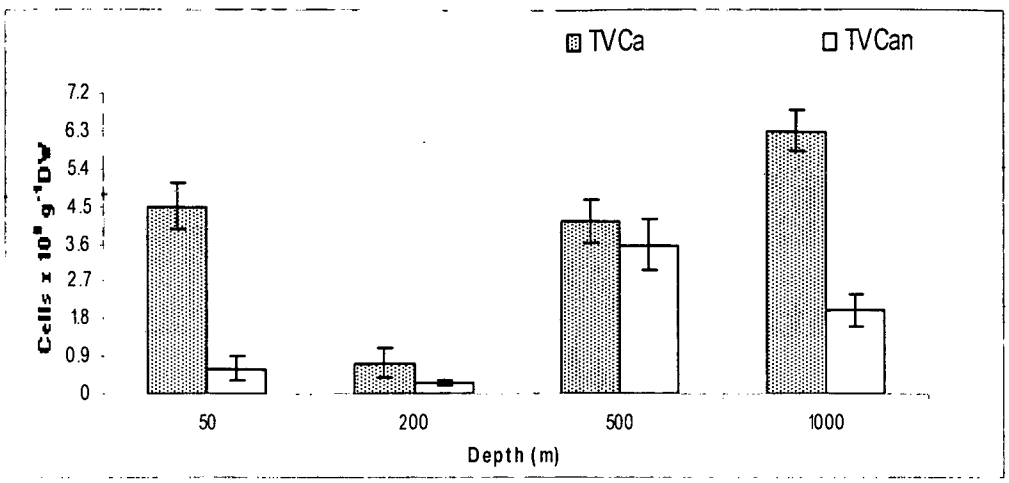


Fig. 19B. Variation in TVCa and TVCan at Ratnagiri transect

At Karwar, TVCa and TVCan were 5.6 ± 3.6 x 10^8 g^{-1}DW and 1.6 ± 1.1 x10^8 g^{-1}DW, respectively. The distribution of TVCa and TVCan at different depths is shown in Figure 19C. As in the other two transects, the aerobic counts were higher than anaerobic counts at all the depths.

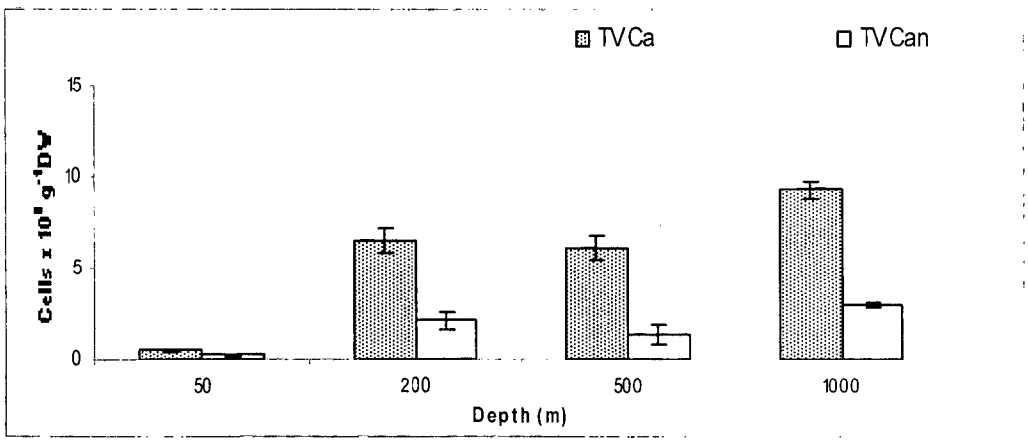


Fig. 19C. Variation in TVCa and TVCan at Karwar transect

TDC was one order more than TVCa and TVCan in all the three transects. Ratio between TVCa, TVCan and TDC of the three transects is given in Table 7A, B&C. In Goa transect, the ratio of TVCa verse TDC decreased with depth and vice versa for TVCan. The ratio of TVCan to TVCa showed an increasing trend with station depths. At 50 m depth, the ratios were the highest showing the dominance of aerobic bacteria.

Depths (m)	Ratios		
	TVCa/TDC	TVCan/TDC	TVCan/TVCa
50	0.17	0.003	0.02
200	0.05	0.02	0.38
500	0.08	0.05	0.6
1000	0.09	0.04	0.5

Table 7A. Ratios of three direct bacterial counts of Goa transect

The aerobic viability decreased with depth while the anaerobic viability did not show any particular trend for Ratnagiri transect (Table 7B). The ratio of TVCan to TVCa though increased with depth, it showed a decrease in 1000 m.

Depths (m)	Ratios		
	TVCa/TDC	TVCan/TDC	TVCan/TVCa
50	0.16	0.02	0.13
200	0.12	0.04	0.37
500	0.08	0.07	0.86
1000	0.06	0.02	0.32

Table 7B. Ratios of three direct bacterial counts of Ratnagiri transect

Ratio between TVCa, TVCan and TDC at Karwar showed the same trend as the other transects (Table 7C). The aerobic viability was the highest at 50 m and 1000 m depths.

Depths (m)	Ratios		
	TVCa/TDC	TVCan/TDC	TVCan/TVCa
50	0.17	0.08	0.44
200	0.12	0.04	0.32
500	0.12	0.03	0.23
1000	0.17	0.06	0.32

Table 7C. Ratios of three direct bacterial counts of Karwar transect

Aerobic viability was higher in OMZ in Karwar with $7.3 \pm 1.7 \times 10^8$ g^{-1}DW while in Goa and Ratnagiri it was observed highest in the non-OMZ region with 5.2 ± 0.59 and $4.5 \pm 0.5 \times 10^8$ g^{-1}DW (Fig. 19D). Anaerobic

viability was observed in the OMZ region compared to non-OMZ in all the transects ranging from 1.96 ± 1.6 to 2.1 ± 0.8 x10^8 g^{-1}DW in Ratnagiri, Goa and Karwar respectively.

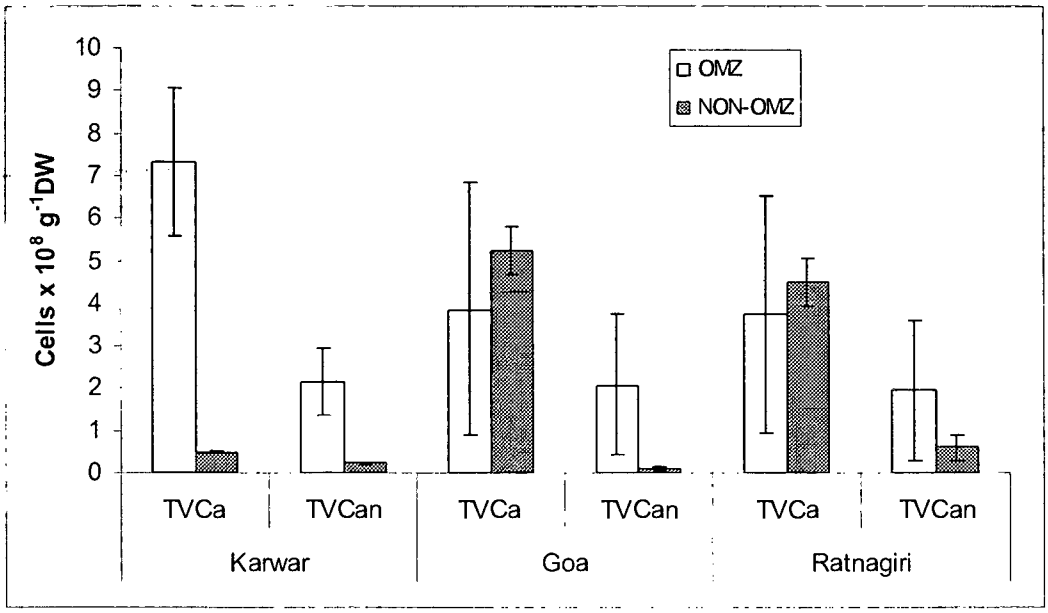


Fig.19D. Comparison of TVCa and TVCan in the OMZ and non-OMZ of Karwar, Goa and Ratnagiri transects.

4.4.2.2. Retrievable Counts

(i) Colony Forming Units-Aerobic and Anaerobic (CFUa and CFUan)

CFUa and CFUan of Goa transect were 1.81 ± 1.25 and 9.81 ± 8.18 x 10^3 g^{-1}DW respectively in high concentration of nutrient (100% NA medium) whereas in low nutrient (0% nutrient sea water agar medium) the abundance were 1.42 ± 2.83 and 3.3 ± 2.55 x 10^3 g^{-1}DW, irrespective of depths (Fig. 20A).

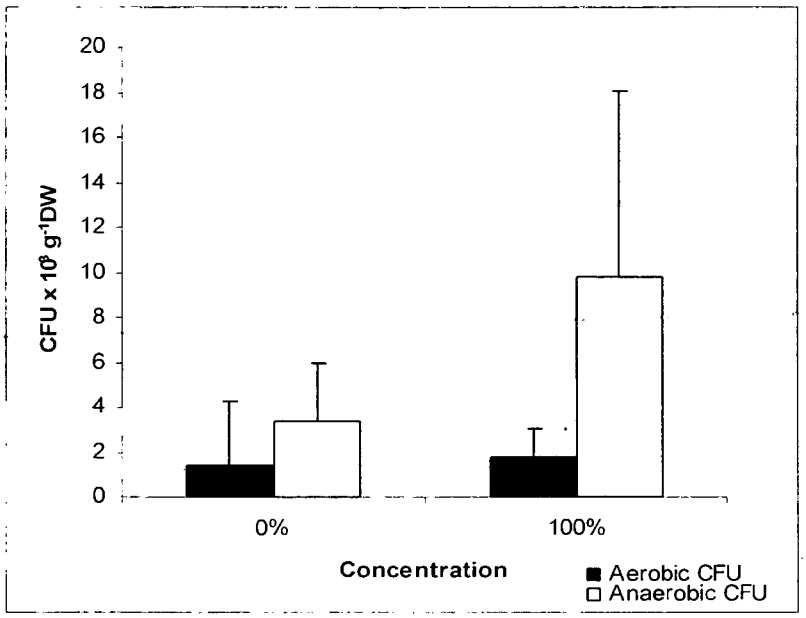


Fig. 20A. CFUa and CFUan of Goa transect

CFUa and CFUan of Ratnagiri transect were 1.64 ± 2.05 and 10.3 ± 5.09 x 10^3 g^{-1}DW in 100% nutrient concentration and 0.65 ± 0.96 and 3.5 ± 2.2 x 10^3 g^{-1}DW at 0% nutrient concentration (Fig. 20B).

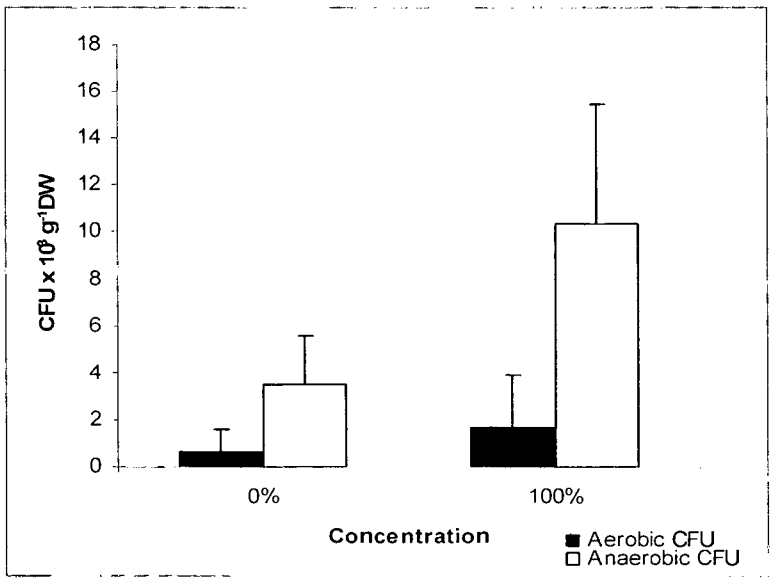


Fig. 20B. CFUa and CFUan of Ratnagiri transect

At Karwar CFUa and CFUan were 1.4 ± 0.4 and 4.9 ± 4.1 x 10^3 g^{-1}DW in 100% nutrient concentration while at 0% nutrient concentration the abundances were 0.89 ± 1.1 and 1.7 ± 0.9 x 10^3 g^{-1}DW, respectively (Fig 20C).

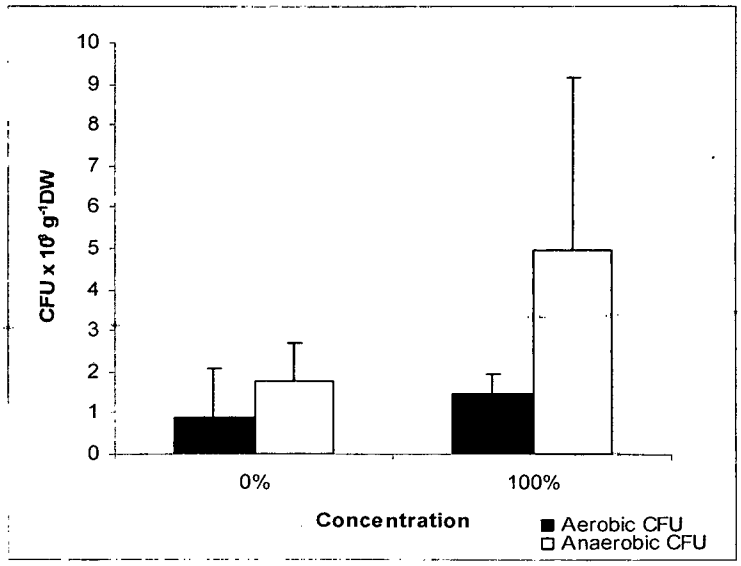


Fig. 20C. CFUa and CFUan of Karwar transect

At low nutrient concentrations of 10 and 25% nutrient agar, CFUa for Goa transect was 11.6 ± 13 and 5.9 ± 7.18 x 10^3 g^{-1} DW, respectively.

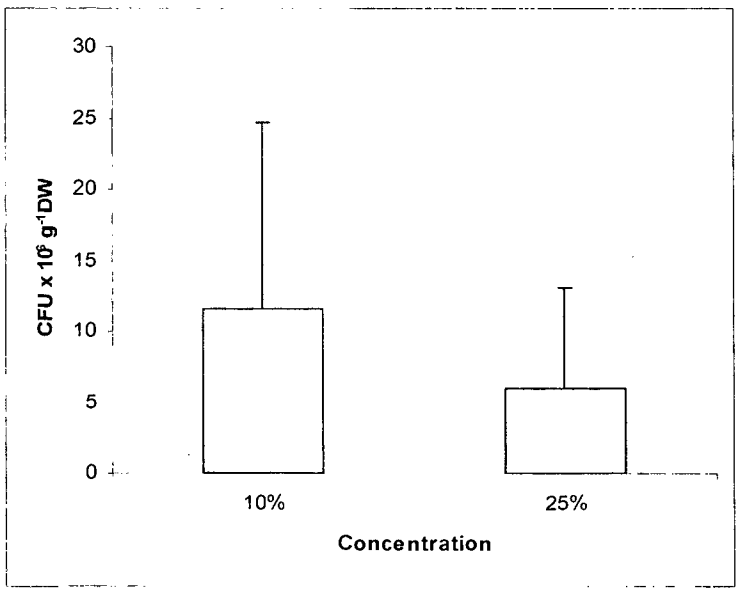


Fig. 21A. CFUa of Goa transect at low nutrient concentrations.

At Ratnagiri transect, CFUa were, 3.6 ± 3.4 and 3.3 ± 1.3 x 10^3 g^{-1}DW at low nutrient concentrations (10 and 25% nutrient agar), respectively. In this transect, there was marginal difference in counts with 10% slightly more than 25% (Fig. 21B).

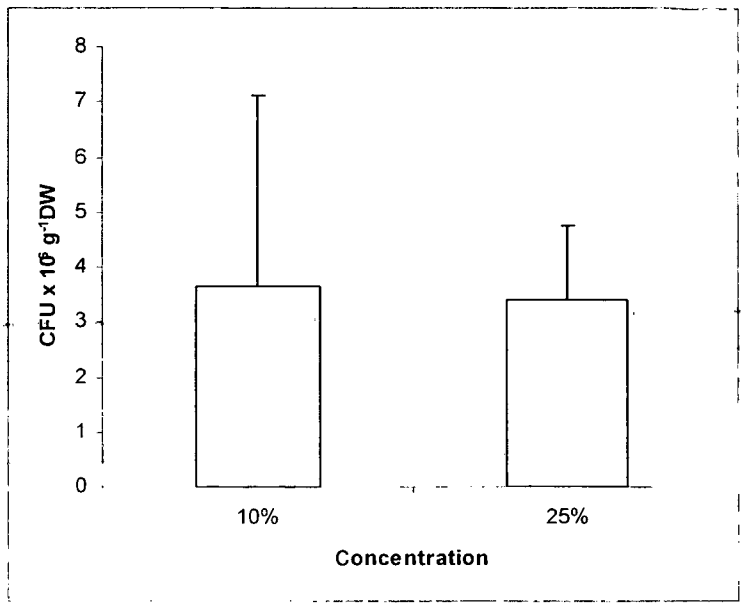


Fig. 21B. CFUa of Ratnagiri transect at low nutrient concentrations

Comparison of the counts at two low concentrations, 10% gave higher counts to 25% at Karwar. CFUa was 5.5 ± 5.5 and 5.5 ± 1.03 x 10^3 g^{-1} DW in 10 and 25% nutrient agar, respectively (Fig. 21C).

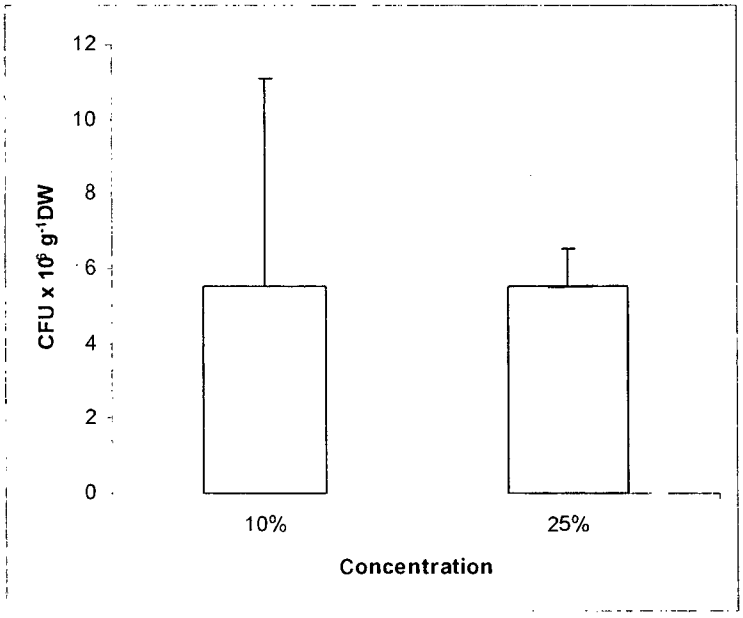


Fig. 21C. CFUa of Karwar transect at low nutrient concentrations

Overall comparison of CFUa in different nutrient concentrations of the three transects is shown in Figure 22. CFUa at all depths ranged between 10^{2-4}. There was no significant variation in bacterial numbers in different nutrient concentrations at all three transects. In non-OMZ and OMZ, low

concentration of nutrient retrieved maximum number of CFUa. The number of colony forming units in different media concentrations based on serial dilutions is given in Appendix-A8.

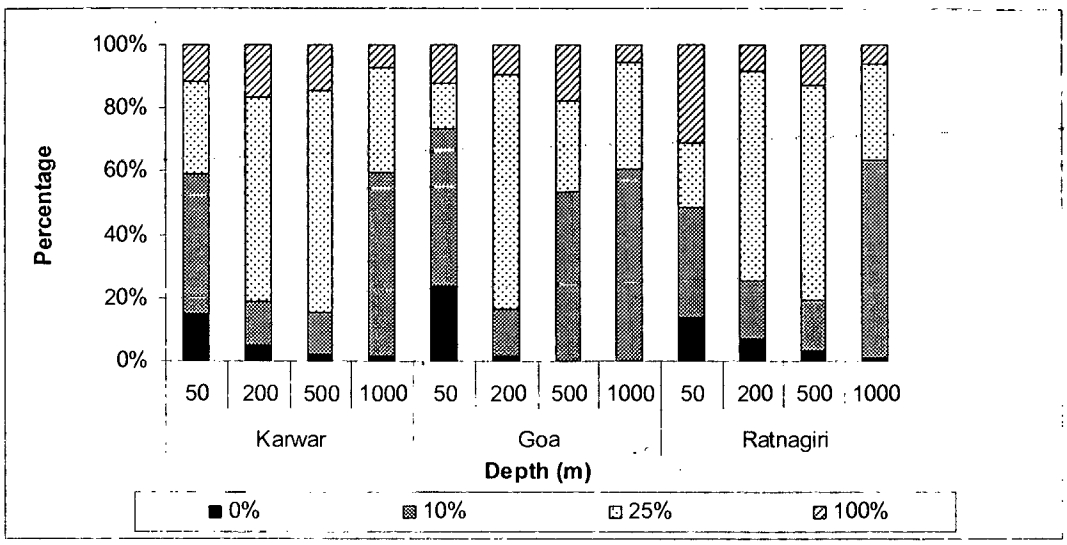


Fig. 22. Transect-wise CFUa in different nutrient concentrations

In the case of CFUan higher concentration retrieved maximum number of bacteria (Fig. 23).

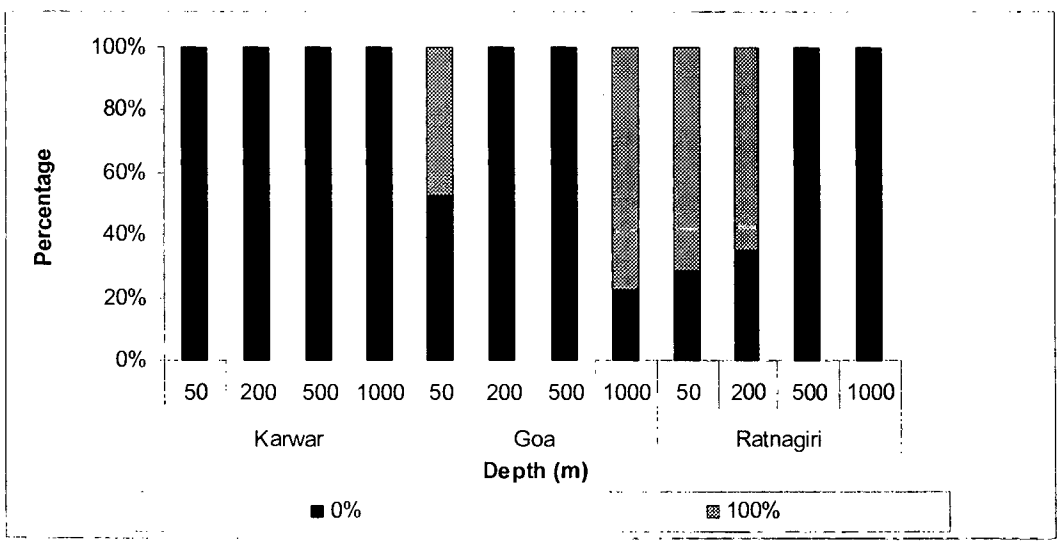


Fig. 23. Transect-wise CFUan in low and high nutrient concentrations.

(ii) Thiobacillus denitrificans like organisms (TDLO)

The average colony forming units of TDLO was 22.7 ± 20.2 x 10^3 g^{-1} DW and ranged from 10^3-10^4 in Goa transect. Depth-wise variation was

observed and higher abundance was seen at deeper stations and 500 m having a maximum CFU of 48.4±0.98 x 10^3 g^{-1} DW (Fig. 24A).

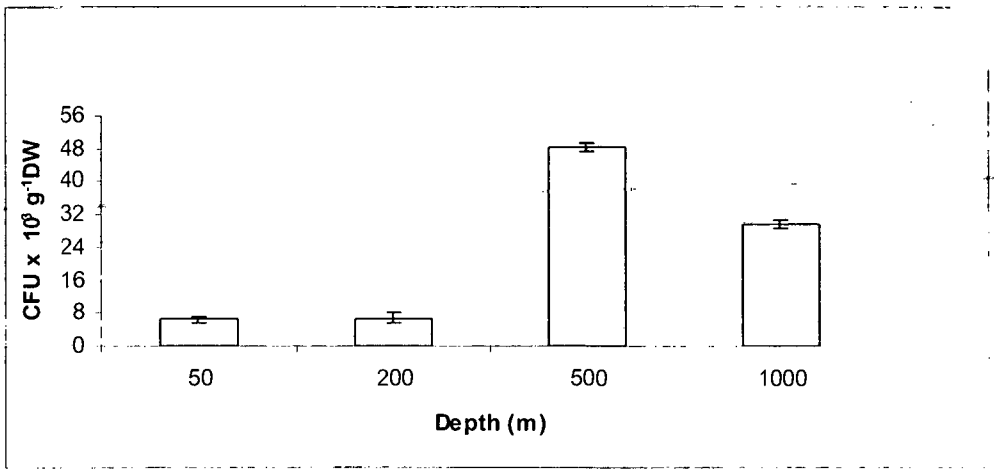


Fig. 24A. Variation in TDLO counts at Goa transect

The average colony forming units of TDLO was 18.6 ± 13.7 x 10^3 g^{-1} DW for Ratnagiri transect. Depth-wise variation observed was very similar to that of Goa transect, with higher abundance of 38.7±1.16 x 10^3 g^{-1} DW at 500 m depth (Fig. 24B).

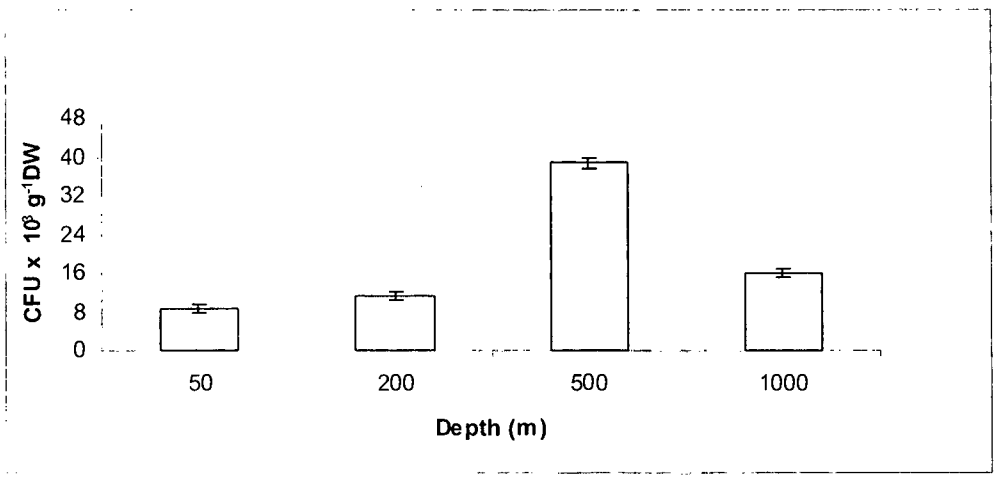


Fig. 24B. Variation in TDLO counts at Ratnagiri transect

The average colony forming units of TDLO at Karwar transect was 1.63 ± 1.6 x 10^3 g^{-1} DW. The number increased with increase in depth with the highest abundance at 1000 m (3.27±0.18 x 10^3 g^{-1} DW). No TDLO counts were obtained at 50 m (Fig. 24C).

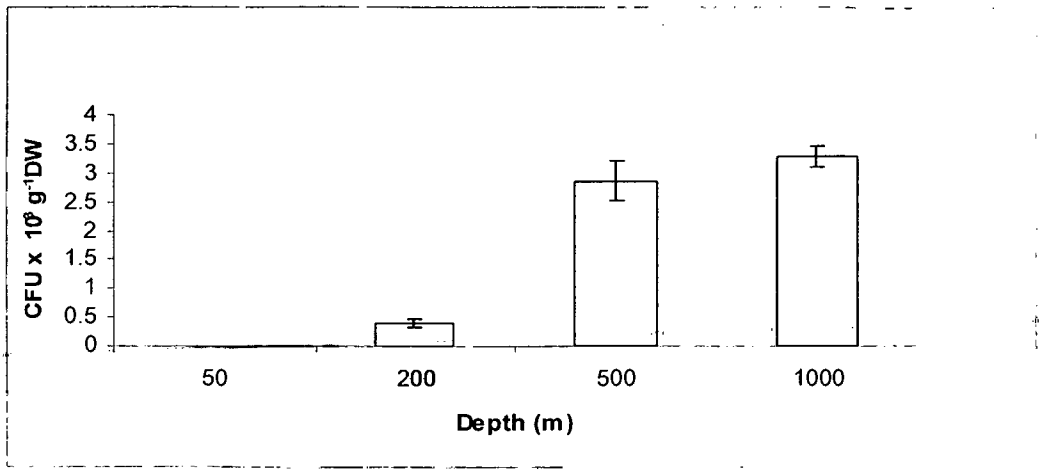


Fig. 24C. Variation in TDLO counts at Karwar transect

(iii) Sulphate Reducing Bacteria (SRB)

The average SRB acetate (act) and SRB lactate (lac) utilizing bacteria were 41.4 ± 33.8 x 10^3 g^{-1}DW and 46.9 ± 37.4 x 10^3 g^{-1}DW, respectively. The abundance was in the order 10^4 at all depths (Fig. 25A). At all depths SRBlac was higher than SRBact. Interestingly, the abundance of both the groups increased with depth, and high numbers were seen at 500 and 1000 m depths.

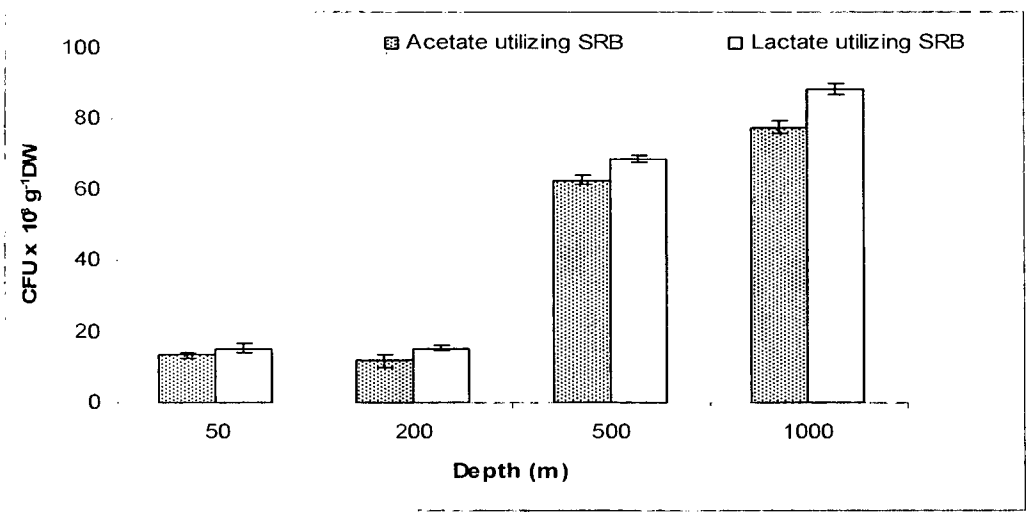


Fig. 25A. Variation in the abundance of SRBact and SRBlac in Goa transect

The average SRBact and SRBlac of Ratnagiri transect were 22.3 ± 9.3 x 10^3 g^{-1} DW and 24 ± 10.2 x 10^3 g^{-1} DW, respectively Fig. 25B. The number was less than that at Goa transect. Like Goa, SRBlac was higher

than SRBact. The abundance of both the groups was the highest at 500 m. Analysis of variance showed that there was significant variation between groups within the various depths ($p < 0.001$).

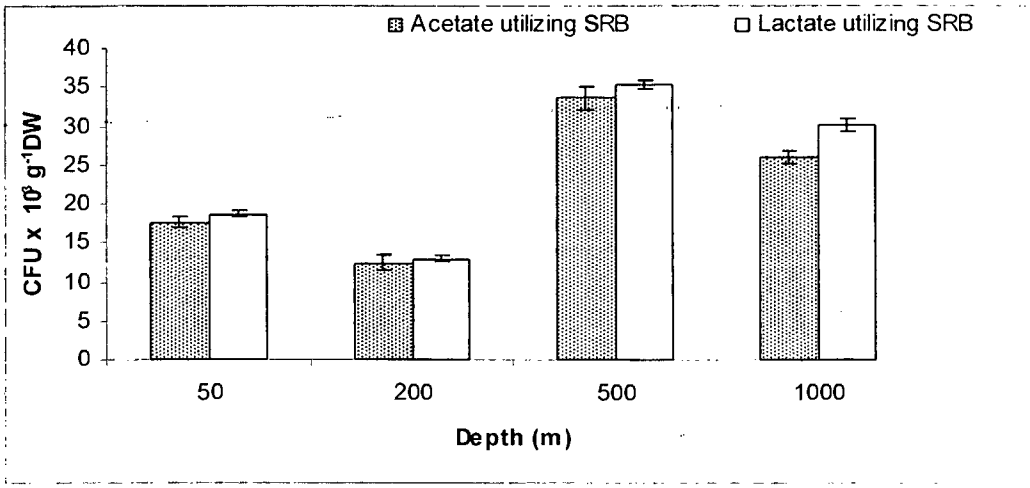


Fig. 25B. Variation in the abundance of SRBact and SRBlac in Ratnagiri transect

The average SRBact and SRBlac were 39.1 ± 9.2 x 10^3 g^{-1} DW and 41.6 ± 12.8 x 10^3 g^{-1} DW respectively in Karwar transect (Fig. 25C). SRBlac was higher than SRBact at all depths except 50 m. The abundance of both the groups was high at 1000 m.

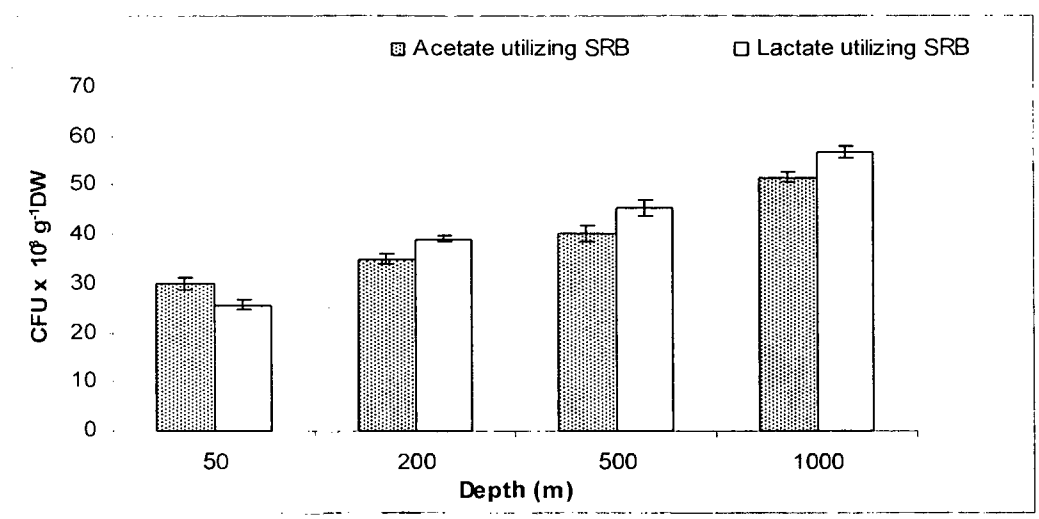


Fig. 25C. Variation in the abundance of SRBact and SRBlac in Karwar transect

Analysis of variance showed that there was significant variation between groups within depths for the three transects ($p < 0.01$). The ratios of

anaerobic groups are given in Table 8. SRBact to SRBlac was constant throughout the depth. The ratio of TDLO to CFUan and the ratio of sulphate reducing bacteria to anaerobic CFU were lowest in 200 m in Goa transect. In Ratnagiri transect, the ratio of SRB to the total CFUan increased from 50 m to 1000 m. Similar trend was also seen at Karwar.

		Ratio					
Transects	Depths (m)	SRBact/ CFUan	SRBlac/ CFUan	TDLO/ SRBact	TDLO/ SRBlac	SRBact/ SRBlac	TDLO/ CFUan
Karwar	50	30.3	26.1	0.0002	0.0002	1.2	0.006
	200	12.6	14	0.01	0.01	0.9	0.1
	500	8.9	10	0.07	0.06	0.9	0.6
	1000	10	10.9	0.06	0.06	0.9	0.6
Goa	50	9.7	11.1	0.5	0.4	0.9	4.6
	200	3.3	4.1	0.6	0.4	0.8	1.8
	500	7.7	8.4	0.8	0.7	0.9	6
	1000	5.9	6.7	0.4	0.3	0.9	2.2
Ratnagiri	50	1.5	1.6	0.5	0.5	0.9	0.7
	200	3.5	3.7	0.9	0.9	1	3.2
	500	5.5	5.8	1.2	1.1	1	6.4
	1000	4.1	4.7	0.6	0.5	0.9	2.5

Table 8. Ratios of the various anaerobic groups at three transects.

4.4.3. Statistical Analysis

All the bacterial parameters of the transects were pooled and subjected to PCA after normalization. High congruencies were observed between abundance obtained from different depth stations as the calculated three eigenvectors, of the direct (TDC) and direct viable counts (TVCa and TVCan) incorporated 91% of the total variance of the data. Table and Figure are given under Appendix-A9 as emphasis is given for OMZ and non-OMZ data. The PCA of the OMZ stations data showed that the first two components brought about 78 and 99% of the variance while in the non-OMZ, PC1 and 2 accounted for 84 and 100% of the variance (Table 9).

Eigenvalues - OMZ

PC	Eigen values	%Variation	Cum. %Variation
1	0.698	78.3	78.3
2	0.186	20.8	99.1

Eigenvalues - non-OMZ

PC	Eigen values	%Variation	Cum. %Variation
1	0.69	84.6	84.6
2	0.126	15.4	100.0

Eigenvectors

(Coefficients in the linear combinations of variables making up PC's)

Variable	OMZ		Non-OMZ	
	PC1	PC2	PC1	PC2
TDC	0.016	0.014	-0.033	0.019
TVCa	0.017	0.019	-0.035	0.021
TVCan	0.02	0.012	-0.007	-0.058
CFUa (0%)	**-0.456**	**0.886**	-0.002	0.061
CFUa (10%)	0.073	0.021	0.004	0.045
CFUa (25%)	0.023	0.015	0.014	0
CFUa (100%)	0.04	0.006	-0.019	-0.021
CFUan (0%)	0.01	-0.056	-0.021	-0.084
CFUan (100%)	0.023	-0.001	**-0.953**	**0.294**
TDLO	0.037	-0.042	-0.009	-0.013
SRBact	0.022	-0.003	0.015	-0.022
SRBlac	0.022	-0.002	0.009	-0.015
ATP	**0.884**	**0.457**	**0.296**	**0.946**

Table 9. Eigen values and coefficient of all variables in the OMZ and non-OMZ

The important biotic factor influencing both the regions was ATP. In the OMZ CFUa (0%) and non-OMZ CFUan (100%) were the other factors responsible for the variation of the two zones. On the PCA plots, the first two vectors of OMZ and non-OMZ are compared to the station depths of the three transects (Fig. 26A&B). As can be seen the OMZ stations clustered but station depth variation was observed within the OMZ.

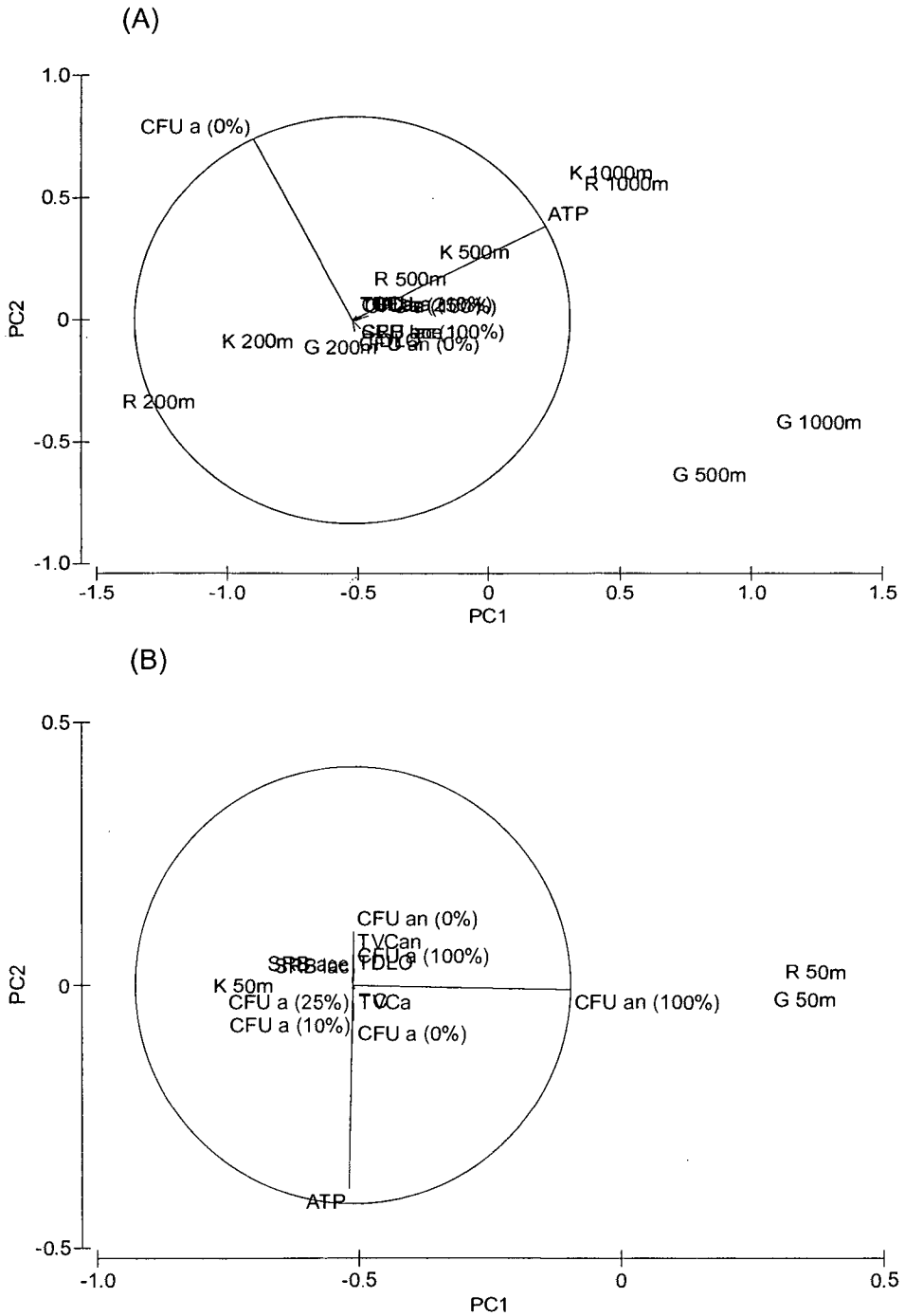


Fig. 26. Principal Component Analysis of variables A) OMZ sites B) non- OMZ with Eigen vectors > 0.25

Multiple correlation analysis of all the variables grouped into OMZ and non-OMZ and only significant variables greater than 99.99% are given in Table10. As seen from the table, direct bacterial abundance was limited by maximum variables followed by the culturable abundance. The SRB groups were limited by the same variables.

	TDC	TVCa	TVCan	CFU a (0%)	CFU a (10%)	CFU a (25%)	CFU a (100%)	CFU an (0%)	CFU an (100%)	TDLO	SRB act	SRB lac	ATP
Temperature	-0.7	-0.66	-0.68		-0.88	-0.65	-0.61		-0.81			-0.59	-0.89
Salinity								0.62					
pH	-0.72	-0.55*	-0.55*								-0.56	-0.61	-0.53
DO		0.49*			0.64			-0.6					0.64
Nitrate	0.63	0.57	0.57	-0.44	0.93	0.76	0.66		0.89		0.66	0.68	0.93
Silicate	0.50*	0.53*			0.88	0.62			0.73				0.87
Water Content	0.82	0.77	0.85		0.69	0.65	0.75		0.72	0.46*	0.79	0.78	0.77
TN	0.73	0.74	0.83				0.73				0.79	0.77	
TS				-0.89									
Carbohydrates	0.63	0.57				0.59							
Lipids	0.66	0.65	0.58		0.88		0.64		0.75		0.69	0.71	0.93
Proteins	-0.6	-0.61	-0.61		-0.67				-0.64				-0.71
Sand	-0.75	-0.86	-0.89		-0.67	-0.72	-0.86					-0.81	-0.59
Silt	0.63	0.58											
Clay						0.56							

Table 10. Significant correlation coefficient ($p<0.001$) of the variables (* $p < 0.01$)

	TDC	TVCa	TVCan	CFU a (0%)	CFU a (10%)	CFU a (25%)	CFU a (100%)	CFU an (0%)	CFU an (100%)	TDLO	SRB act	SRB lac	ATP
Temperature	0.98	0.99				-0.92	0.65		0.89	0.57	-0.99	-0.98	
Salinity	0.96	0.96		0.59		-0.87	0.56		0.83		-1.00	-1.00	
pH	-0.89	-0.89		-0.73		0.76			-0.71		0.98	0.99	
DO	-0.66	-0.65	-0.78		0.71	0.82	-0.99	-0.91	-0.86	-1.00			0.94
Nitrate	0.99	0.99				-0.92	0.65		0.89	0.58	-0.99	-0.98	
Nitrite	1.00	1.00				-0.98	0.79		0.96	0.73	-0.95	-0.93	
Phosphate	0.73	0.72	0.72		-0.65	-0.86	1.00	0.87	0.90	1.00			-0.90
Silicate	0.63	0.63	0.80		-0.74	-0.79	0.98	0.92	0.84	1.00			-0.95
Eh	0.97	0.97		0.57		-0.88	0.58		0.85		-1.00	-1.00	
Water Content			1.00	-0.95	-1.00		0.61	0.95		0.68			-0.92
TC	-0.99	-0.99				0.99	-0.83		-0.98	-0.77	0.92	0.90	
TN	0.97	0.97				-1.00	0.90		1.00	0.85	-0.86	-0.83	-0.57
TH	0.63	0.62	0.81		-0.74	-0.79	0.98	0.92	0.83	0.99			-0.95
TS	0.83	0.84		0.81	0.60	-0.68			0.63		-0.96	-0.97	
TOC	-1.00	-1.00				0.98	-0.80			-0.74	0.94	0.92	
Carbohydrates	0.96	0.96		0.60		-0.86			0.82			-1.00	
Lipids	0.73	0.73	-0.65	0.89	0.73						-0.90	-0.92	
Proteins	0.95	0.96		0.60		-0.86			0.82		-1.00	-1.00	
Sand	-1.00	-1.00				0.98	-0.80		-0.97	-0.74	0.94	0.92	
Silt			-0.89	1.00	0.93			-0.76			-0.66		0.71
Clay	0.82	0.81	0.62			-0.93	1.00	0.79	0.95		-0.61		-0.83

Table 11. Significant correlation coefficient ($p<0.001$) of the variables

In the case of non-OMZ, most of the significant variables were the same except that carbon and nitrate were the additional variables influencing the culturable fractions of the anaerobic groups (Table 11.)

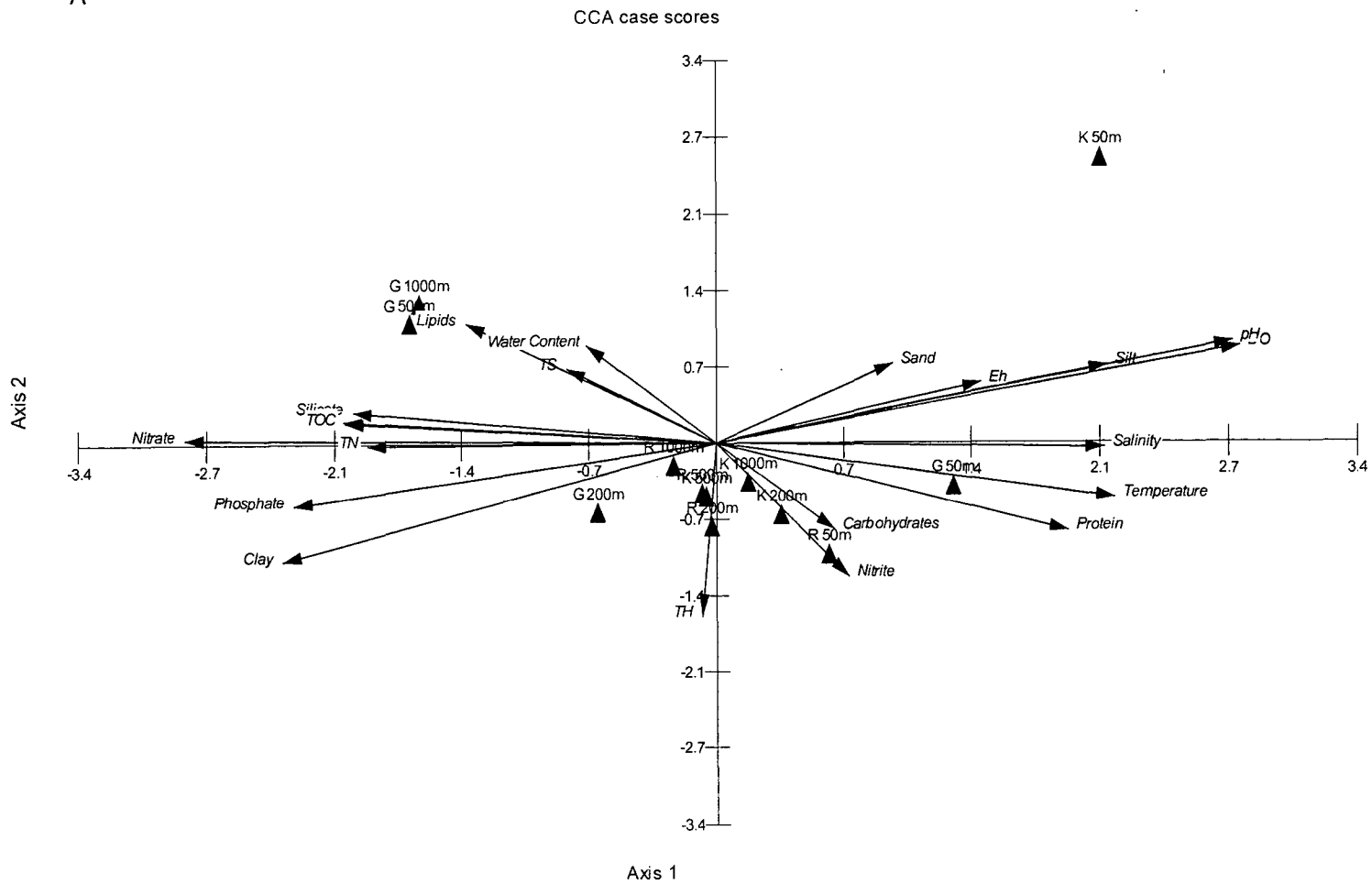
Ordination analysis was performed to analyze and demonstrate the relationship between bacterial abundance and environmental parameters of the overlying water and sediment. CCA revealed high values of variance inflation factors (>20) for variables like temperature, pH salinity, nitrate, nitrite silicate, protein, etc indicating collinearity with other variables (Table 12). Despite, collinearity, all variables were included in CCA to achieve a preliminary insight into the relationship between abundance and environmental parameters. The sum of all eigenvalues of variables indicated an overall variance of the data set. Total variance that could be explained by environmental variation accounted for 83% of the variables as indicated by the sum of all canonical eigenvalues (Table12). The CCA for all the sites using abundance data and environmental parameters revealed that the first canonical axis is a significant function of a dissolved oxygen gradient (r= 0.77), pH (r=0.76) and sediment temperature (r=0.59). Also it is a significant function of a nitrate gradient (r=-0.786), followed by clay content (r=-0.642) and phosphate (r=-0.626). The second axis is a function of parameters such as water content (r=0.243), carbohydrates (r=-0.214), nitrite (r=-0.331) and TH (r=-0.428). The biplot scaling of CCA related 50 m station off Goa and Karwar to the first axis (Figure 27). Parameters associated with the first axis had a positive influence on the 50 m station off Goa, where as at 50 m station at Ratnagiri was influenced negatively by carbohydrates and nitrite. Interestingly the shallow station of Karwar (50 m) was not influenced by many of the factors. The OMZ stations were associated with canonical axes 1 and 2 with the former having high weightage. Interestingly, the deeper stations of Goa transect was positioned distinctly with the labile lipid representing the significant relationship with the first axes ordination.

	Eigen values	Abundance-environmental correlations	Cumulative Percentage variance of environmental data
Axis 1	0.012	1	62.26
Axis 2	0.004	1	83.339

Interset correlations between environmental variables and site scores		
	Envi. Axis 1	Envi. Axis 2
Temperature	0.587	-0.136
DO	0.771	0.238
Salinity	0.572	-0.012
pH	0.761	0.251
Nitrate	-0.786	0.012
Nitrite	0.195	-0.331
Phosphate	-0.626	-0.153
Silicate	-0.537	0.078
Eh	0.392	0.149
Water Content	-0.192	0.243
TC	-0.552	0.056
TN	-0.516	-0.004
TH	-0.021	-0.428
TS	-0.221	0.186
TOC	-0.55	0.052
Carbohydrates	0.174	-0.214
Lipids	-0.369	0.298
Proteins	0.518	-0.217
Sand	0.262	0.196
Silt	0.577	0.192
Clay	-0.642	-0.29

Table 12. Eigen values and variance decomposition for CCA and interset correlation coefficients

A



Vector scaling: 3.63

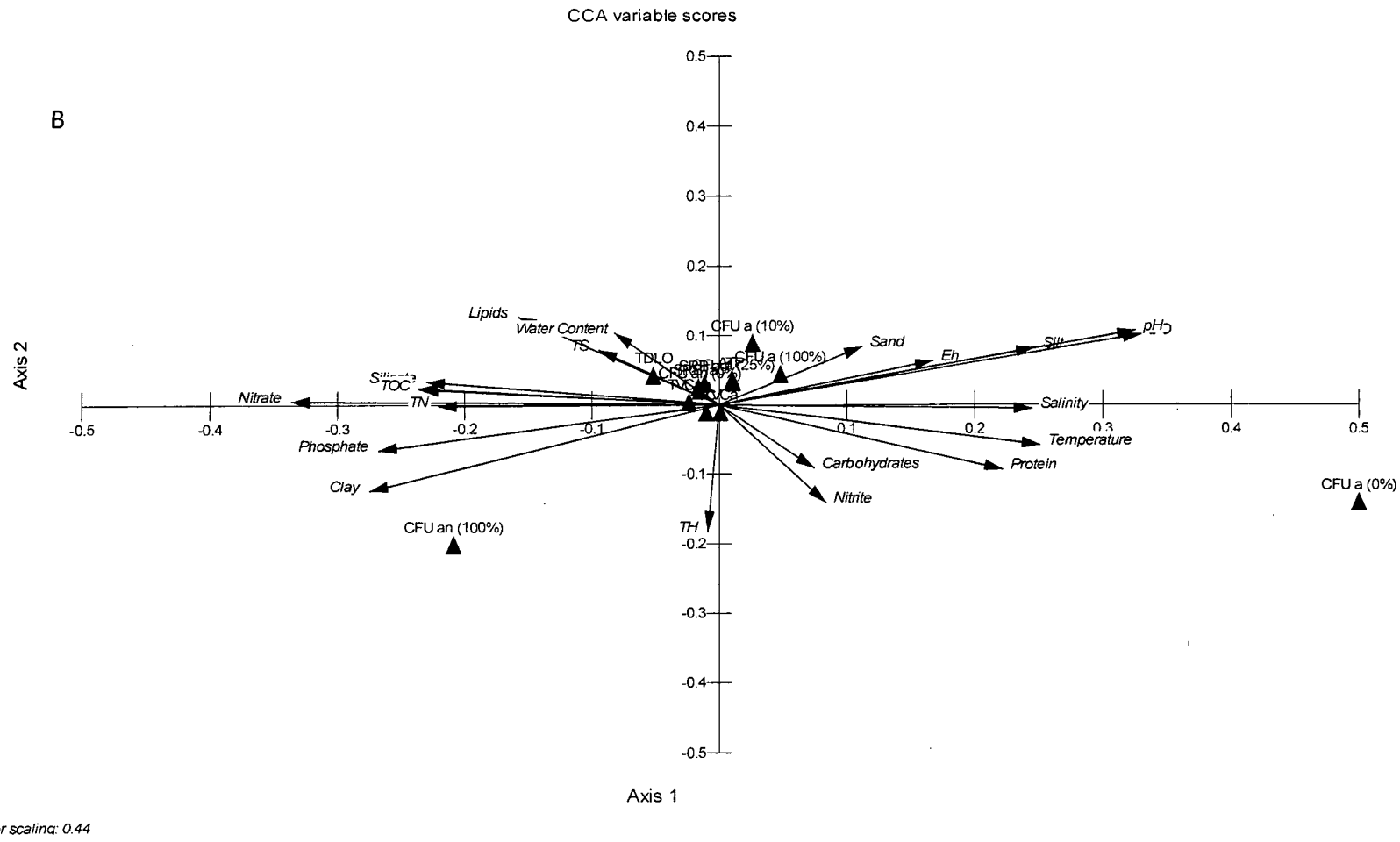


Fig. 27. Ordination diagrams for (A) Stations and (B) Abundance based on CCA of bacterial abundance and environmental variables. Arrows indicate the direction of the respective variables and length of the arrow indicates the degree of correlation of the variables with bacterial abundance

4.5. BACTERIAL DIVERSITY

For estimating the total bacterial diversity, sediment DNA was extracted by 6 different methods. DNA estimated by nanodrop spectrophotometer showed that Steffan method yielded 2.65 ng/µl, Yeates method 8.3 ng/µl, Purohit method 81ng/µl, Gray and Lovell methods 260 ng/µl and Zhou method yielded 565 ng/µl of DNA. Inspite of getting DNA, PCR amplification failed for all methods demonstrating the presence of PCR inhibitors (Appendix-A5). Modified Zhou method yielded good amplification product in 1:5 dilution of the DNA extract (Fig. 28).

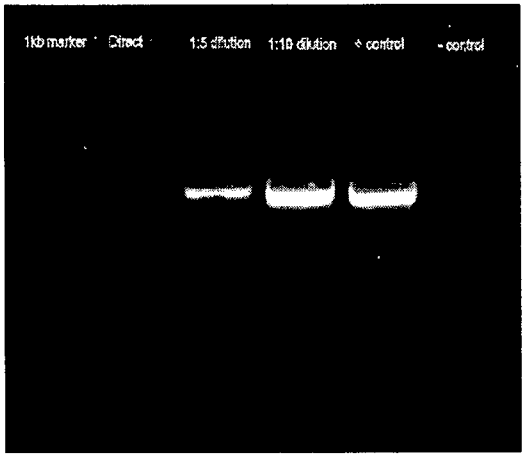


Fig. 28. Gel picture showing 16S rRNA amplified product genomic DNA extracted from sediments using Zhou method (Lane 1- 1000bp ladder, Lane 2- Direct sample, Lane 3- 1:5 dilution of sample, Lane 4- 1:10 dilution of sample, Lane 5- Positive control (MTCC 434 - *Stenotrophomonas maltophila*), Lane 6 -Negative control)

Genomic DNA of the sediment extracted by modified Zhou method has been used for investigating the diversity of bacterial community by DGGE and clone libraries (16S rRNA and *dsr*B).

4.5.1. Taxonomic diversity (DGGE)

The fingerprinting of the bacterial community structure was studied to understand the spatial variation of bacterial diversity between OMZ and non-OMZ. The PCR-DGGE analysis of the bands of the transect showed that there was variation in the number of bands/phylotypes within transects. The

total numbers of bands recorded were 648. Maximum bands were observed at Ratnagiri (57±5) (Fig. 29).

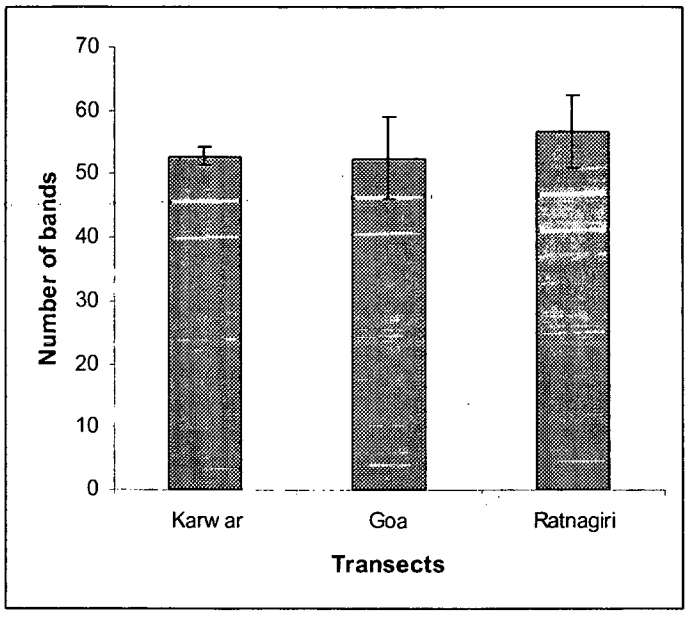


Fig. 29. Number of bands in the three transects

The number of bands encountered between stations varied between 47 and 62 and on an average was 54. Deeper stations recorded higher bands than 50 m stations. In Karwar transect the difference in bands was relatively less (Table 13).

Depths	Number of Bands			
	50 m	200 m	500 m	1000 m
G - Goa	47	51	62	50
K - Karwar	51	52	54	54
R - Ratnagiri	49	60	56	62

Table 13. Number of bands obtained in the three transects

Few bands were detected in all the stations indicating the presence of widespread phylotypes in these transects of the AS. Though there was no significant variation between transects, OMZ sites (200 m, 500 m and 1000 m) had higher number of bands than the non-OMZ sites in all the transects with an average of 54 (SD=± 2.3) and 49 (SD=± 2) respectively (Fig 30).

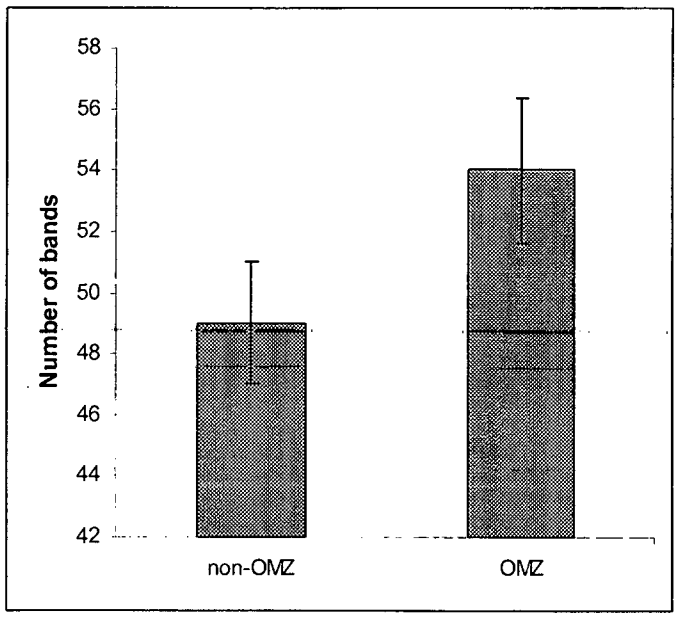


Fig. 30. Number of bands in the OMZ and non-OMZ

4.5.1.1. Phylogenetic Analysis

The data in DGGE gel shown in Figure 31 were extracted by image analysis which resulted in a binary matrix (presence or absence of bands in different samples) and intensity matrix (binary matrix information plus band intensity information).These matrices were used for statistical comparison of the different samples later on in Statistical Analysis Section 4.5.3.1. Based on the position of the bands, 82 band classes were discerned. Twenty two bands belonging to different band classes were sequenced in order to identify the the dominant members of the microbial community. This represented the structure of the most numerically dominant bacterial population at that point in time. The bands with equivalent position always corresponded with the sequence. The image of the DGGE gel and the position of the 22 excised bands are shown in Figure 31.

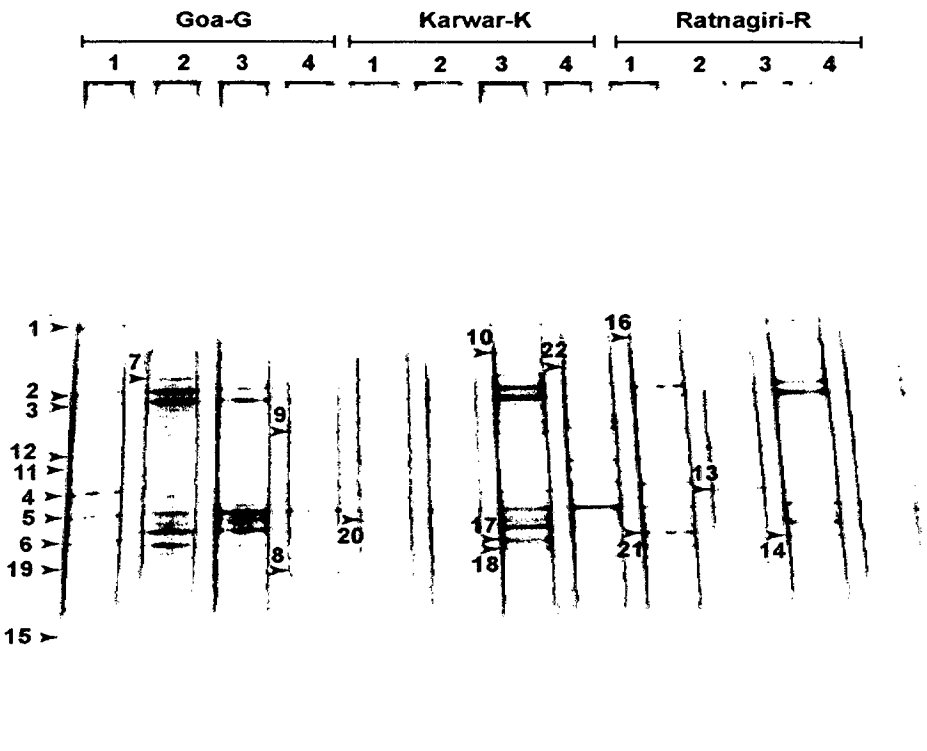


Fig. 31. DGGE profiles 16S rRNA gene diversity in the OMZ (200 m, 500 m, 1000 m) and non-OMZ (50 m) sites. Lanes 1, 2, 3 and 4 represents 50 m, 200 m, 500 m and 1000 m depths respectively. The positions and band numbers of excised bands are indicated by numbers in the DGGE gel.

Sequencing analysis showed that the band similarity ranged from 91-100%. About 90% of the sequenced bands were closely related (>98% similarity) to the sequences deposited in the GenBank while the remaining exhibited lower sequence similarity (<96% similarity). The sequences have been submitted to the GenBank (Accession numbers: GU968618-GU968638) and sequences presented in Appendix-A11. The nearest neighbour sequences with similarity values and their source is given in the Table 14. About 90% of the sequences encountered were of marine origin. Interestingly, most of the sequences were similar to the sequences reported from sediments of continental shelves, hydrothermal vents, methane seeps, coastal regions, polluted sites, fish farms and even fresh water sediments. Interestingly, a few of the sequences showed similarity to sequences from the extreme environments such as hydrothermal vents and methane seeps. Only two sequences namely EB 19 and EB 22 were marine organism-associated. The DGGE bands were mostly related to uncultured bacteria

(91-99% similarity). These results indicate that there are several uncultured bacteria and their functions are also not understood.

Band	Accesion No.	Phylum	Nearest Neighbour	Source	Similarity
EB1	GU968632	Deltaproteobacteria	Uncultured delta proteobacterium (FM179874)	Methane seeps sediment	98
EB2	GU968638	Unaffiliated	Uncultured bacterium clone 2C74 (FN431232)	Sediments of the Bizerte Lagoon	99
EB3	GU968620	Unaffiliated	Uncultured bacterium gene Clone (AB429785)	Fish farm sediments	99
EB4	GU968633	Unaffiliated	Uncultured bacterium clone (AB305483)	Hydrothermal sediments Okinawa Trough	98
EB5	GU968622	Unaffiliated	Uncultured bacterium clone Out12bac60 (GU302485)	Marine sediments Mississippi Canyon	99
EB6	GU991352	Unaffiliated	Uncultured bacterium clone BD72BR7 (GU362949)	Marine sediment South China Sea	100
EB7	GU968631	Unaffiliated	Uncultured bacterium clone Sd1-56 (GQ246337)	North Yellow Sea sediments	100
EB8	GU968626	Deltaproteobacteria	Uncultured delta proteobacterium clone KorMud-V8C107 (DQ112462)	Intertidal mudflatsediment	98
EB9	GU968636	Unaffiliated	Uncultured bacterium clone BD72BR7 (GU362949)	Marine sediment South China Sea	100
EB10	GU968627	Acidobacteria	Uncultured Acidobacteria bacterium clone ARTE12_204 (GU230431)	Atlantic Ocean coastal sediment	98
EB11	GU968637	Unaffiliated	Uncultured bacterium clone CK_1C4_62 (EU488D91)	Sea grass bed siliciclastic sedment	99
EB12	GU968630	Unaffiliated	Uncultured bacterium clone Ucp15492 (AM997638)	Surface sediments South Atlantic Ocean	96
EB13	GU968624	Unaffiliated	Uncultured bacterium clone ARD50 (GQ86D262)	PCB-Spiked Ohio River sediments	98
EB14	GU968621	Unaffiliated	Uncultured bacterium clone C36 (EU234271)	Wang Yang River receiving waste water	91
EB 15	GU968618	Unaffiliated	Uncultured bacterium clone Sd1-56 (GQ246337)	North Yellow Sea sediments	100
EB16	GU968629	Unaffiliated	Uncultured bacterium clone 661199 (DQ4D4921)	Contaminated sediment	99
EB 17	GU968619	Chloroflexi	Uncultured Dehalococcoides sp. clone JJB207 (GQ143769)	Yellow Sea continental shelf sediment	100
EB18	GU968625	Gammaproteobacteria	Uncultured gamma proteobacterium clone A072 (FJ358865)	Marine reef sandy sediment	100
EB19	GU968628	Unaffiliated	Uncultured bacterium clone RefTOc8_D04 (GQ413428)	Coral-associated bacteria	98
EB 20	GU968634	Unaffiliated	Uncultured bacterium clone UncDee20 (AM997522)	Surface sediments South Atlantic Ocean	99
EB 21	GU968623	Actinobacteria	Uncultured actinobacterium clone ARTE12_237 (GU230446)	Coastal sediment	98
EB22	GU968635	Actinobacteria	Uncultured actinobacterium clone TAA-10-01 (AM259898)	Sponge-associated bacteria	100

Table 14. Comparison of sequenced DGGE bands (16S rRNA gene) to closest relative in GenBank.

Further, phylogenetic analysis with the neighbour sequences and other reference sequences revealed that the sequences belonged to Proteobacteria, Acidobacteria, Actinobacteria, Chloroflexi, Nitrospira and Bacteroidetes (Fig. 32). Proteobacteria was the dominant phylum at all depths with Deltaproteobacteria and Gammaproteobacteria forming the major classes. This was followed by the Actinobacteria group.

Proteobacteria was the major phyla observed at all depths (Range=42-61%) and stations followed by the Actinobacteria (Range=17-30%). Though qualitatively there was no difference in the groups observed at different stations, there were significant differences in the density of these groups at various stations and transects. In the Karwar transect Proteobacteria was the highest in 200 m with 59% of the population followed by 1000 m (55%), whereas Actinobacteria was the highest at 50 m (28%) (Fig. 33A). However, the percentage of Actinobacteria increased from 200 m to 1000 m. Interestingly, Nitrospira was higher in the OMZ sites (200 m, 500 m and 1000 m) of Karwar, forming around 10-11% of the population which was double than that observed at 50 m off Karwar. Bacteroidetes was not observed in the deepest station and Acidobacteria in 200 m. In Goa, transect 50 m station had 67% of the Proteobacteria and 60% at 1000 m (Fig. 33B). Actinobacteria increased from 50 to 500 m ranging from 17-22%, with an immediate decrease (13%) at 1000 m. At 1000 and 200 m, station the highest abundance of Nitrospira (13% each) and Chloroflexi (7%) was observed. In the Ratnagiri transect (Fig. 33), Proteobacteria was dominant at 500 m (61%) followed by 1000 m (56%). Percentage of Actinobacteria ranged from 17 to 21% with the highest observed in 200 m with 21%. Nitrospira, Bacteriodetes, Acidobacteria and Chloroflexi were high at 50 m compared to other depths. Acidobacteria was absent at 500 and 1000 m.

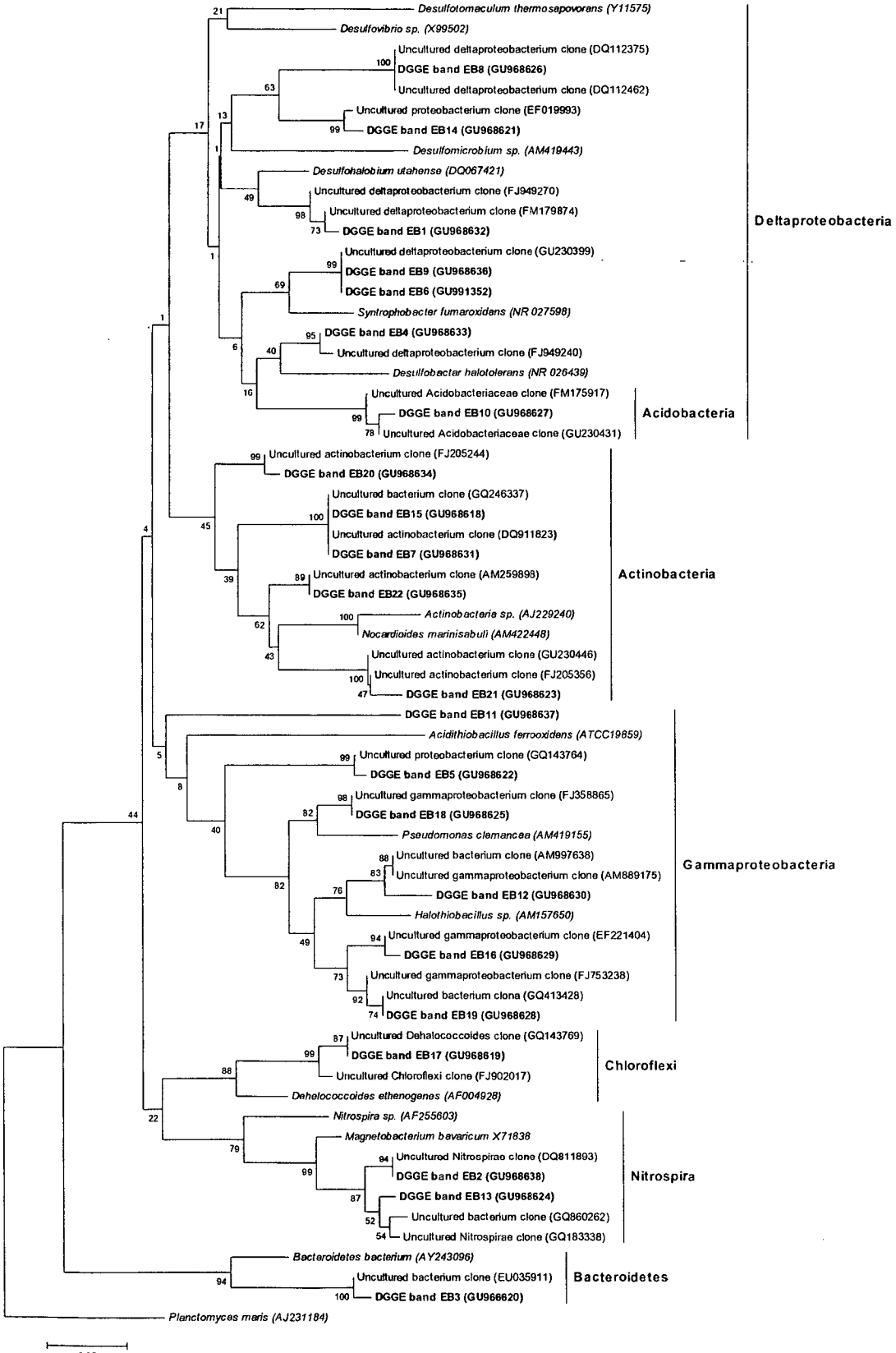


Fig. 32. Neighbor-joining tree showing the phylogenetic affiliations of bacteria derived from the sequences of excised DGGE bands. The sequences obtained in this study are shown in bold. The bar represents 5% sequence divergence. *Planctomyces maris* was used as the outgroup.

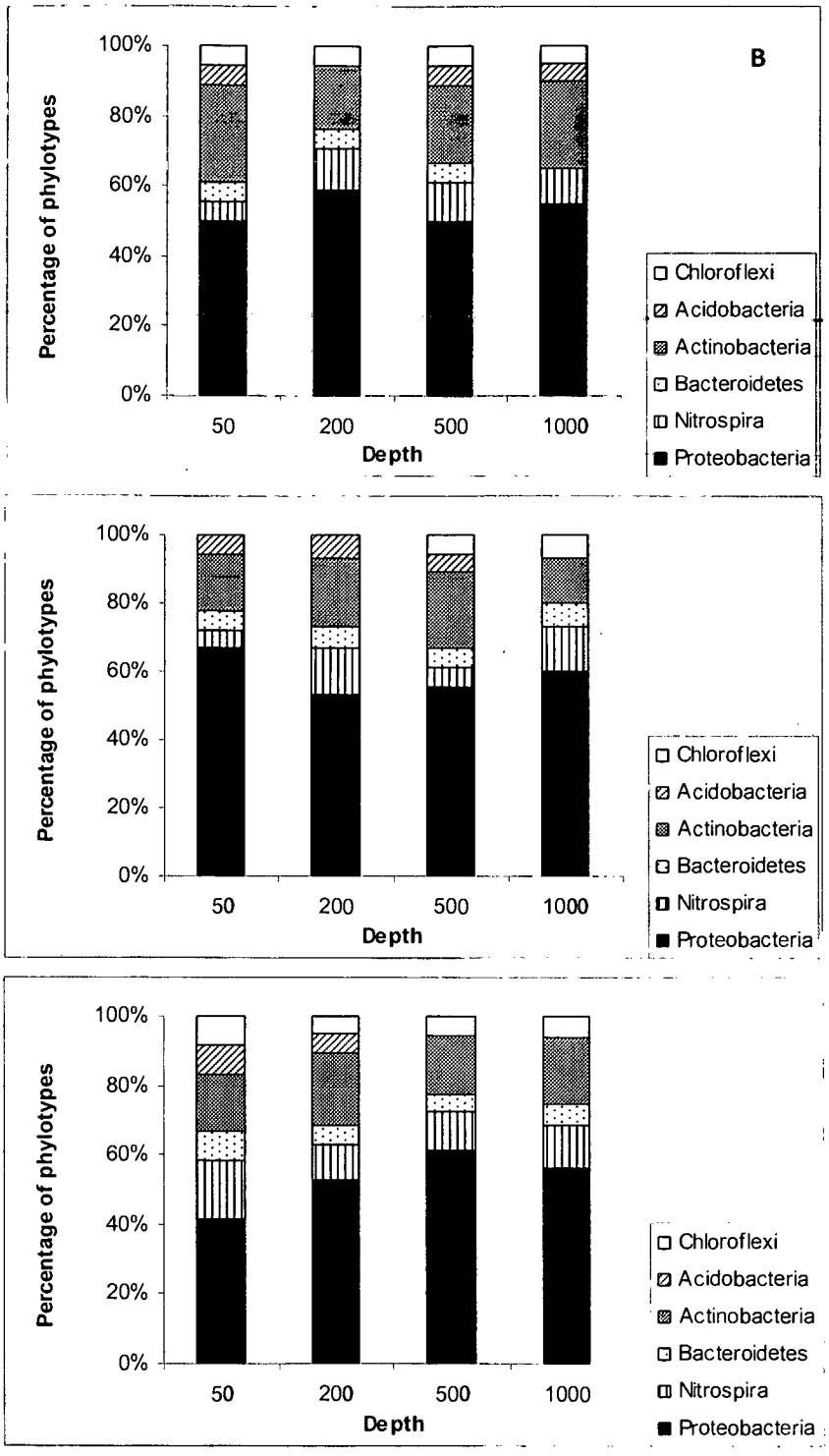


Fig. 33. Percentage of phylogenetic groups in three transects (A) Karwar, (B) Goa (C) Ratnagiri

All the groups were invariably observed at all the depths without any differentiation of the OMZ and non-OMZ regions. In the OMZ region the density of the phyla such as Proteobacteria, Nitrospira and Chloroflexi were

higher than the non-OMZ region (Fig. 34). Proteobacteria accounted for 44% of the population in the OMZ as compared to 39% in the non-OMZ. Nitrospira and Chloroflexi comprised of 9% and 4% respectively in the OMZ and 6% and 3% respectively in the non-OMZ region. However, in the non-OMZ region Bacteroidetes and Acidobacteria, each contributing to 5% of the population. The abundance of Actinobacteria were similar (15%) in both the regions.

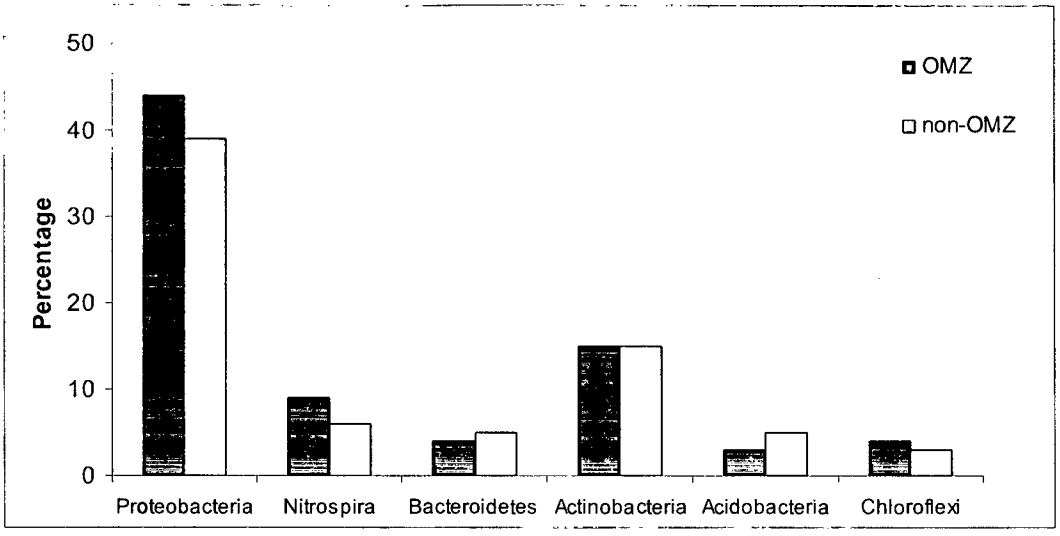


Fig. 34. Percentage of phylogenetic groups in OMZ and non-OMZ sites

4.5.1.2. Species Diversity Index

All the indices showed slight variation among transect and stations (Table 15). Interestingly, almost all of the indices estimated were higher at 500 m and 1000 m stations, off Goa and Ratnagiri. The Shannon-Weiner diversity index (H') recorded a value of about 3.82. However, the Fisher's index was higher in Ratnagiri at 1000 m station with 69 compared to that in Goa 500 m station which is 66.4. Species richness (J') and evenness were about 13 and 0.9 respectively in both the stations. In the Goa transect the Shannon-Weiner index (H') ranged from 3.5 to 3.8, Karwar from 3.5 to 3.7, Ratnagiri 3.6 to 3.8 (Fig. 35). The lowest species richness of 9.9 and species eveness of 0.89 was recorded at 50 m station off Goa.

Depth	Abundance	Richness	Evenness	Brillouin	Fisher	Shannon	Simpson
G 50m	47	9.9	0.92	2.97	34.5	3.55	0.97
G 200m	51	10.8	0.89	2.95	41.6	3.51	0.96
G 500m	61	13.02	0.92	3.16	66.4	3.82	0.98
G 1000m	50	10.6	0.89	2.9	39.7	3.5	0.97
K 50m	51	10.8	0.92	3.06	41.6	3.6	0.97
K 200m	52	11.1	0.90	2.99	44.7	3.57	0.97
K 500m	54	11.5	0.89	3.02	48.1	3.58	0.97
K 1000m	54	11.5	0.93	3.13	47.8	3.7	0.98
R 50m	49	10.4	0.92	3.08	37.9	3.6	0.97
R 200m	60	12.8	0.92	3.14	63.3	3.7	0.98
R 500m	56	11.9	0.89	2.99	52.5	3.6	0.97
R 1000m	62	13.2	0.92	3.16	69.6	3.8	0.98

Table 15. Diversity indices of three transect Goa (G), Karwar (K) and Ratnagiri (R)

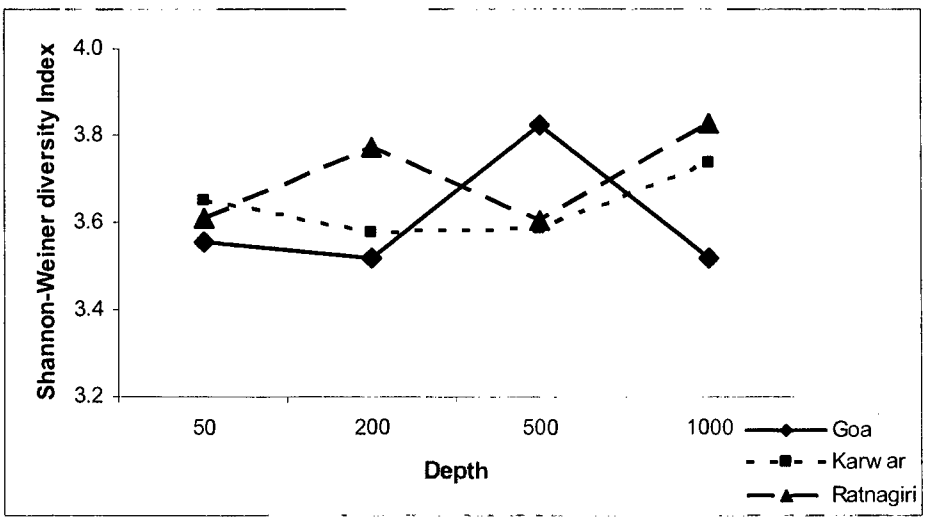


Fig. 35. Variation in Shannon-Weiner diversity index at various depths (m)

The 16S rRNA gene based DGGE fingerprinting provided a general insight into the bacterial community of the ASOMZ. To understand more indepth community analysis of the composition using clone library was done for the Goa transect which is given below in detail.

4.5.1.3. Diversity in Goa Transect

This transect was studied in detailed for total and culturable bacterial diversity.

4.5.1.3.1. 16S rRNA Clone Library Analysis

Clone libraries were generated using the total DNA extracted from sediments with average insert size of 1kb. These clones were sequenced and the phylogenetic analysis was carried out.

(i) Phylogenetic analysis of 16S rRNA clone library

Of the 115 16S rRNA gene clones sequenced, 103 were of sufficient length and devoid of any sequence anomalies. Therefore, the remaining 12 sequences with anomalies were omitted from further analysis. The sequences have been submitted to the GenBank (Accession numbers: EU071379-EU071386, EU445347-EU445355, FJ268480-FJ268573) and sequences presented in Appendix-A11. The analysis of the phylogeny of the 103 clones is illustrated in Figure 36. These clone sequences were designated as PL 'N', where 'N' represents the number of the clones. Of the 103 sequences of clones analyzed, 14 different groups were obtained. The dominant group was the Proteobacteria (52%) followed by the Planctomycetes (12.7%). The Deltaproteobateria formed the major class forming about 62.5%. The other classes of Proteobacteria such as Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria, had fewer representatives with 3.9%, 1.9% and 7.8% respectively. These clones showed close resemblance to marine sediments from different ecosystems. The relative abundance of each taxonomic group is depicted in Figure 37. Firmicutes, Bacteroidetes and Verrucomicrobia each formed 0.9% of the total sequences. Acidobacteria and Spirochaetes each had four representatives (3.9%). Also 2.9% of sequences were represented by Nitrospira. An interesting observation was the presence of three uncultured candidate divisions OP3, OP8 and OP11 accounting for 3.9%, 2.9% and 0.9% of the sequences respectively. Fifteen clones were not assigned to any phyla and were placed in two unidentified clusters.

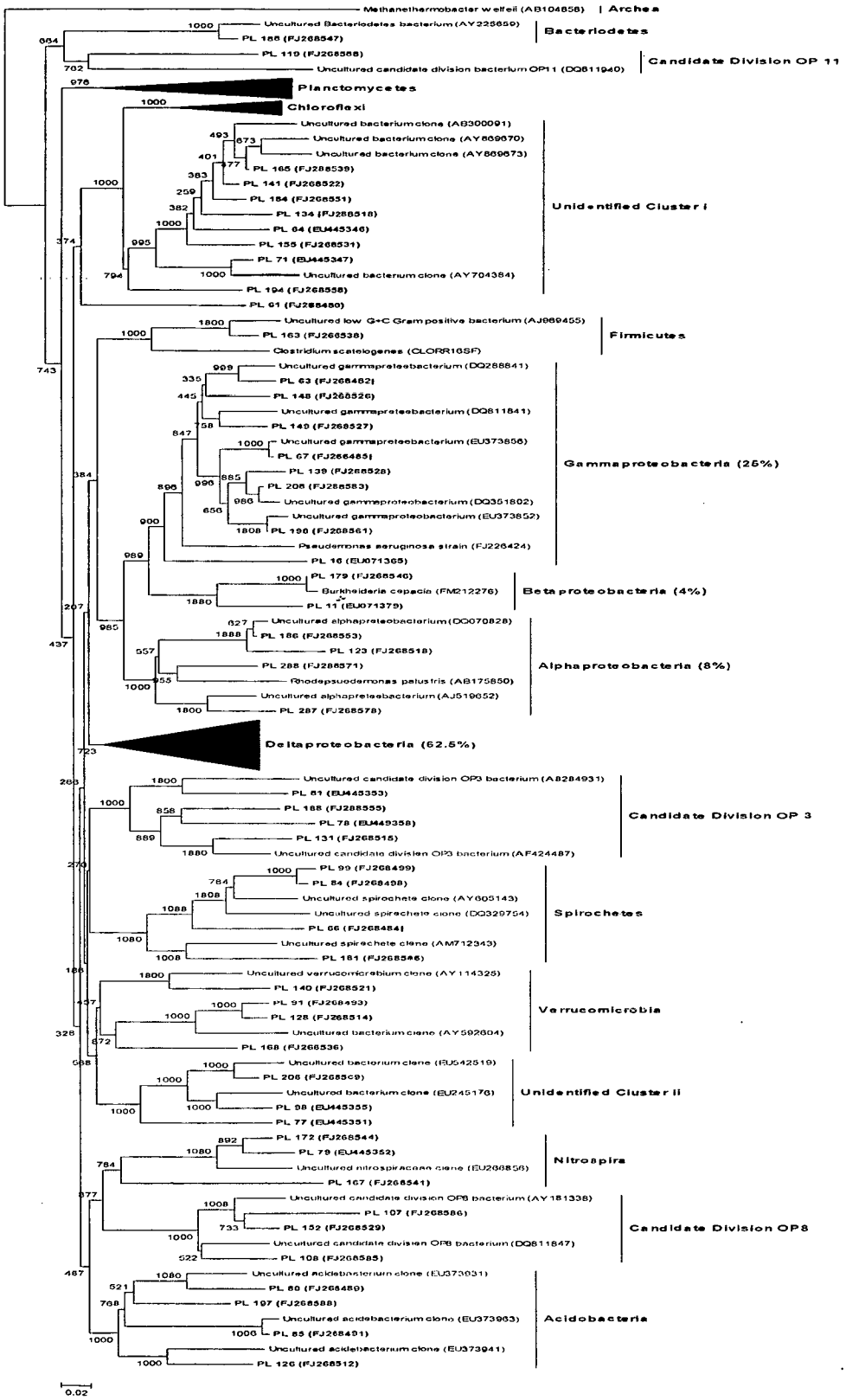


Fig.36. Phylogenetic tree of 16S rRNA gene sequences of Arabian Sea sediments. Sequences with prefix 'PL' are results of the present study. Tree topology was inferred by neighbour-joining method, using 1000 bootstrap iterations. *Methanothermobacter wolfeii* is the outgroup. Scale bar shows 2% sequence divergence.

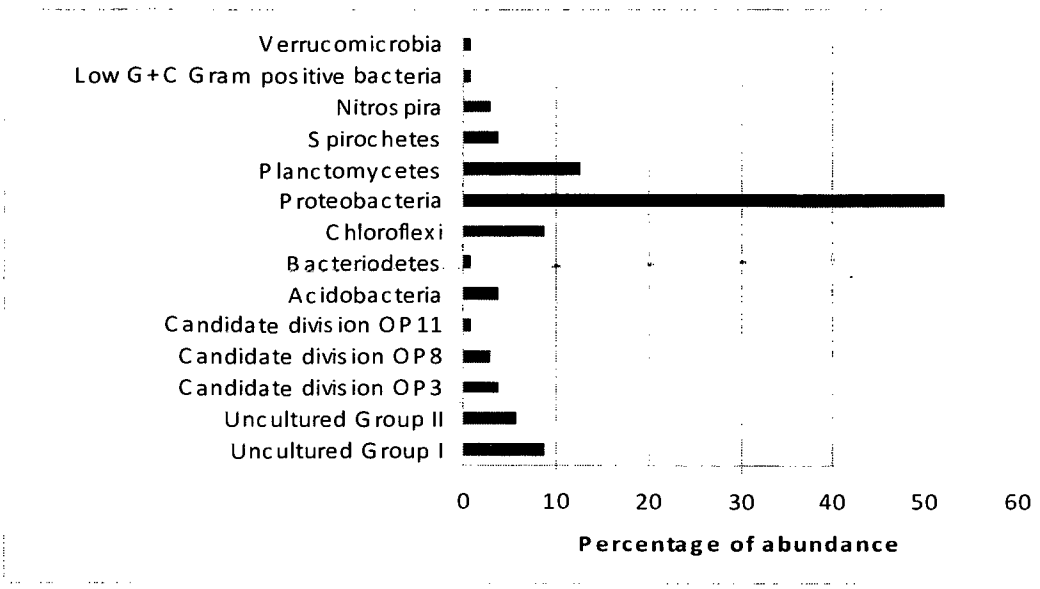


Fig. 37. Abundance of phylogenetic groups in OMZ sediment library

ii. Phylogenetic analysis of the major groups

a.Deltaproteobacteria

In the phylum Proteobacteria, 62.5% of the sequences belonged to the Class Deltaproteobacteria, members of which are known for sulphate reduction potential (Fig. 38). The clones fell into orders Desulfovibrionales and Syntrophobacterales while a large number was not affiliated to any orders and were classified as uncultured Delta proteobacteria. The clones also showed close similarity to the sequences found in ridge flank crustal fluids, marine sediments and mangrove soils.

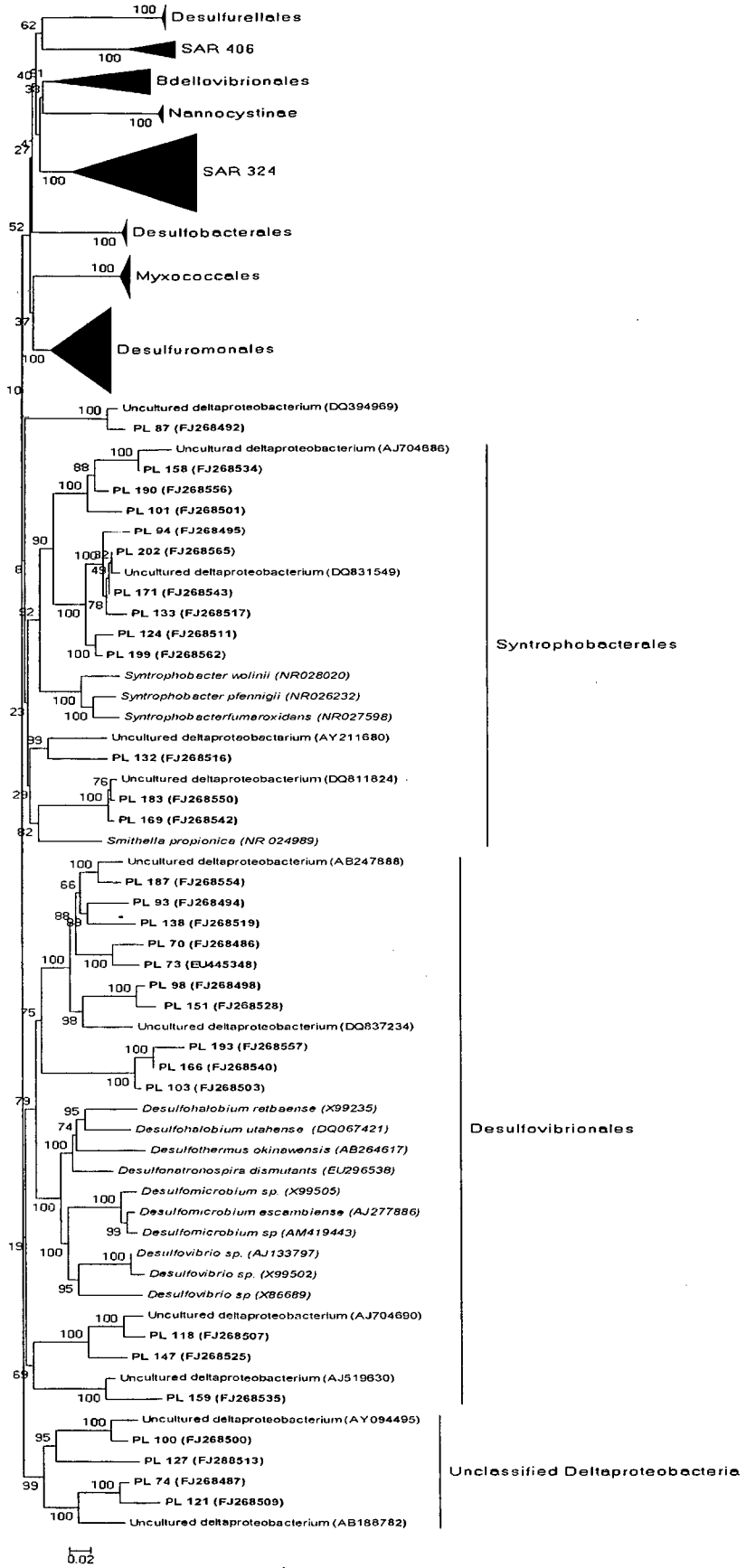


Fig. 38. Phylogenetic tree of 16S rRNA gene sequences of OMZ sediments showing affiliation within the Deltaproteobacteria.

b. Planctomycetes

Interestingly, the Planctomycetes group had 13 sequences (12.7%) and formed the second dominant group after Deltaproteobacteria, suggesting its importance in the OMZ (Fig. 39). Majority of the clones were not classified into any order and formed the unidentified Planctomycete clones. The sequences also showed similarity to sequences reported from marine sediments, mangrove soils and octacorals. None of the sequences showed similarity to the Candidatus *Scalindua* cluster.

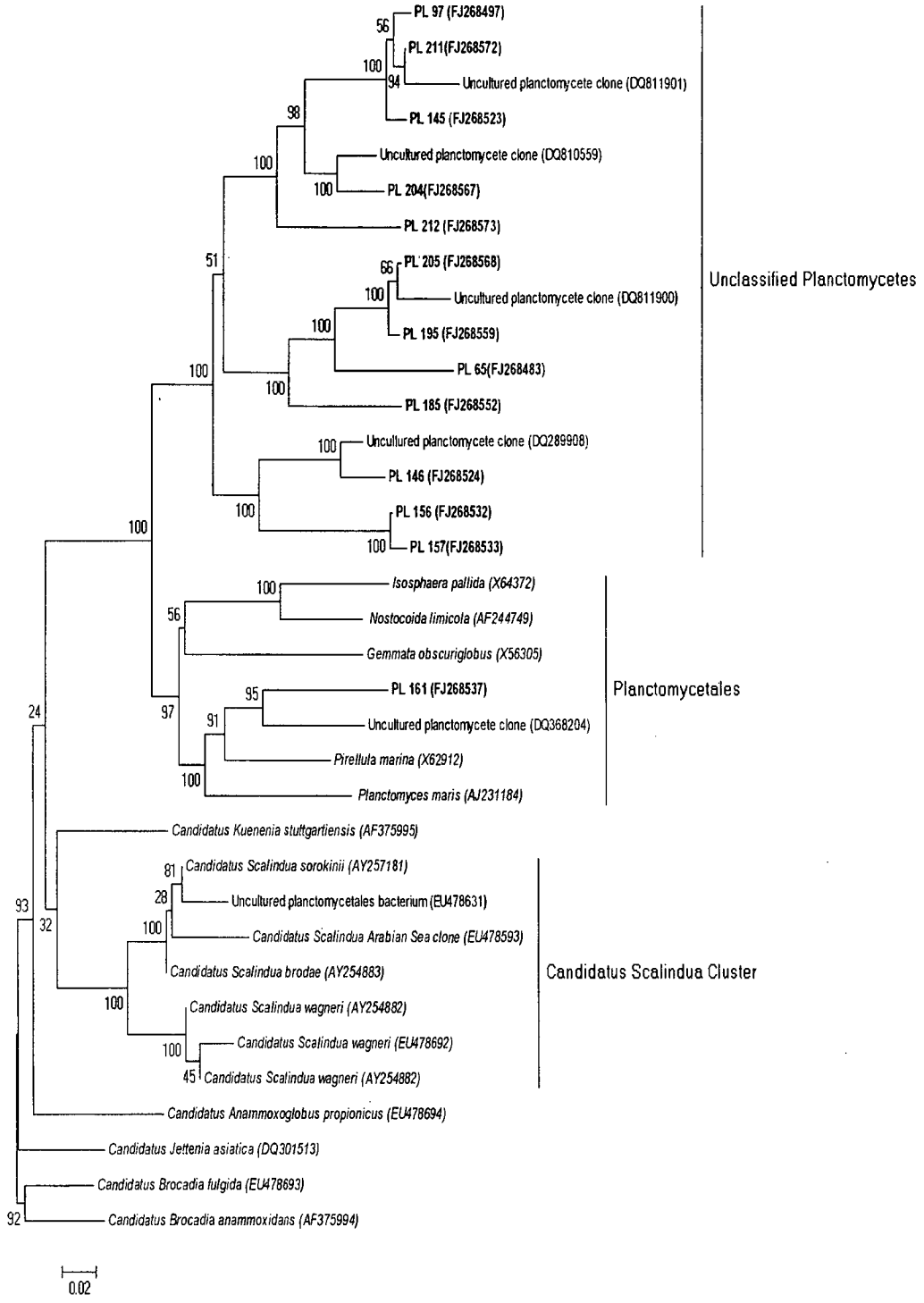


Fig. 39. Phylogenetic tree of 16S rRNA gene sequences of OMZ sediments showing affiliation within the Planctomycetes.

c.Chloroflexi

The Phylum Chloroflexi also formed a cluster with 9 representatives (8.8%) and classified into Family Anaerolinae (Fig. 40). The nearest neighbours were affiliated to shallow and deep marine sediments and mangrove soils.

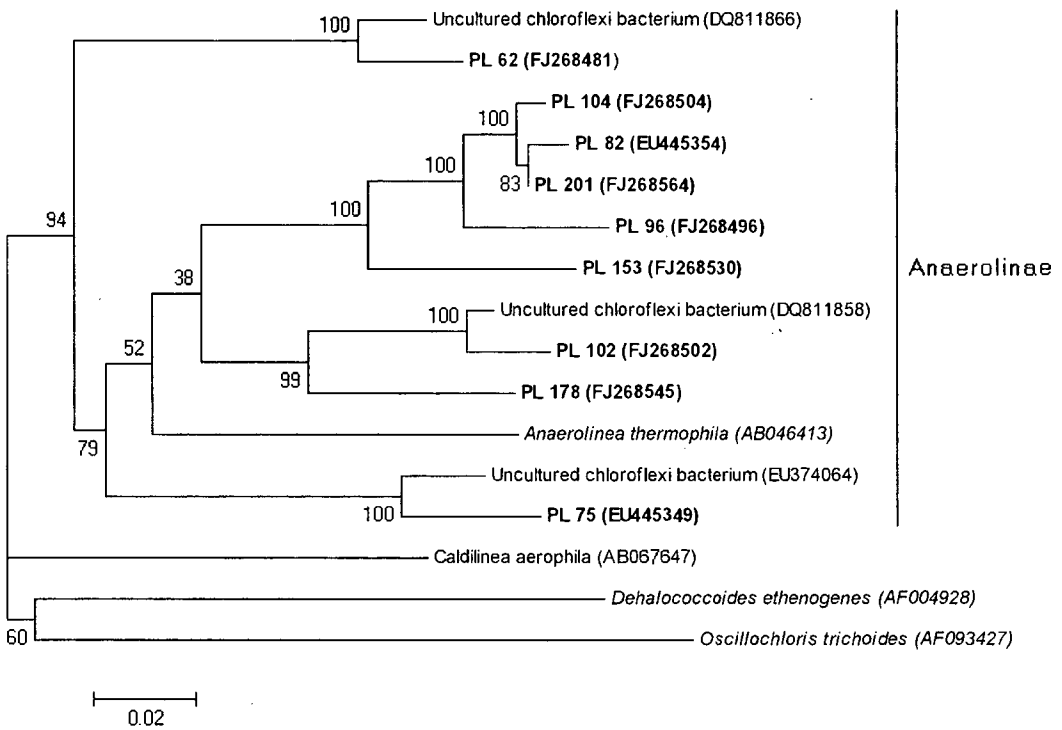


Fig. 40. Phylogenetic tree of 16S rRNA gene sequences of OMZ sediments showing affiliation within the Chloroflexi.

(iii) Statistical analysis of clone library

DOTUR analysis showed that the 16S rRNA ASOMZ clone library had 90 OTUs at an evolutionary distance of $\geq$ 0.03 (species level). The Good's library coverage for sediment clone library was 22%. The Shannon-Weiner index was high with a value of 4.4 indicating that the overall diversity was high while Simpson's index of the clone library was 0.003. Analysis of rarefaction curve at different dissimilarity levels, which is measured as the number of observed OTUs as a function of sequences sampled, enabled the comparison of the extent of samples at various taxonomic levels (**Fig. 41).** At the species level (3% diff) and at class level (10% diff) steep curves were

obtained. However, at the phylum level the curve was almost reaching an asymptote. Estimates of OTUs increased with the number of sequences. ASOMZ sediment library showed a steep curve. Estimates of OTUs increased with the number of sequences analysed.

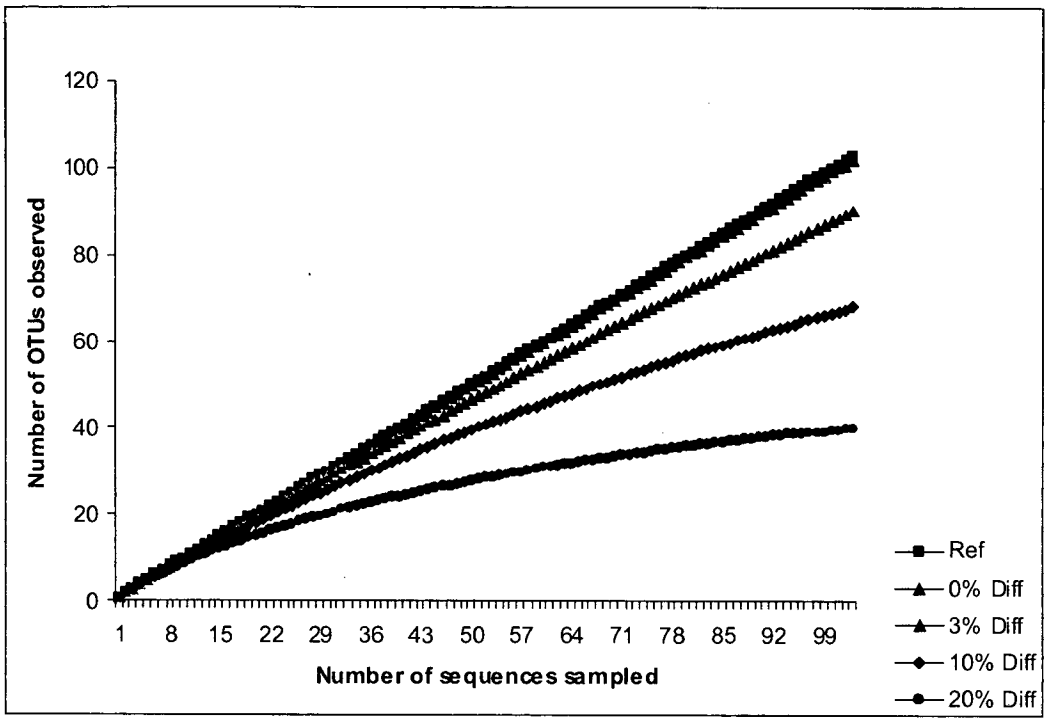


Fig. 41. Rarefaction analysis of 16S rDNA sequence heterogeneity. The diagonal line (reference) represents the 1:1 relationship where each sequence is unique.

The Shannon-Weiner diversity index was 4.4 (Table 16). The abundance based coverage estimators (ACE) and Chao1 richness estimate were 484.6 and 485, respectively.

Statistical parameters	Evolutionary distance/ %Dissimilarity				
	Unique	0	3	10	20
Unique OTUs	103	102	90	68	40
Abundance-based coverage estimator (ACE)	0	5253	484.6	170.4	59.3
Bootstrap estimate	140.7	139	120	87.4	47.7
Chao 1 richness estimate	5356	2627	485	154.8	49
Jackknife estimate	206	203	754.9	164.2	56
Shannon–Weiner Index	4.6	4.6	4.4	4.05	3.4
Simpson Index	0	2E-04	0.003	0.011	0.03

Table 16. Diversity indices and statistical parameters of the clone library

iv. Statistical comparison with other clone libraries

The comparison of clone libraries from North Sea, South China Sea, Gulf of Mexico and Arabian Sea water with that of the present study showed that Arabian Sea OMZ sediment clone library had the highest number of OTUs with 90 at a evolutionary distance of ≥0.03 (species level), followed by South China Sea sediment library with 74 unique OTUs. However, the lowest number was found in Gulf of Mexico sediment with only 23 OTUs. The Good's library coverage was highest for ASOMZ water clone library (66%) and lowest for Arabian Sea OMZ sediment clone library (22%). The diversity indices and estimators were highest for the Arabian Sea OMZ sediment library as shown in Table 17.

	No. of unique OTUs	Abundance-based coverage estimator (ACE)	Chao 1 richness estimate	Shannon -Weaver Index (H)	Simpson Index (1/D)	Good's coverage (%)
ASOMZ sediment	90	484.6	485	4.4	0.003	22
ASOMZ water	36	73.3	64.9	3.3	0.028	66
North Sea	47	139.7	121	3.7	0.009	38
South China Sea	74	757	442.5	4.1	0.007	23
Gulf of Mexico	23	114.8	118	3.04	0.018	28

Table 17. Comparison of diversity indices and statistical parameters of the various 16S rDNA clone libraries

Analysis of rarefaction curve which is measured as number of observed OTUs as a function of sequences sampled is depicted in the Figure 42. The figure shows that the ASOMZ water library reaching almost an asymptote while the ASOMZ sediment library showed a steep curve compared to the other libraries.

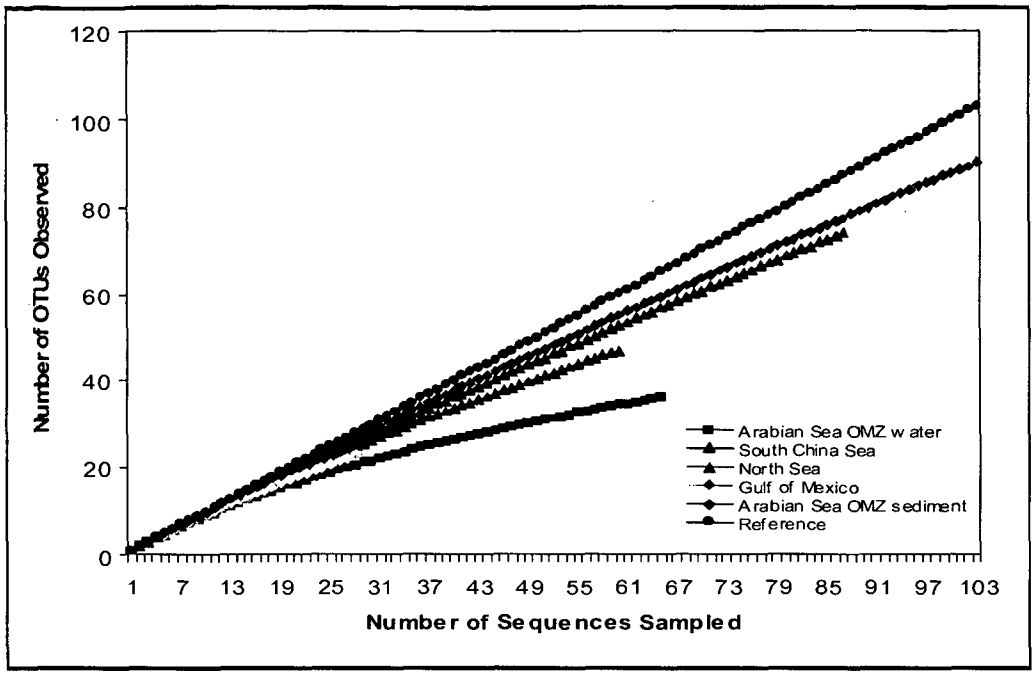


Fig. 42. Rarefaction analysis of 16S rDNA sequence heterogeneity in the five clone libraries. The diagonal line (reference) represents the 1:1 relationship where each sequence is unique.

The comparison of library of the present study with other libraries revealed that bacterial community composition differed significantly between the four clone libraries ($p=0.001$) (Fig. 43.). The differences between the libraries were greatest at genetic distance $D \leq 0.2$ for all combination of libraries. The distribution of Delta C as a function of evolutionary distance (D) showed that delta C was maximum at 0.2 genetic distances in all the combinations.

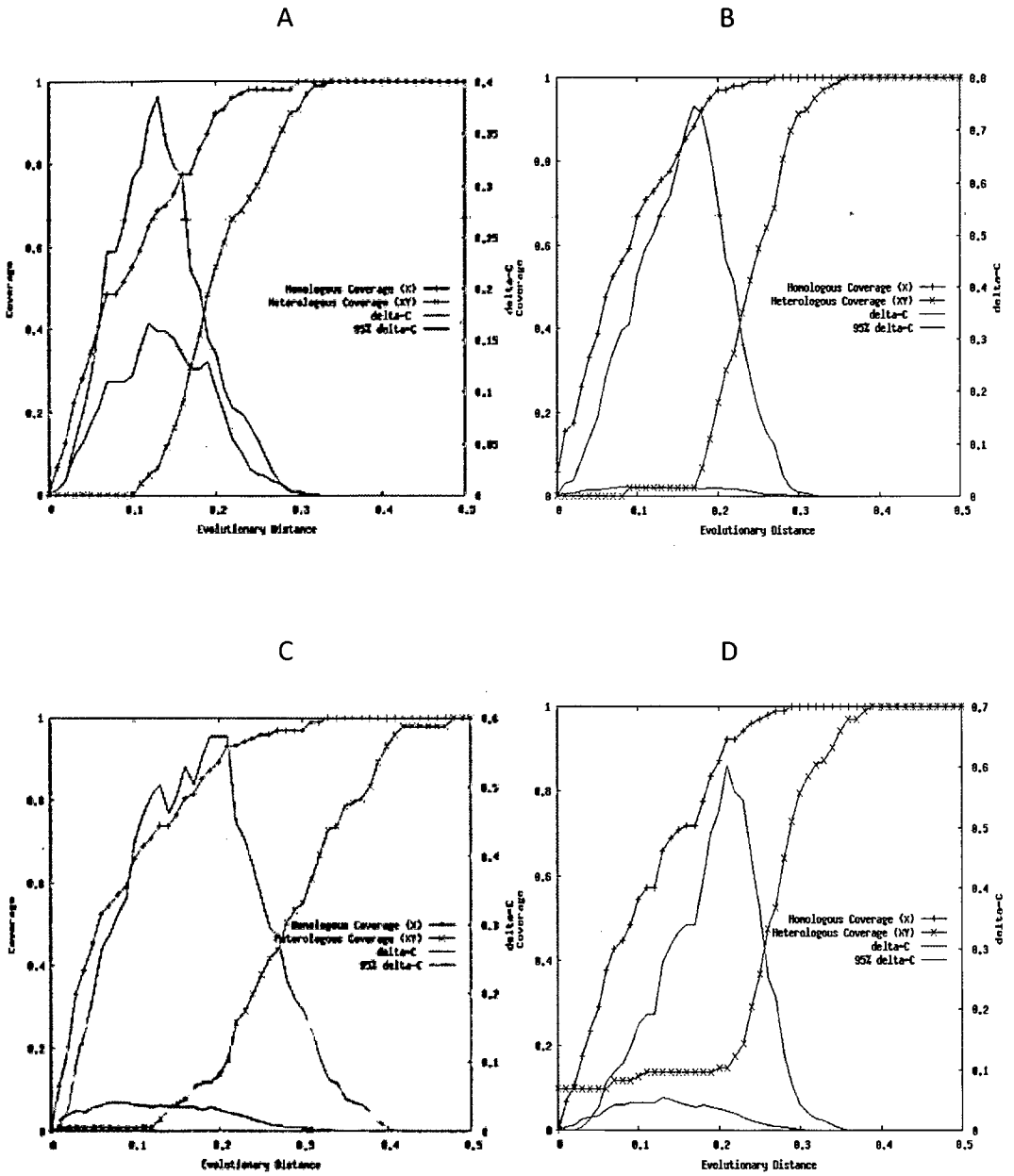


Fig. 43. Results of LIBSHUFF comparisons of ASOMZ sediment clone library with A) South China Sea sediment, B) Gulf of Mexico sediments, C) North Sea sediment, D) ASOMZ water clone libraries. Homologous (red) and heterologous (green) coverage curves are depicted. Blue line indicates the value of $(C_X-C_{XY})^2$ for the original samples at each value of evolutionary distance (D). Violet line indicates the 950th value (p=0.05) of $(C_X-C_{XY})^2$ for the randomized sample.

4.5.1.3.2. Culturable Diversity

(i)Aerobic Bacteria

A total of 105 bacterial strains having visibly different colony morphologies isolated from both non-OMZ and OMZ sediments (Number of isolates: 50 m-31; 200 m-19; 500 m-23; 1000 m-32) were subjected to ARDRA analysis. The characteristics of the bacterial colonies have been given in the Appendix section-A8. This method helped in rapid and reliable screening of isolates compared to the more laborious techniques for microbial identification and characterization. Figure 44 shows with the discrete DNA band devoid of any mispriming. These were analysed by amplified rDNA restriction analysis using restriction enzyme *Hae* III and banding patterns were obtained.

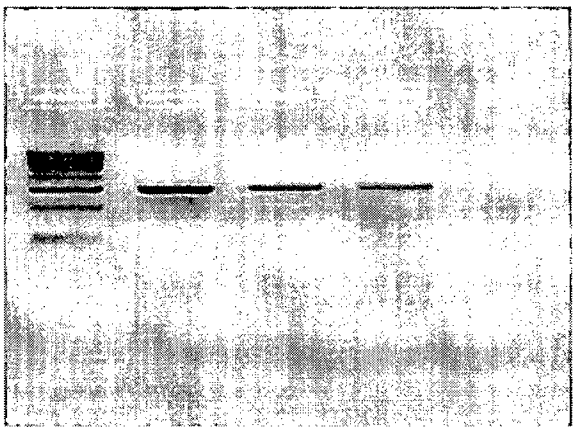


Fig. 42. Gel picture showing amplification of 16S rRNA gene. (L-500bp ladder, 1-Sample 1, 2-Sample 2, 3-Positive control (*Stenotrophomonas maltophila*), 4-Negative control)

The ARDRA patterns were analysed using Bionumerics software and thus grouped the isolates of different depths into a particular phylotype which included all isolates with similar banding patterns. When all the ARDRA patterns of all isolates from Goa at all the depths were analysed using UPGMA clustering, 56 phylotypes were obtained, thus exhibiting high diversity and 16S rRNA gene heterogeneity. Of these, 47 were unique phylotypes with only one representative, while the remaining 9 had 2-4 representatives which included members across the depth. The groups clustered at 30% similarity and formed three major clusters (Fig. 46).

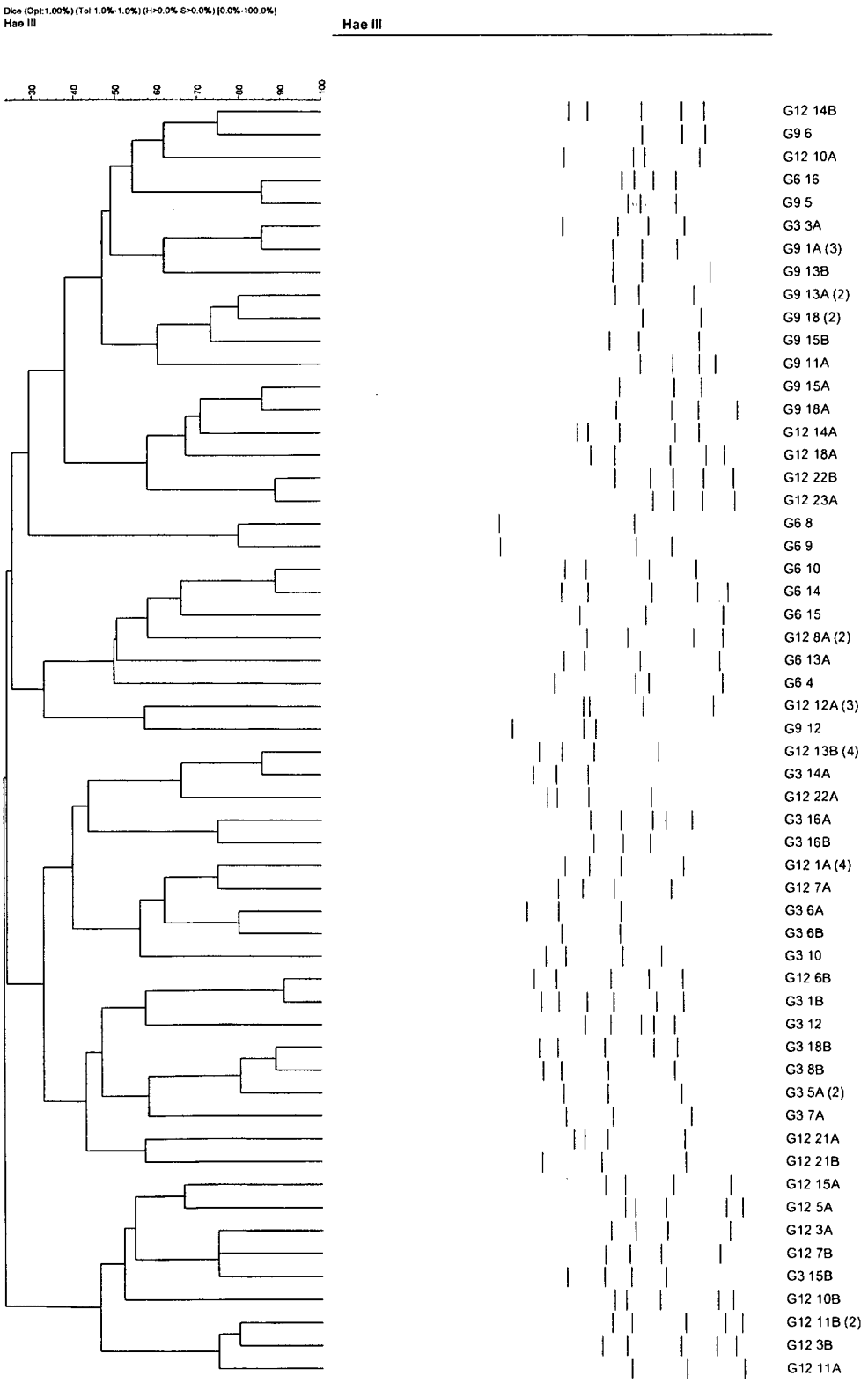
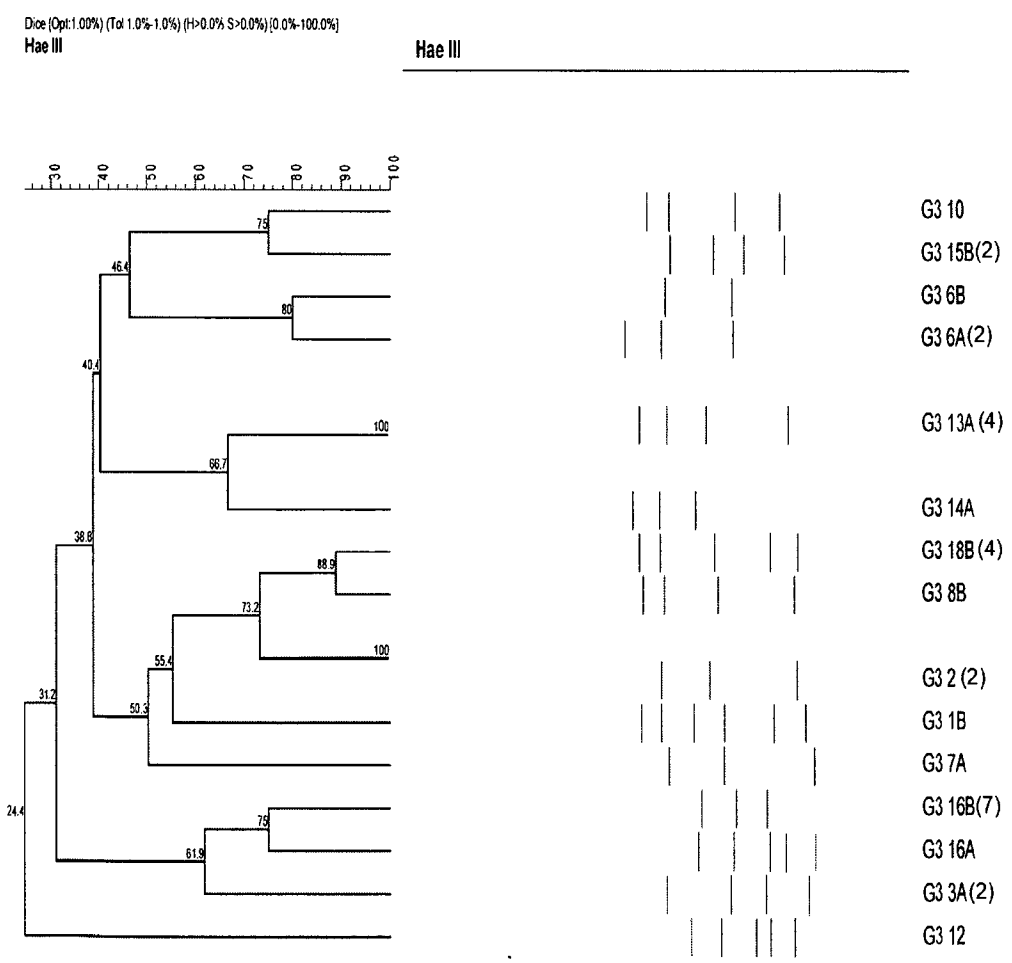


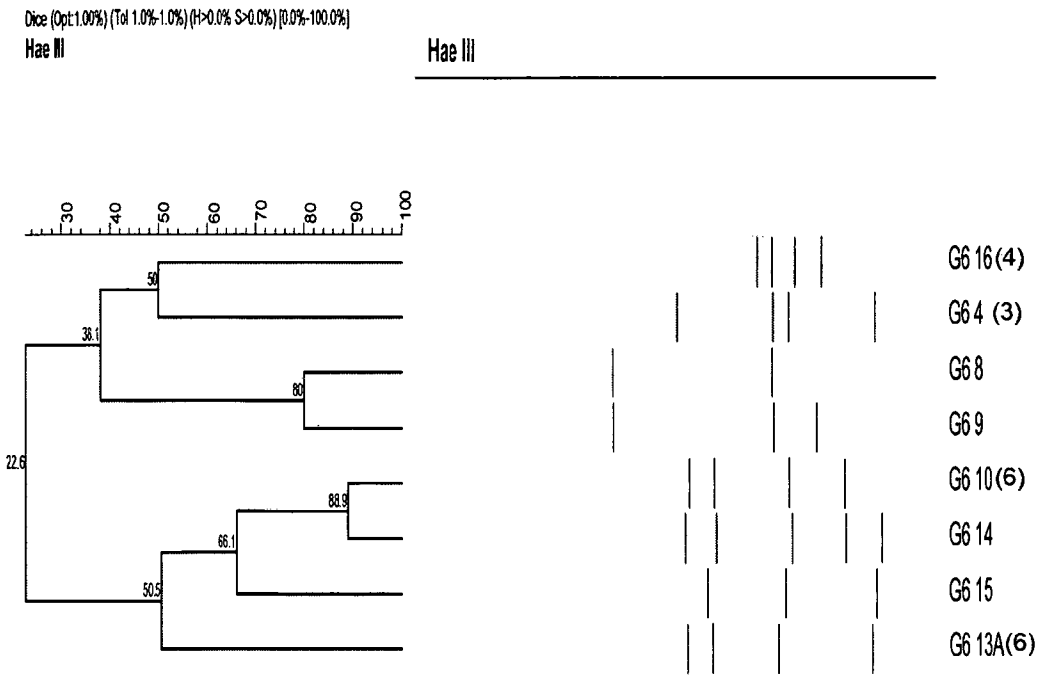
Fig. 46. UPGMA clustering of ARDRA patterns of culturable bacteria at Goa transect

ARDRA analysis of the isolates from distinct depths revealed differences between the depths. The cluster analysis of the ARDRA patterns of the isolates of various depths in the Goa transects is depicted in the Figures 44 A-D. The isolates from 1000 m clustered into 22 phylotypes, while those in 50 m clustered into 15 phylotypes. This was followed by 500 m with 11 phylotypes. The lowest number of phylotypes was observed in 200 m with only 8 phylotypes. Thus there was variation in the phylotype diversity depending on station depth. However the core of the OMZ (1000 m) showed maximum number of phylotypes.

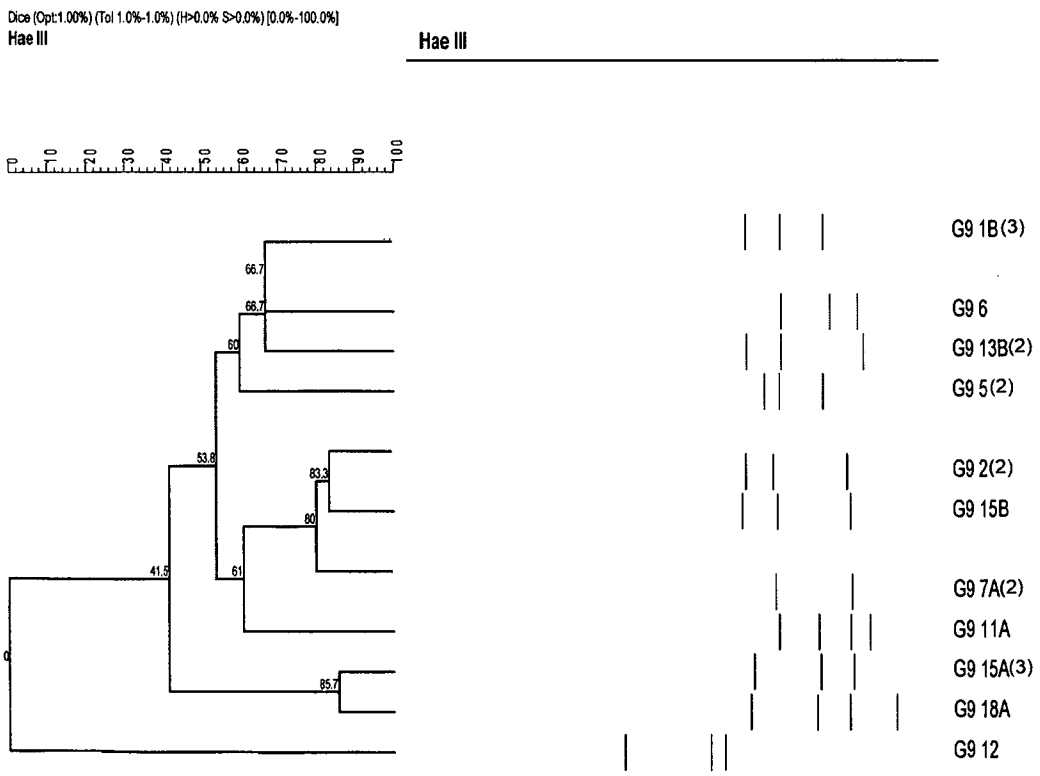
A)



B)


Dice (Opt:1.00%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
Hae III
Hae III
30
40
50
60
70
80
90
100
50
38.1
80
22.6
88.9
66.1
50.5
G6 16(4)
G6 4 (3)
G6 8
G6 9
G6 10(6)
G6 14
G6 15
G6 13A(6)

C)


Dice (Opt:1.00%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
Hae III
Hae III
0
10
20
30
40
50
60
70
80
90
100
66.7
66.7
60
53.8
83.3
80
41.5
61
85.7
G9 1B(3)
G9 6
G9 13B(2)
G9 5(2)
G9 2(2)
G9 15B
G9 7A(2)
G9 11A
G9 15A(3)
G9 18A
G9 12

D)

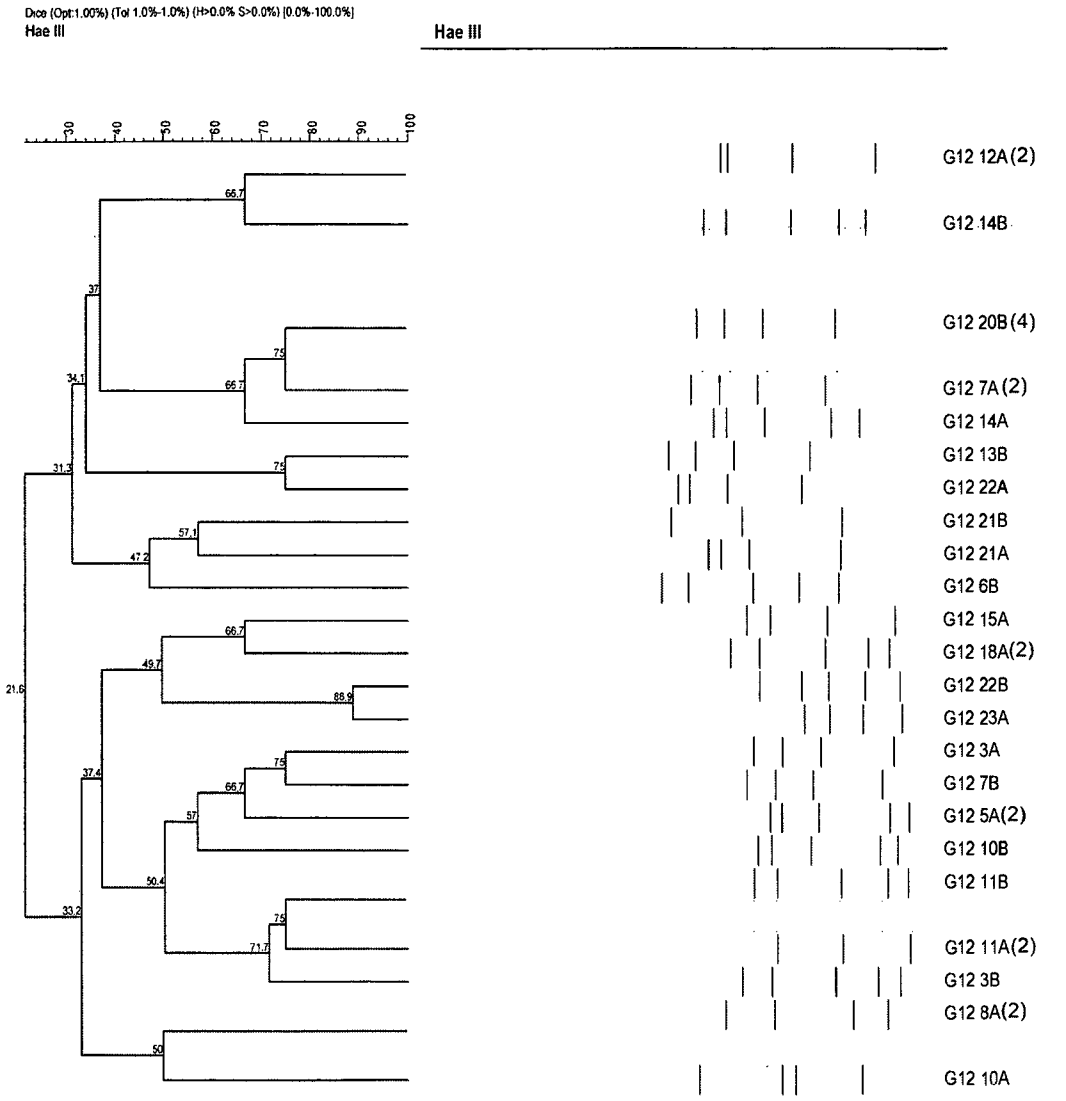


Fig. 43. Cluster analysis of ARDRA patterns of bacterial isolates from (A) 50 m (B) 200 m (C) 500 m (D) 1000 m off Goa. (Codes to the right represent isolate numbers).

The restriction analysis of the 16S rRNA gene (ARDRA) showed highest number of phylotype at 1000 m (39%) and the lowest number of phylotypes at 500 m (14%) in Goa transect (Fig. 44a).

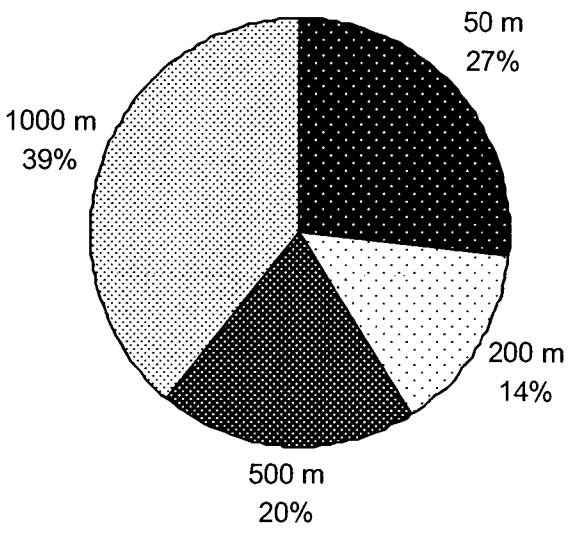


Fig. 44a. Depth-wise distribution of the phylotypes at Goa transect

A similar trend was observed in the case of unique phylotypes (An ARDRA pattern that occurred only once) with about 38% in 1000 m, followed by 28% in 50 m. The intermediate depths ie, 200 and 500 m had about 17% each of unique phylotypes (Fig. 44b.).

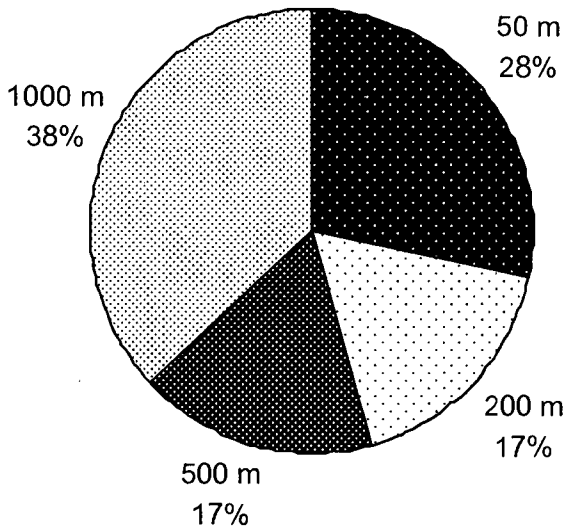


Fig. 44b. Depth-wise distribution of unique phylotypes at Goa transect

When the OMZ and non-OMZ regions were compared the number of total phylotypes and unique phylotypes were found to be higher in the OMZ region (Fig. 45).

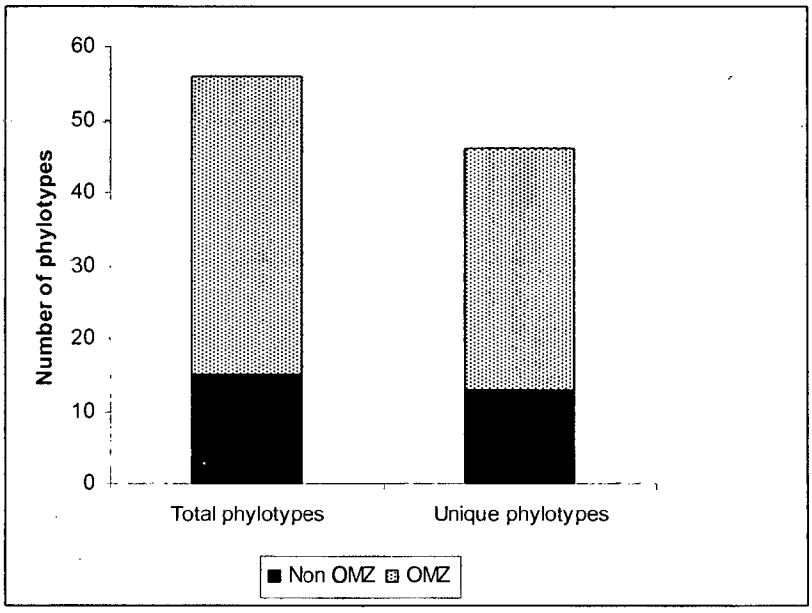


Fig. 45. Comparison of OMZ and non-OMZ phylotypes

The recovered population from all the depths off Goa was dominated by Firmicutes. The generic representatives of this Phylum were *Bacillus, Halobacillus, Marinilactibacillus, Paenibacillus* and *Pullulanibacillus*. The station wise affiliation of the phylotypes along with their nearest neighbour in the GenBank is represented (Table 18A). The sequences have been submitted in the GenBank and the accession numbers are GQ900674-GQ900701.

Phylotype representative	Accession numbers	Phylogenetic Group	Nearest neighbour	Similarity (%)
G12-11A	GQ 900674	Unaffiliated bacteria	Glacial ice bacterium (AF479338)	99
G12-15A	GQ 900675	Firmicutes	Halobacillus (AB305183)	99
G12-23A	GQ 900676	Actinobacteria	Kytococcus sedentarius (AY881239)	98
G12-22B	GQ 900677	Actinobacteria	Micrococcus sp.(EF491952)	97
G12-10A	GQ 900678	Firmicutes	Bacillus licheniformis (FN298317)	99
G12-21B	GQ 900679	Unaffiliated bacteria	Uncultured bacterium (FJ479457)	99
G12-21A	GQ 900680	Unaffiliated bacteria	Uncultured compost bacterium clone (DQ346631)	99
G12-11B	GQ 900681	Firmicutes	Bacillus sp.(DQ078995)	96
G12-1A	GQ 900682	Unaffiliated bacteria	marine sediment bacterium (AY936960)	95
G12-14A	GQ 900683	Low G + C Gram positives	Low G+C gram positive organism (AB002642)	92
G12-12A	GQ 900684	Low G + C Gram positives	Low G+C gram positive organism (AB002643)	94
G12-8A	GQ 900685	Firmicutes	Halobacillus mangrovi strain (DQ888316)	96
G9-1A	GQ 900686	Firmicutes	Halobacillus sp (AB305183)	99
G9-2	GQ 900687	Firmicutes	Bacillus sp (FJ977613)	89
G9-6	GQ 900688	Firmicutes	Bacillus sp. (DQ868675)	99
G9-15A	GQ 900689	Firmicutes	Virgibacillus sp (FJ477404)	97
G9-18B	GQ 900690	Firmicutes	Halobacillus sp (AB305183)	99
G9-15B	GQ 900691	Unaffiliated bacteria	Uncultured compost bacterium (DQ346631)	97
G9-5	GQ 900692	Unaffiliated bacteria	Marine sediment bacterium (AY911022)	94
G9-12	GQ 900693	Firmicutes	Paenibacillus sp.(DQ854976)	99
G6-4	GQ 900694	Firmicutes	Uncultured Firmicutes (EU753617)	98
G6-15	GQ 900695	Low G + C Gram positives	Low G+C Gram-positive bacterium (AF348730)	99
G6-13A	GQ 900696	Firmicutes	Halobacillus sp. (AB198765)	96
G6-8	GQ 900697	Unaffiliated bacteria	Uncultured bacterium (AM183073)	93
G6-10	GQ 900698	Firmicutes	Marinilactibacillus sp (DQ344859)	96
G6-14	GQ 900699	Unaffiliated bacteria	Uncultured compost bacterium (DQ346631)	97
G6-16	GQ 900700	Proteobacteria	Halomonas sp (EU781513)	98
G6-9	GQ 900701	Proteobacteria	Alteromonas hispanica (EU529840)	99

Table 18A. Comparison of representative phylotype sequences (16S rDNA) to closest relative in GenBank.

The other groups included Actinobacteria, Proteobacteria and the unaffiliated bacteria. Interestingly, the unaffiliated group formed a major fraction at all depths and 1000 m had the highest diversity represented by all

the above groups except Proteobacteria. Surprisingly, the Proteobacteria was observed only at 200 m site (Fig. 47).

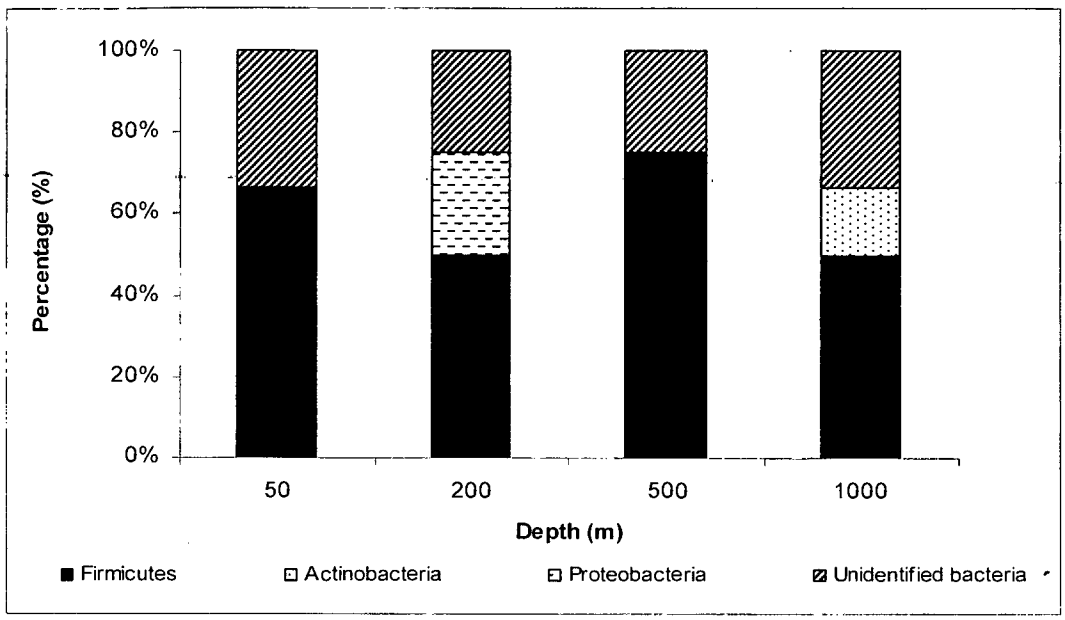


Fig. 47. Percentage of distribution of bacterial groups at different depths of Goa transect

(ii) Anaerobic Bacteria

Anaerobic heterotrophic bacteria were identified using 16S rRNA sequencing and most of them were facultative anaerobes. They belonged to *Staphylococcus, Pseudomonas, Bacillus* and *Vibrio* sp (Table 18B). The accession numbers are EU407222-25, EU407227, EU407229-33. The sequences are given in Appendix-A11.

Isolate No.	Accession No.	Nearest Neighbour	Similarity
AN2	EU407222	Vibrio communis strain J821(JF836185)	100
AN3	EU407223	Vibrio communis strain J821(JF836185)	100
AN4	EU407224	Bacillus sp. DHXJ18 (JN244988)	100
AN6	EU407225	Vibrio communis strain J821(JF836185)	100
AN13	EU407227	Bacillus sp. MB66 (AB518978)	100
AN22	EU407229	Bacillus sp. m8-51 (HM587934)	100
AN37	EU407230	Staphylococcus sp. KMSZP2 (JN230423)	100
AN38	EU407231	Bacillus sp. MB66 (AB518978)	100
AN41	EU407232	Pseudomonas sp. PCA_D6_5 (JF710992)	100
AN42	EU407233	Staphylococcus gallinarum JDM2_3A (JN644523)	100

Table 18B. Comparison of anaerobic bacterial sequences (16S rDNA) to closest relative in GenBank.

(iii) Phenotypic Diversity

The phenotypic characterization of the 105 isolates based on morphological ad biochemical characterization grouped the organisms into different taxonomic groups such as Firmicutes, Actinobacteria, Proteobacteria and unidentied bacteria (Fig. 48). Firmicutes was the most dominant group at all the depths such as 50 (59%), 500 (67%) and 1000 m (71%) except at 200 m where most of the bacteria were not identified and categorized into the unaffiliated bacteria which formed 52%. Proteobacteria was observed only at 50 m (13%), 200 m (5%) and 500 m (4%) and accounted for only a minor fraction of the total population.

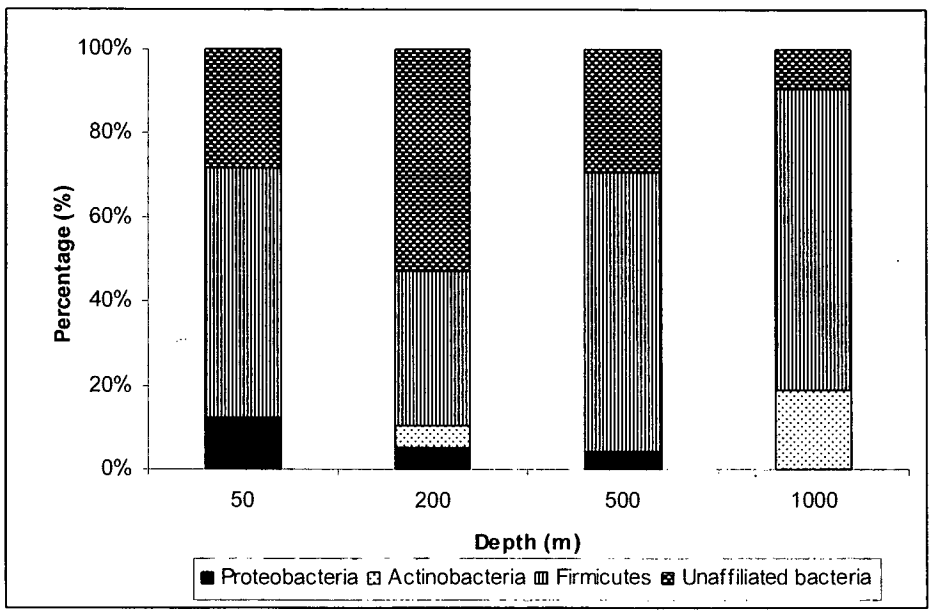


Fig. 48. Percentage of phyla at various depth in Goa transect

4.5.2. Functional Diversity (DGGE)

Effective analysis of the microbial community reqiures that the link be established between the structure and function of the microbial community. These links between strucutral and functional biodiversity is particularly difficult when the phylogenetically diverse function such as sulphate reduction is of interest.

The *dsr*B gene sequences were amplified from the total community DNA and the resulting amplicons was subjected to DGGE. The sulphate reducers are not only versatile in their metabolism but also in the environmental condition under which they thrive in the marine sediments. Thus SRB present not only a large phylogenetic diversity but also a large range of habitats with variation ie. high and low levels of sulphate. Due to their key role in marine carbon and sulphur cycle it is of considerable significance in understanding diversity and metabolic activity in organic-rich OMZ by quantification of *dsr*B gene ie. the functionl diversity of SRB. The total number of bands obtained were 266 and ranged from 10 to 31 and on an average was 22 within the transects. Among the three transects studied, the average number of bands was highest in the Goa transect with 24 ± 2 bands followed by Ratnagiri with 22 ± 3.8 bands (Fig. 49).

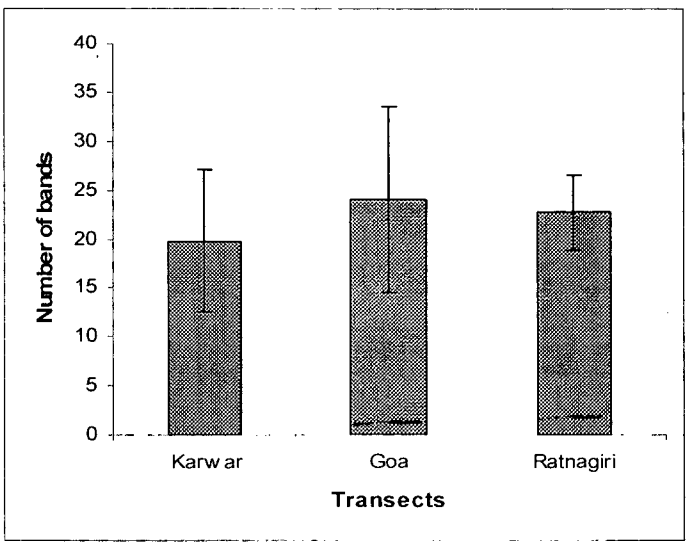


Fig. 49. Transect-wise distribution of bands

In the Goa and Karwar transects the number of bands generally increased from 50 to 500 stations and decreased at 1000 m station. However, in the Ratnagiri transect the number of bands in 50, 200 and 500 m were more or less similar but decreased at 1000 m station. However, in all the transects the number of bands were lesser in the 1000 m at station (Table 19).

Transects	Number of Bands			
	50 m	200 m	500 m	1000 m
G – Goa	47	51	62	50
K - Karwar	51	52	54	54
R -Ratnagiri	49	60	56	62

Table 19. Number of bands obtained in the three transects

Some of the bands were detected in all the stations indicating the presence of SRBs.Since there was very low number of bands at 1000 m depth station in all the three transects the OMZ site showed lesser number of bands compared to non-OMZ. The number of bands was slightly higher in the non-OMZ on average with value of 23.6 ±1.5 as compared to 22±1.5 in the OMZ region (Fig. 53). However some unique bands were seen in the OMZ region.

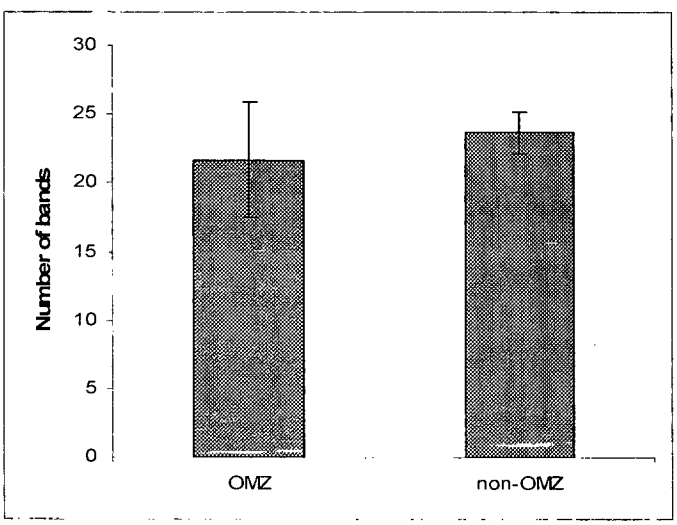


Fig. 53. Distribution of bands in the OMZ and non-OMZ region

4.5.2.1. Phylogenetic analysis

The data of the *dsr*B gel were image analysed and the resulting binary matrix were used for statistical comparison. The image and the positon of the bands are given below. The DGGE analysis of *dsr*B gene revealed that there was variation in the band patterns between stations and transects. In all the samples 40 band classes were obtained (Fig. 50).

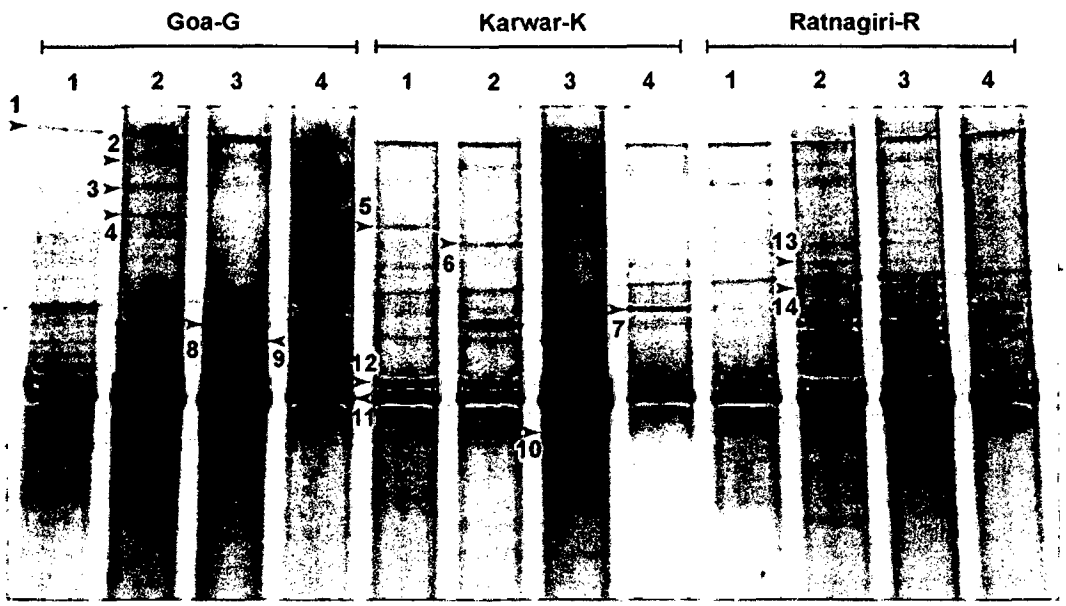


Fig. 50. DGGE profiles *dsr*B gene diversity in the OMZ (200 m, 500 m, 1000 m) and non-OMZ (50 m) sites. Lanes 1, 2, 3 and 4 represents 50 m, 200 m, 500 m and 1000 m depths respectively. The positions and band numbers of excised bands are indicated by numbers in the DGGE gel.

The 14 bands eluted belonging to various band classes were sequenced. All the sequences showed similarity values less than 97% to the GenBank sequences. The sequences have been submitted in the GenBank (JF906894-JF906907) and are presented in the Appendix section-A11. The nearest neighbour sequences with similarity values and sequence length are given in the Table 20. The sequences showed similarity to sequences reported from continental margins of Mexico, Nankai Trough, non-sulfidogenic sediment and mangrove sediments.

Band ID	Accession Number	Closest relative	Origin/Source	Similarity
dDSR1	JF906894	Uncultured sulfate-reducing bacterium clone M306268 (AY3371 37.1)	Continental Margin Habitats Pacific offcoast of Mexico	93
dDSR2	JF906895	Uncultured sulfate-reducing bacterium clone M300232 (AY3372 18.1)	Continental margin sediment off the Pacific coast of Mexico	93
dDSR3	JF906896	Uncultured sulfate-reducing bacterium NTd-V03 (AB263174.1)	Nankai Trough deep-sea sediment	86
dDSR4	JF906897	Uncultured bacterium clone ng3d1014 (AY753127.1)	Nonsulfidogenic deltaic mobile mud sediment	94
dDSR5	JF906898	Uncultured sulfate-reducing bacterium clone LGWN06 (EF065093.1)	Groundwater polluted by leachate from landfil	94
dDSR6	JF906899	Uncultured sulfate-reducing bacterium NTd-V05 (AB263176.1)	Nankai Trough deep-sea sediment	93
dDSR7	JF906900	Uncultured sulfate-reducing bacterium clone Nye-0_2-dsrG10 (HM004751.1)	Marine sediment Norway: Storegga Slide, Nyegga region	97
dDSR8	JF906901	Uncultured prokaryote clone DOF07_11(GU395960.1)	Mangrove sediment	96
dDSR9	JF906902	Uncultured bacterium clone ng7d1029	Nonsulfidogenic tropical mobile	93

Band ID	Accession Number	Closest relative	Origin/Source	Similarity
dDSR1	JF906894	Uncultured sulfate-reducing bacterium clone M3D6268 (AY337137.1)	Continental Margin Habitats Pacific off coast of Mexico	93
dDSR2	JF906895	Uncultured sulfate-reducing bacterium clone M3D0232 (AY337218.1)	Continental margin sediment off the Pacific coast of Mexico	93
dDSR3	JF9D6896	Uncultured sulfate-reducing bacterium NTd-V03 (AB263174.1)	Nankai Trough deep-sea sediment	86
dDSR4	JF906897	Uncultured bacterium clone ng3d1D14 (AY753127.1)	Nonsulfidogenic deltaic mobile mud sediment	94
dDSR5	JF906898	Uncultured sulfate-reducing bacterium clone LGWND6 (EFD65D93.1)	Groundwater polluted by leachate from landfill	94
dDSR6	JF906899	Uncultured sulfate-reducing bacterium NTd-VD5 (AB263176.1)	Nankai Trough deep-sea sediment	93
dDSR7	JF9069D0	Uncultured sulfate-reducing bacterium clone Nye-0 2-dsrG1D (HM0D4751.1)	Marine sediment Norway: Storegga Slide, Nyegga region	97
dDSR8	JF9069D1	Uncultured prokaryote clone DDFD7 11(GU39596D.1)	Mangrove sediment	96
dDSR9	JF906902	Uncultured bacterium clone ng7d1029 (AY753136.1)	Nonsulfidogenic tropical mobile muds	93
dDSR10	JF906903	Uncultured Syntrophobacteraceae bacterium clone dsr-5 (EU437396.1)	Gas hydrate-bearing sediment from Gulf of Mexico	94
dDSR11	JF906904	Uncultured bacterium clone fg8d11D6 (AY753099.1)	Nonsulfidogenic deltaic mobile mud sediment	84
dDSR12	JF906905	Uncultured bacterium clone ng7d1029 (AY753136.1)	Nonsulfidogenic deltaic mobile mud sediment	94
dDSR13	JF906906	Uncultured sulfate-reducing bacterium EBext-2 (FM212282.1)	Sediment contaminated by petroleum	93
dDSR14	JF906907	Uncultured sulfate-reducing bacterium clone PIM02A11 (AY741563.1)	Plum Island Estuary sediment	90

Table 20. Comparison of sequenced DGGE bands (dsrB gene) to closest relative in GenBank.

The PCR-DGGE analysis of the *dsr*B gene diversity in the three transects showed the presence of two dominant groups (dDSR 11 and dDSR 12) at all the stations which belonged to uncultured sulphate reducing bacteria, reported in nonsulfidogenic deltaic mobile mud sediment (Fig. 51). Phylogenetic analysis of the excised community bands showed that most of the sequences showed similarity to Deltaproteobacteria, Firmicutes and Uncultured sulphate reducing bacteria.

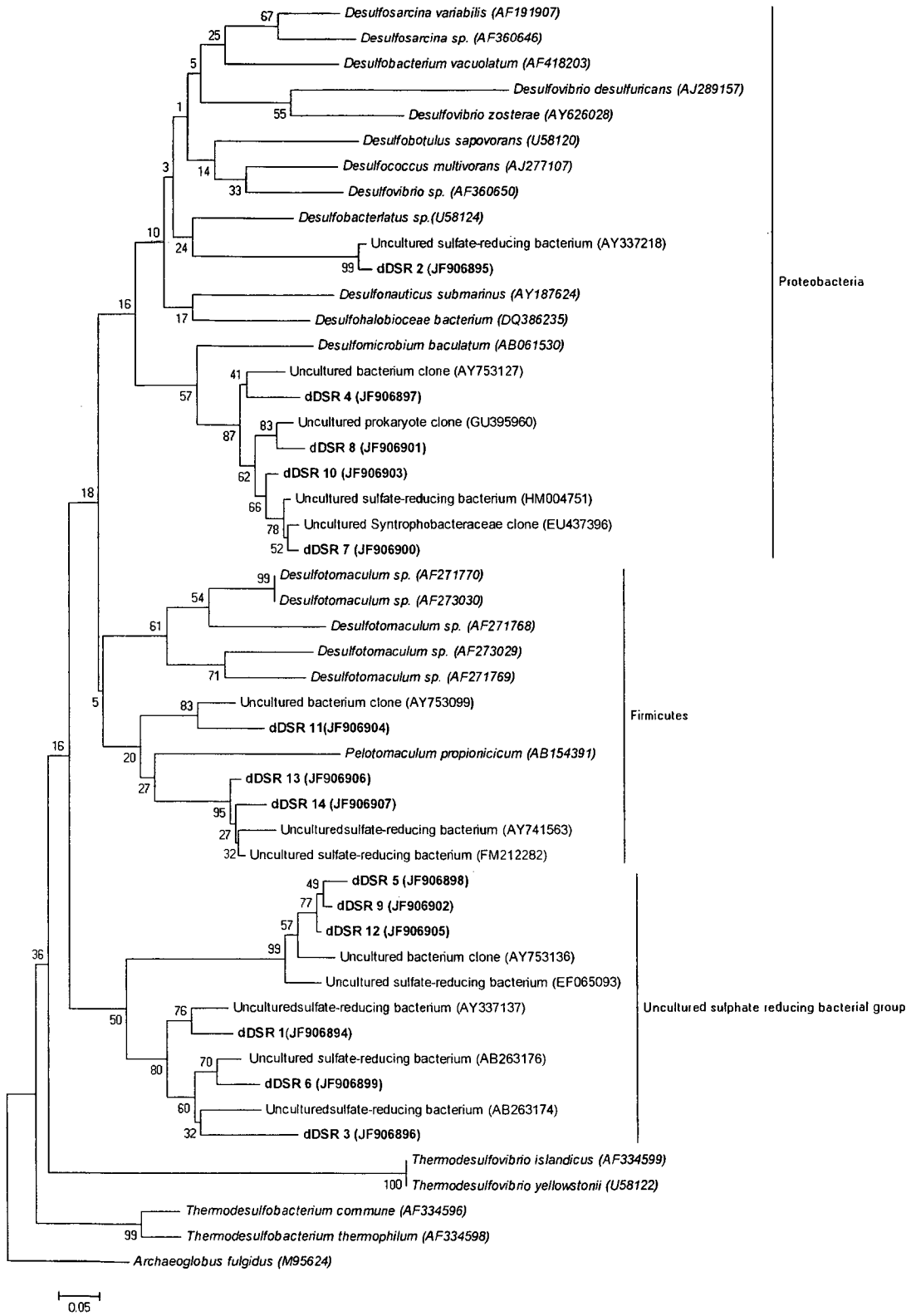


Fig. 51. Phylogenetic tree of *dsr*B gene sequences of Arabian Sea sediments. Sequences with prefix 'DSR' are results of the present study. Tree topology was inferred by neighbour joining method, using 1000 bootstrap iterations. *Archaeoglobus fulgidus* is the outgroup. Scale bar shows 5% sequence divergence.

About 43% of the sequences belonged to the uncultured sulphate reducing bacteria, followed by Proteobacteria (36%) and Firmicutes (21%) (Fig. 55).

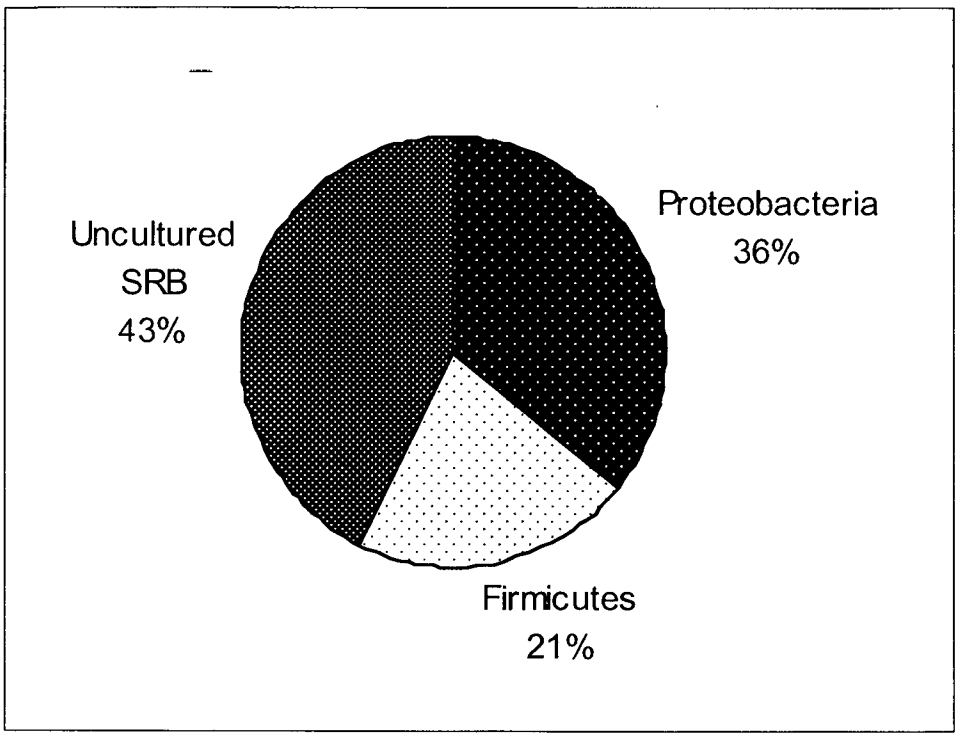


Fig. 55. Percentage of phylogenetic groups from the *dsr*B clone library

In the Karwar transect, Proteobacteria was the dominant group in the deeper stations such as 200, 500 and 1000 m while at 50 m the unaffiliated group was dominant. In the Goa transect, the Proteobacteria was the dominant group at all depths except at 50 m where unaffiliated group was the maximum. However, at 1000 m the unaffiliated group was also dominant along with Proteobacteria. In the Ratnagiri transect at 50 m and 200 m the dominant group was the unaffiliated group while at 500 m and 1000 m Proteobacteria was the dominant group (Fig. 56 A,B&C)

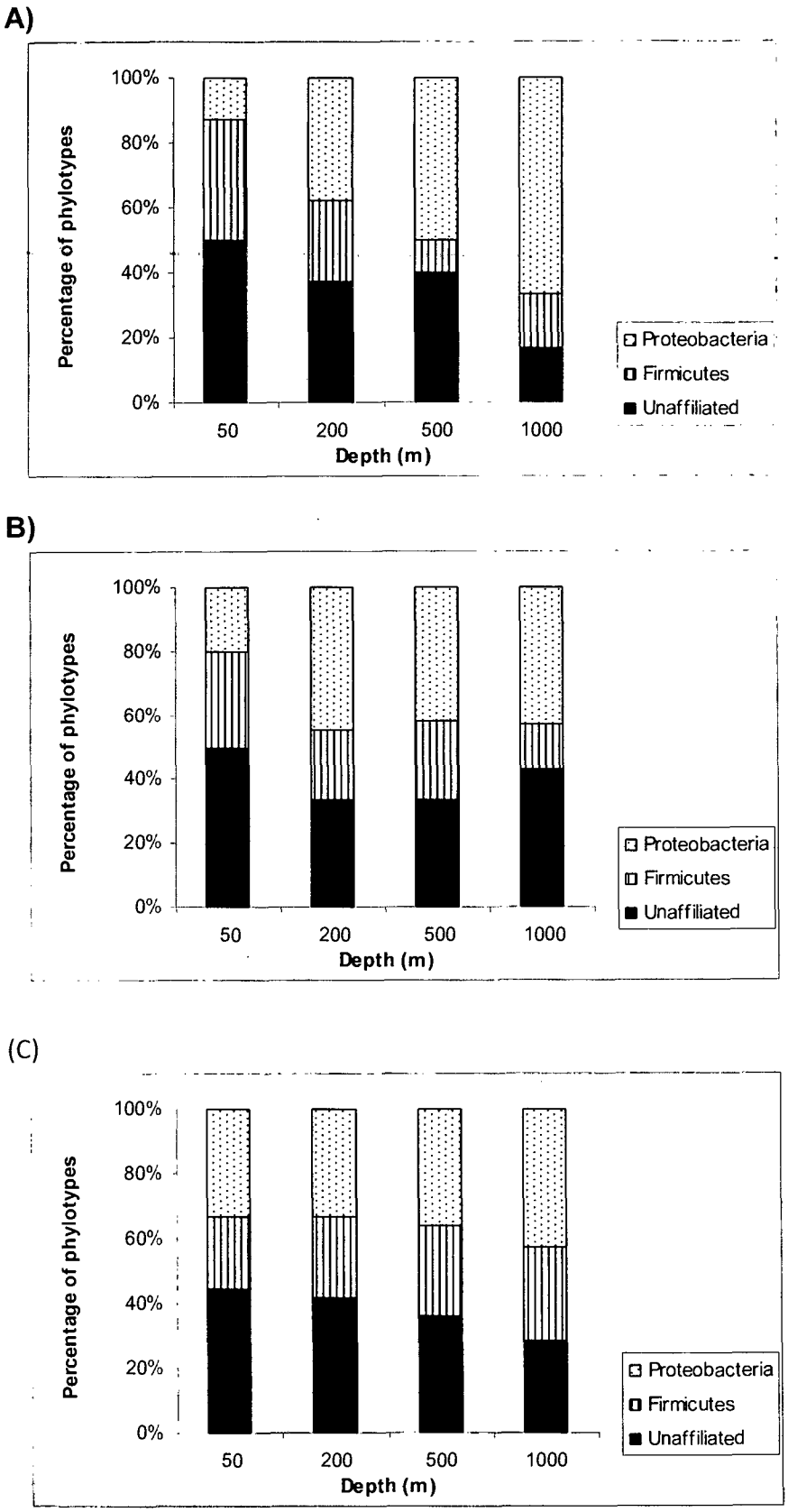


Fig. 56. Percentage of phylotypes in (A) Karwar (B)Goa and (C) Ratnagiri

Proteobacteria was the major group in the OMZ site and accounted for 45% of the population, while in the non-OMZ region Firmicutes and the unaffiliated group were dominant with 30 and 48% respectively (Fig. 57).

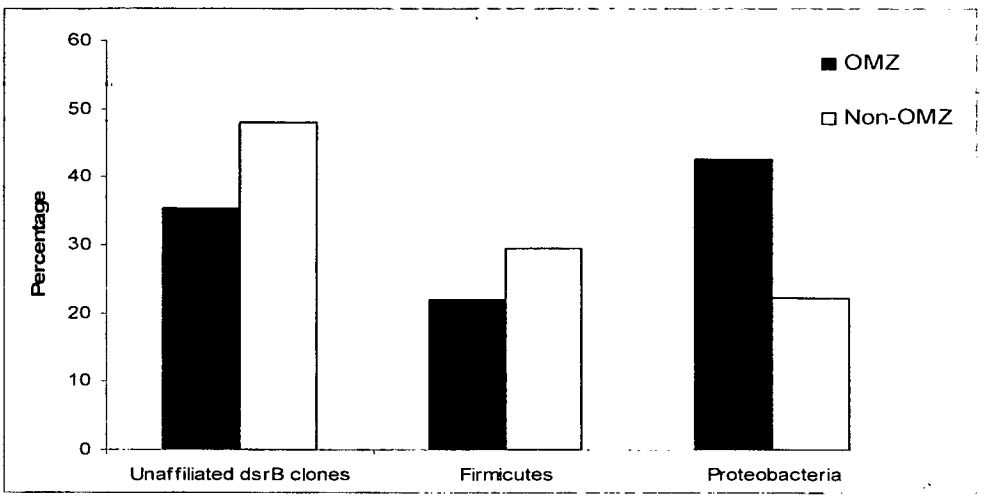


Fig. 57. Percentage distribution of various groups in OMZ sites

4.5.2.2. Species diversity index

Species abundance (d) was the highest at 500 m depth in Goa and Karwar transects, while in the Ratnagiri transect it was the highest at 50 m and 500 m depths (Table 21). The lowest species abundance was observed in the 1000 m in all transects. The same trend was observed for richness (J') and Shannon-Weiner Diversity Index (H').

Depth	Abundance	Richness	Eveness	Brillouin	Fisher	Shannon	Simpson
G 50m	27	5.66	0.79	2.3	12.25	2.61	0.9
G 200m	28	5.86	0.71	2.03	12.92	2.35	0.84
G 500m	31	6.51	0.71	2.07	15.38	2.43	0.85
G 1000m	10	1.95	0.53	1.08	2.77	1.23	0.6
K 50m	19	3.91	0.58	1.5	6.95	1.7	0.69
K 200m	17	3.47	0.71	1.82	5.88	2.02	0.8
K 500m	30	6.3	0.65	1.88	14.53	2.21	0.79
K 1000m	13	2.61	0.55	1.25	3.99	1.41	0.65
R 50m	25	5.21	0.64	1.78	10.7	2.06	0.77
R 200m	24	4.99	0.71	1.99	10.01	2.26	0.82
R 500m	25	5.21	0.7	1.97	10.7	2.25	0.82
R 1000m	17	3.47	0.63	1.57	5.88	1.78	0.72

Table 21. Diversity indices of Goa (G), Karwar (K) and Ratnagiri (R)

The Shannon-Weiner index (H') ranged from 1.4 to 2.4 for *dsr*B genes at all transect (Fig. 59). The highest was found at 500 m depth station off Goa and the lowest at 1000 m depth station off Karwar. The diversity index of *dsr*B gene dropped at 1000 m sites at all transects.

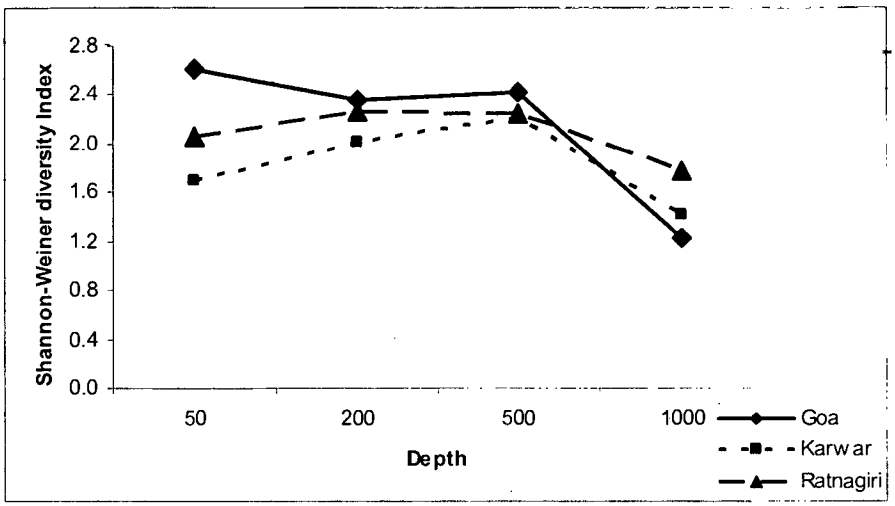


Fig. 59. Shannon-Weiner index for *dsr*B genes at all transect

The *dsr*B gene based DGGE fingerprinting provided a general insight into the sulphate reducing bacterial community of the ASOMZ. To understand more indepth community analysis of the composition using clone library was done for the Goa transect which is given below in detail.

4.5.2.3. Diversity in Goa transect

4.5.2.3.1. *dsr*B clone library analysis

The phylogenetic analysis of the clones showed that sequences fell into Uncultured sulphate reducing bacteria, Deltaproteobacteria and Firmicutes (Fig. 60). The sequences have been submitted in the GenBank (JF906908-JF906975) and are presented in the Appendix-A11. About 34 clones (DSR cluster I) formed one cluster without showing any similarity to any known sequences and formed a separate cluster. Also 10 clones were clustered together (DSR cluster II) and were grouped into the Firmicutes. The nearest neighbour sequences with similarity values and sequence length are given in the Table 22.The sequences of Uncultured sulphate reducing bacteria and Deltaproteobacteria were showing similarity to the sequences from mangrove, deep sea and continental margin sediments.

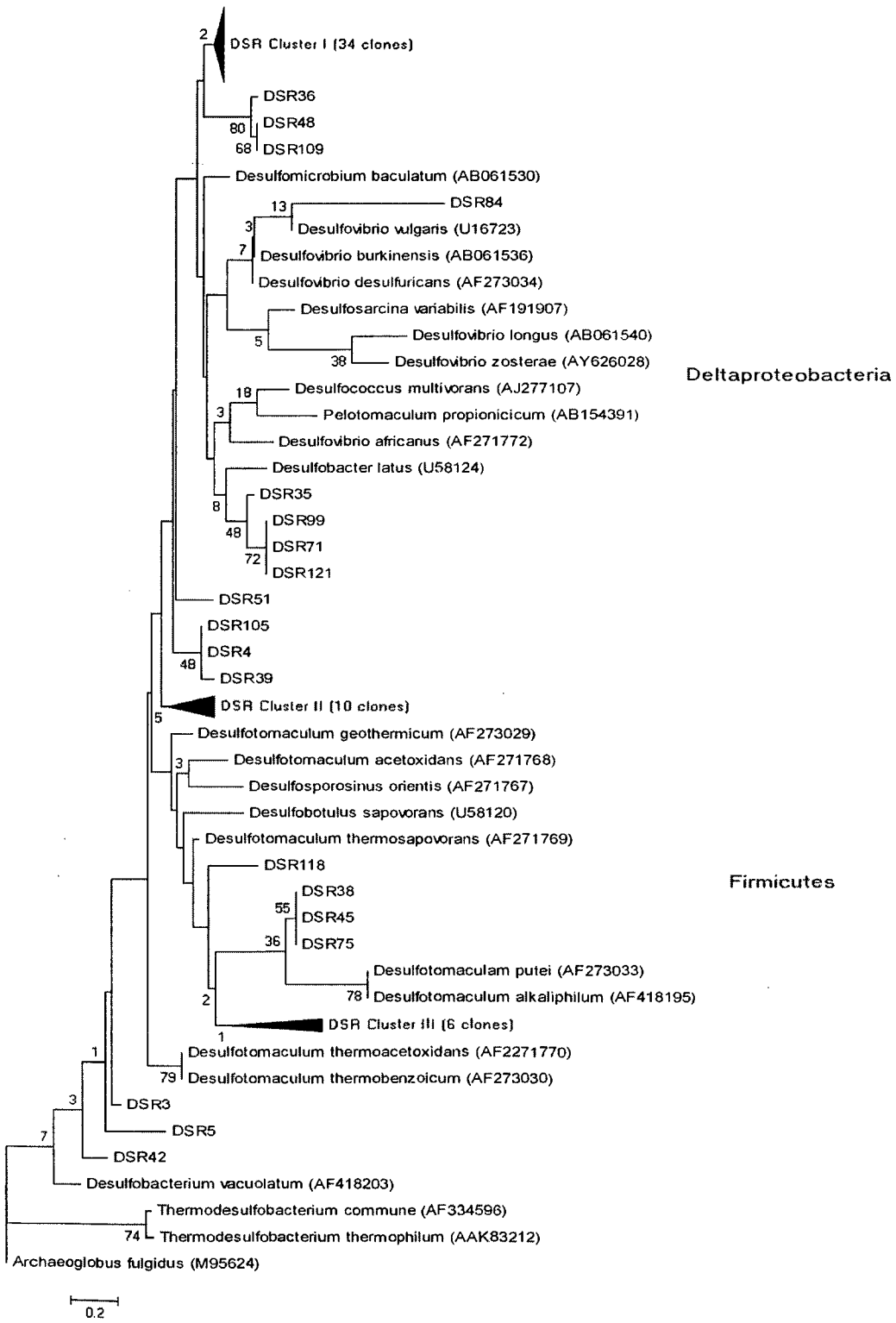


Fig. 60. Phylogenetic tree of *dsr*B gene sequences of Arabian Sea sediments. Sequences with prefix 'DSR' are results of the present study (accession numbers given in the table below). Tree topology was inferred by neighbour joining method, using 1000 bootstrap iterations. *Archaeoglobous fulgidus* is the outgroup. Scale bar shows 2% sequence divergence.

Clone	Accession Numbers	Nearest Neighbour	Similarity
DSR 49	JF906927	Uncultured prokaryote clone D0F07_11 (dsrB) GU395960.1	92
DSR 116	JF906970	Uncultured prokaryote clone D0F07_11 (dsrB) GU395960.1	93
DSR 34	JF906914	Uncultured prokaryote clone D0F07_11 (dsrB) GU395960.1	93
DSR 64	JF906935	Uncultured prokaryote clone D0F07_11 (dsrB) GU395960.1	91
DSR 43	JF906921	Uncultured prokaryote clone D0F07_11 (dsrB) GU395960.1	92
DSR 77	JF906944	Uncultured bacterium clone ng9d1016 (dsrB) AY753142.1	88
DSR 9	JF906912	Uncultured prokaryote clone D0F07_11 (dsrB) GU395960.1	90
DSR 101	JF906961	Uncultured srb clone Nye-0_2-dsrG10 dsr B HM004751.1	91
DSR 57	JF906932	Uncultured bacterium clone ng9d1016 (dsrB) AY753142.1	91
DSR 90	JF906953	Uncultured prokaryote clone D0F07_11 (dsrB) GU395960.1	92
DSR 82	JF906947	Uncultured prokaryote clone D0F07_11 (dsrB) GU395960.1	92
DSR 44	JF906922	Uncultured prokaryote clone D0F07_11 (dsrB) GU395960.1	91
DSR 89	JF906952	Uncultured prokaryote clone D0C12_06 (dsrB) GU395957.1	91
DSR 119	JF906973	Uncultured bacterium clone ng9d1016 (dsrB) AY753142.1	91
DSR 113	JF906967	Uncultured prokaryote clone D0F07_11 (dsrB) GU395960.1	92
DSR 91	JF906954	Uncultured srb clone M306064 dsr AB AY337188.1	98
DSR 117	JF906971	Uncultured srb clone M306064 dsr AB AY337188.1	99
DSR 86	JF906951	Uncultured prokaryote clone D0D09_07 (dsrB) GU395958.1	94
DSR 112	JF906966	Uncultured bacterium clone ng9d1016 (dsrB) AY753142.1	91
DSR 63	JF906934	Uncultured bacterium clone ng2d984 (dsrB) AY753117.1	82
DSR 104	JF906962	Uncultured bacterium clone ng9d1016 (dsrB) AY753142.1	91
DSR 115	JF906969	Uncultured bacterium clone ng9d1016 (dsrB) AY753142.1	91
DSR 81	JF906946	Uncultured prokaryote clone D0F07_11(dsrB) GU395960.1	92
DSR 85	JF906950	Uncultured prokaryote clone D0D09_07(dsrB) GU395958.1	91
DSR 59	JF906933	Uncultured prokaryote clone D0C12_06 (dsrB) GU395957.1	92
DSR 40	JF906919	Uncultured prokaryote clone D0C12_06 (dsrB) GU395957.1	94
DSR 54	JF906931	Uncultured prokaryote clone D0F07_11 (dsrB) GU395960.1	91
DSR 65	JF906936	Uncultured prokaryote clone D0C12_06 (dsrB) GU395957.1	94
DSR 53	JF906930	Uncultured prokaryote clone D0F07_11(dsrB) GU395960.1	94
DSR 100	JF906960	Uncultured srb clone Nye-0_2-dsrG10 dsr B HM004751.1	91
DSR 73	JF906942	Uncultured srb Nye-0_2-dsrG10 dsr B HM004751.1	95
DSR 92	JF906955	Uncultured prokaryote clone D0F07_11 (dsrB) GU395960.1	94
DSR 46	JF906924	Uncultured srb clone Nye-0_2-dsrG10 dsr B HM004751.1	92
DSR 124	JF906975	Uncultured prokaryote clone D0F07_11 (dsrB) GU395960.1	94
DSR 36	JF906916	Uncultured srb dsrA, dsrB genes clone: NTd-I20 AB263144.1	90
DSR 48	JF906926	Uncultured srb dsrA, dsrB genes clone: NTd-I20 AB263144.1	91
DSR 109	JF906965	Uncultured srb dsrA, dsrB genes clone: NTd-I20 AB263144.1	91
DSR 84	JF906949	Uncultured srb dsr AB Clone Ebext-25 FM212302.1	93
DSR 35	JF906915	Uncultured srb clone M300232 AY337218.1	92
DSR 99	JF906959	Uncultured srb clone M300232 AY337218.1	88
DSR 71	JF906940	Uncultured srb clone M300232 AY337218.1	89
DSR 121	JF906974	Uncultured srb clone M300232 AY337218.1	89
DSR 51	JF906928	Uncultured srb clone: NTd-I22 AB263146.1	89
DSR 105	JF906963	Uncultured Desulfobacteraceae bacterium clone dsr-190 EU496888.1	95
DSR 4	JF906909	Uncultured Desulfobacteraceae bacterium clone dsr-190 EU496888.1	97
DSR 39	JF906918	Uncultured srb dsrA&B clone: NTd-I11 AB263135.1	94
DSR 52	JF906929	Uncultured srb clone VHS119 DQ112212.1	83

DSR 72	JF906941	Uncultured srb clone PIMO2A11 AY741563.1	90
DSR 83	JF906948	Uncultured srb clone PIMO2A11 AY741563.1	90
DSR 7	JF906911	Uncultured srb dsrb Nye-0_2-dsrB10 HM004730.1	88
DSR 94	JF906956	Uncultured srb dsrA, dsrB clone: NTd-I08 AB263132.1	85
DSR 106	JF906964	Uncultured srb clone LGWG24 dsr a, dsr b EF065029.1	83
DSR 69	JF906938	Uncultured prokaryote clone D40F08_12 dsr b GU395858.1	80
DSR 68	JF906937	Uncultured bacterium dsrA, dsrB YS16dsrAB03 AB451524.1	80
DSR 70	JF906939	Uncultured bacterium dsrA, dsrB YS16dsrAB03 AB451524.1	80
DSR 79	JF906945	Uncultured srb clone LGWG24 EF065029.1	81
DSR 118	JF906972	Uncultured srb clone TopDsr35 FJ748832.1	85
DSR 38	JF906917	Uncultured srb clone LGWG01 EF065021.1	77
DSR 45	JF906923	Uncultured srb clone LGWG01 EF065021.1	83
DSR 75	JF906943	Uncultured srb clone LGWG01 EF065021.1	77
DSR 47	JF906925	Uncultured srb partial dsrA and dsrB; DSRI-7 AM408827.1	93
DSR 114	JF906968	Uncultured srb a70 AF388267.1	87
DSR 28	JF906913	Uncultured srb clone AM236162.1	94
DSR 97	JF906958	Uncultured bacterium clone fg8d1100 AY753097.1	93
DSR 95	JF906957	Uncultured bacterium clone ng3d1010 AY753125.1	
DSR 3	JF906908	Uncultured srb clone M300209 AY337136.1	92
DSR 5	JF906910	Uncultured srb clone B04P017 AY197454.1	88
DSR 42	JF906920	Uncultured srb FW-005-066 AY885522.1	94

Table 22. Comparison of sequenced clones (dsrB gene) to closest relative in GenBank.

*dsr*B clone library analysis of the 1000 m station showed that the clones belonged to three main groups Uncultured sulphate reducing bacteria, Deltaproteobacteria and Firmicutes which is shown in Figure 61.

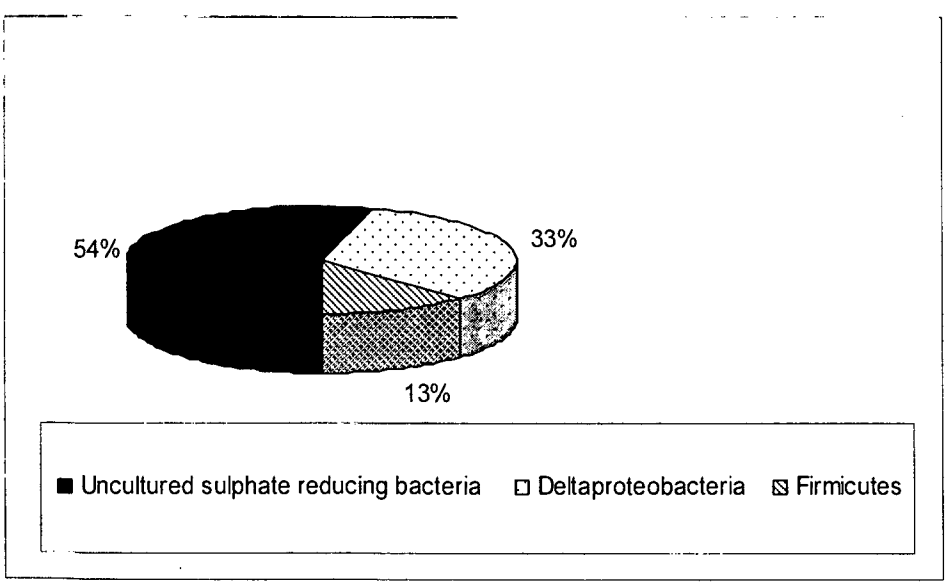


Fig. 61. Abundance of groups in *dsr*B sediment library

4.5.2.3.2. Enzymatic Diversity

(i)Respiratory enzymes

The bacterial isolates were subjected to various physiological tests like oxidase and catalase. Most of the bacteria analyzed at all the depths were oxidase positive. The highest fraction of 68% occurred at 1000 m depth and the lowest at 500 m (34%). However, isolates showing catalase activity was the highest in the shallowest depths and decreased with increasing in depth (Fig. 62).

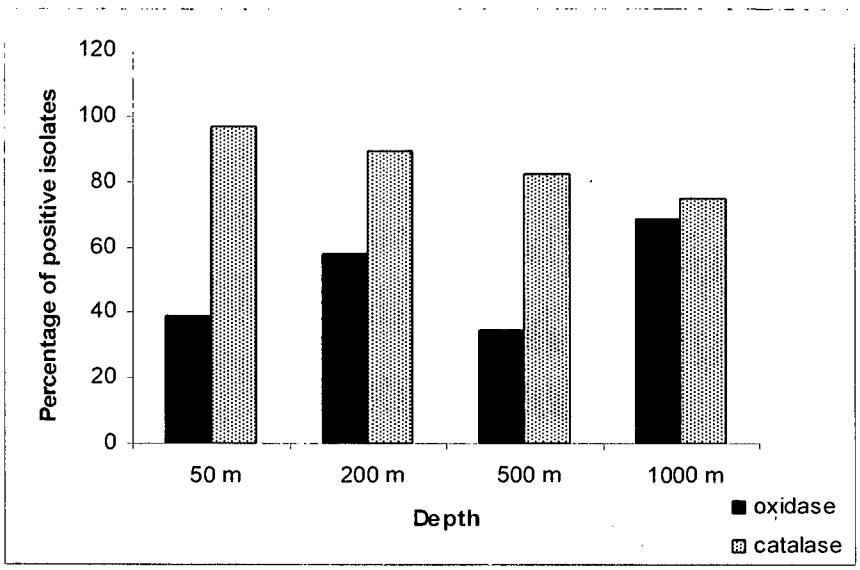


Fig. 61. Variation in the activity of respiratory enzymes with depth off Goa

(ii) Amino acid utilization

Majority of the isolates from the shallowest site (50 m) could utilize the amino acid lysine (54%) compared to the isolates from the deeper sites. On the contrary, the utilization of ornithine (36%) and tryptophan (18%) was the highest in the deepest site while no tryptophan positive isolates were obtained from 50 m and 500 m (Fig. 62).

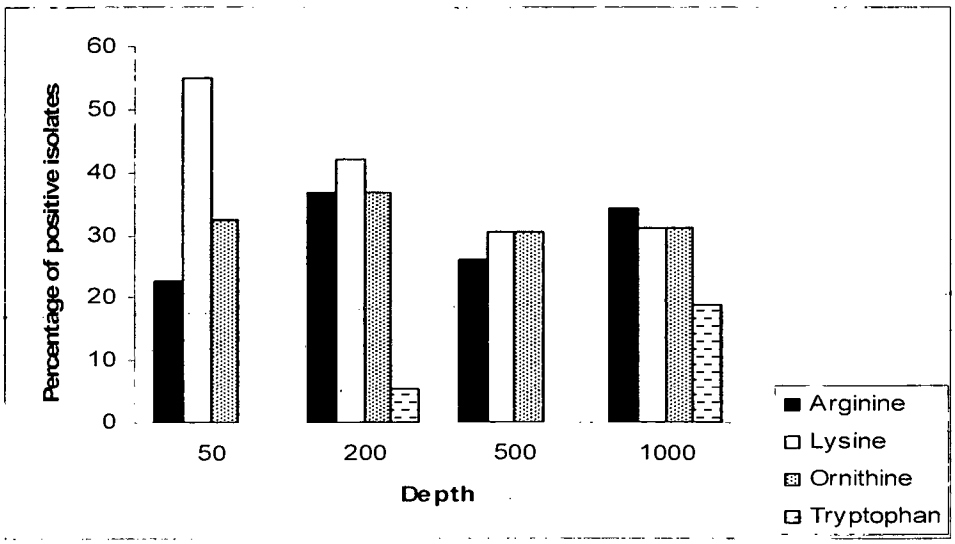


Fig. 62. Variation in the aminoacid utilizing bacteria in station depths (m) off Goa.

(iii) Enzymes in the Carbon cycle

(a) Amylase

Amylase activity was about twice at the 50 m depth (70%) than that of the isolates from the 1000 m depth (34%). However, the lowest activity was shown at 200 m depth (26%) (Fig. 63).

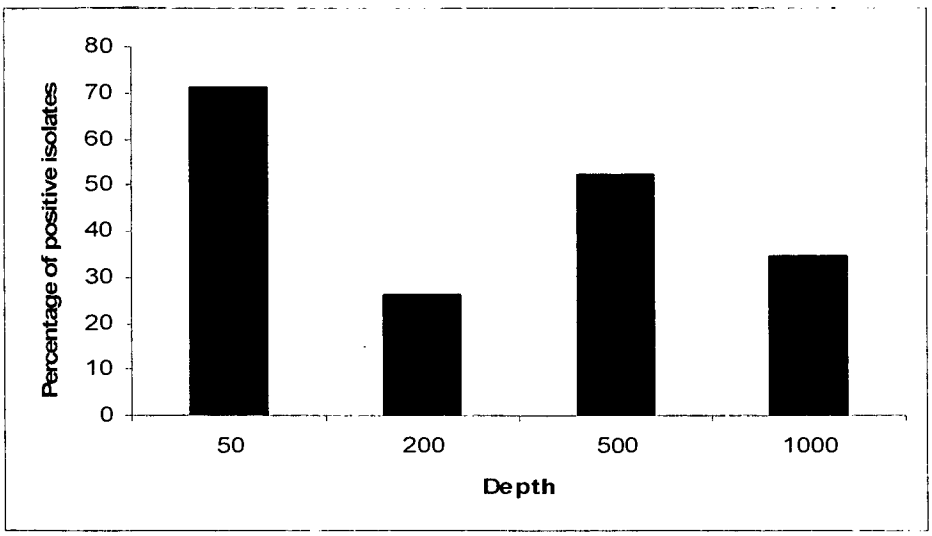


Fig. 63. Variation in the activity of amylase enzymes with depth (m)

(b) Lipase and Esterase

Hydrolysis of tween 80 by the isolates were shown to be 41% and 15% respectively at 50 m and 1000 m whereas that of tween 20 was the highest in the deepest site (31%) followed by the shallowest site.

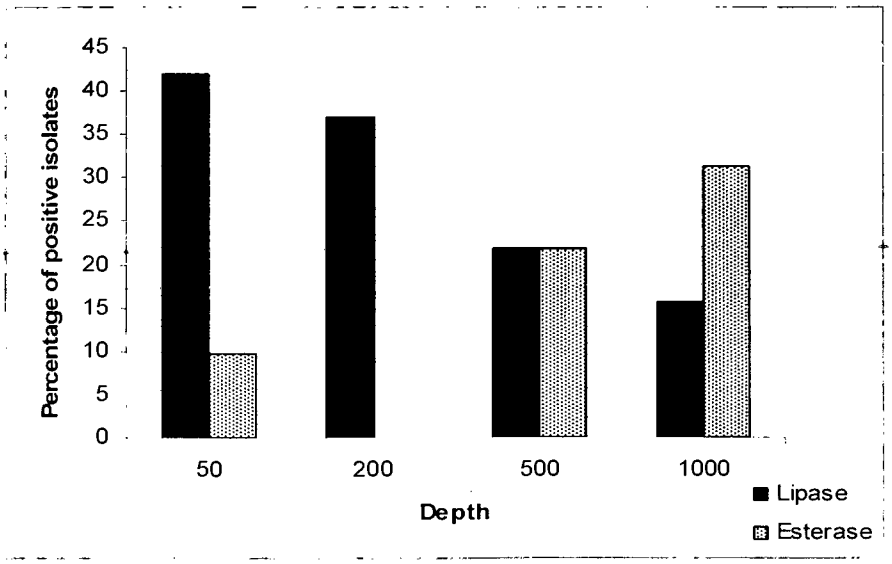


Fig. 64. Variation in the activity of lipase and esterase with depth (m)

(c)Citrate permease

The highest citrate permease or citrase activity was shown by isolates of 1000 m depth with a value of 43%, followed by those from 50 m depth (19%), whereas isolates of 500 m and 200 m showed similar activity of around 5% (Fig. 65).

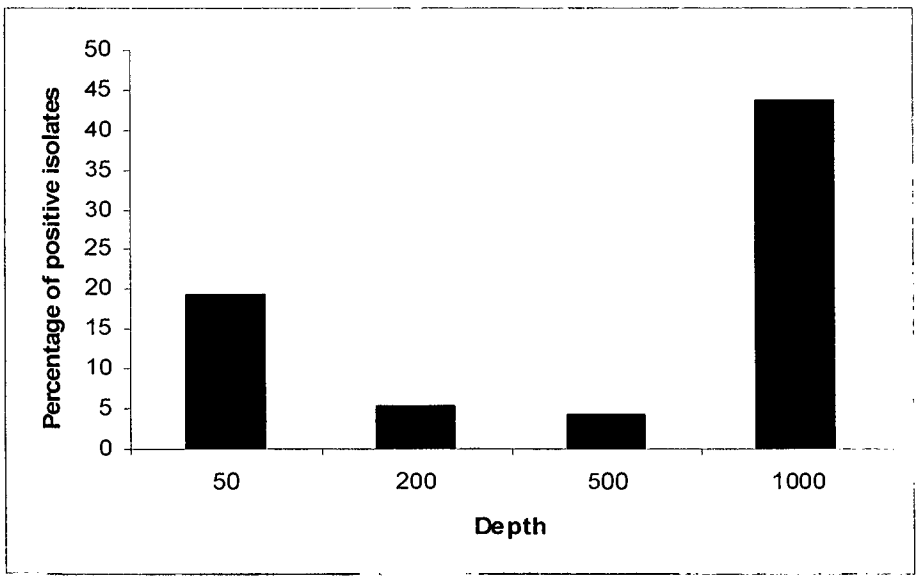


Fig. 65. Variation in the activity of citrate permease with depth (m)

(iv) Enzymes in the Nitrogen cycle

(a) Gelatinase

About 74% of the isolates from the shallowest site were able to degrade gelatin while only 15% of the isolates could utilize the substrate from the deepest region (Fig. 66).

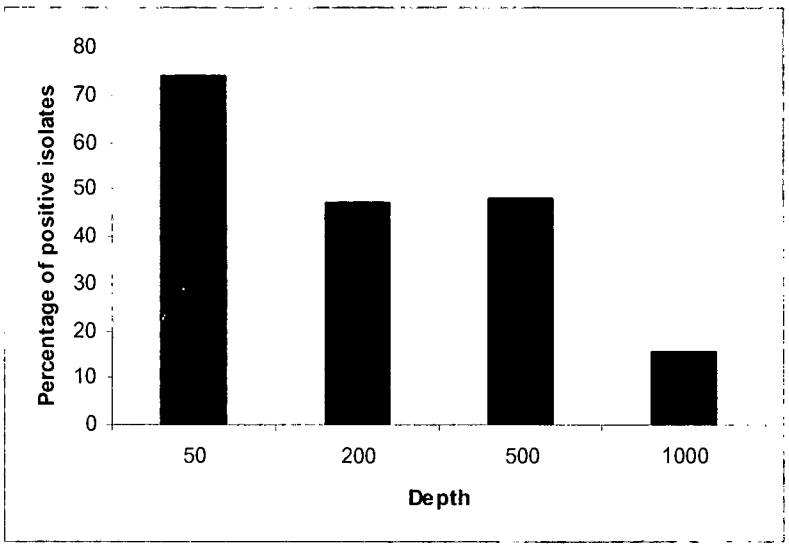


Fig. 66. Variation in the activity of gelatinase with depth (m)

(b) Urease

The highest urease activiry was observed at 500 m (17%) followed by 1000 m (15%). The lowest was seen at 200 m (5%) (Fig. 67).

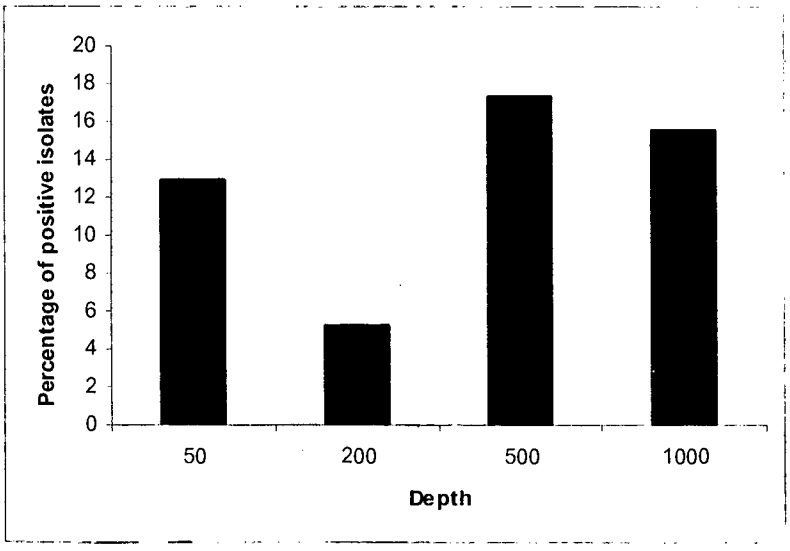


Fig. 67. Variation in the activity of urease with depth (m)

(c) Nitrate reductase

About 65% of the isolates from 1000 m showed nitrate reductase activity followed by 200 m and 500 m (52%). The lowest was observed at 50 m station (38%) (Fig. 68).

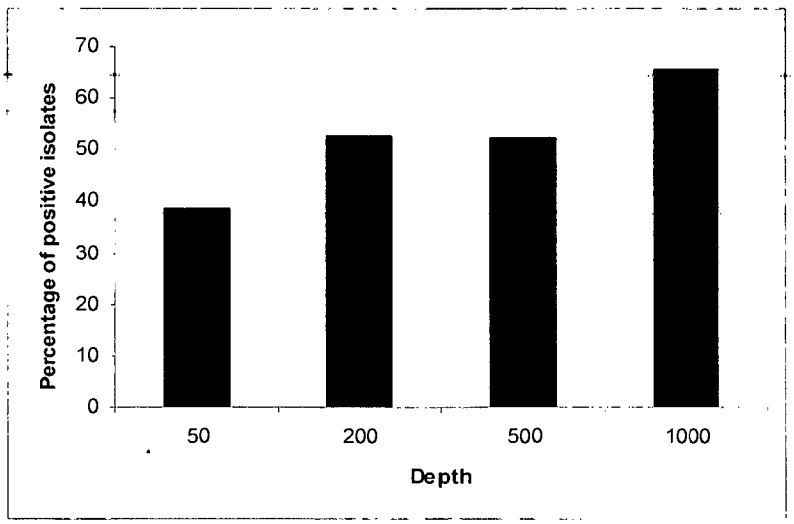


Fig. 68. Variation in the nitrate reductase with depth (m)

(v) Enzymes in the Phosphorus cycle

(a) DNase

The highest DNase activity was shown by bacteria from 1000 m depth (37%) followed by isolates of 200 m (15%), and lowest activity was recorded by those of 500 m depth (9%) (Fig. 69).

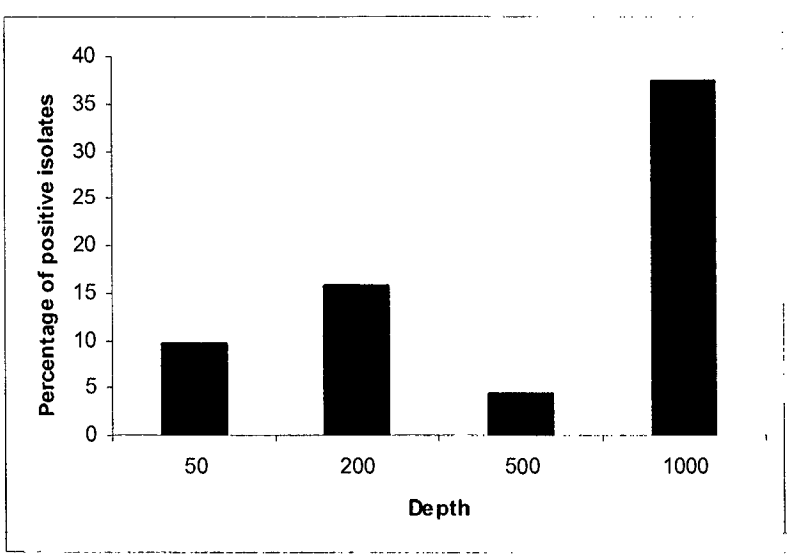


Fig. 69. Variation in the activity of DNase with depth (m)

(b)Phosphatase

About half the isolates (56%) from 1000 m depth could express phosphatase enzyme and the lowest (47%) was obtained at 500 m (Fig.70).

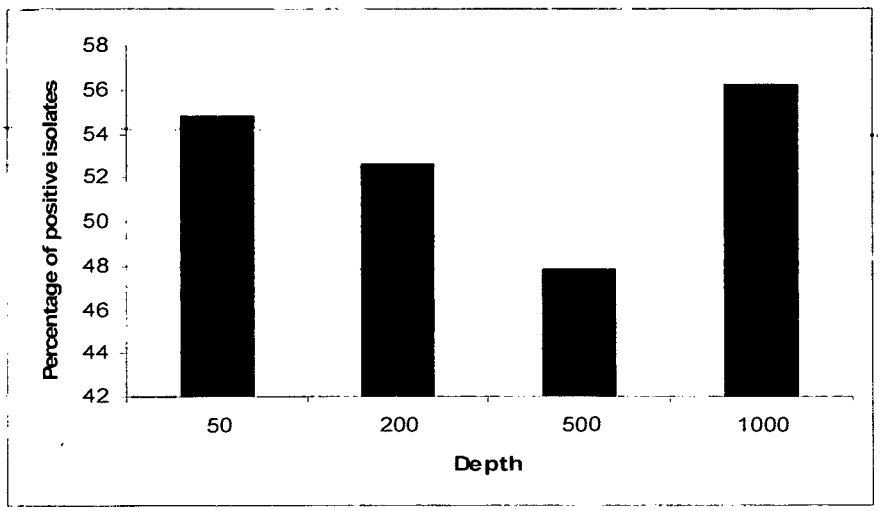
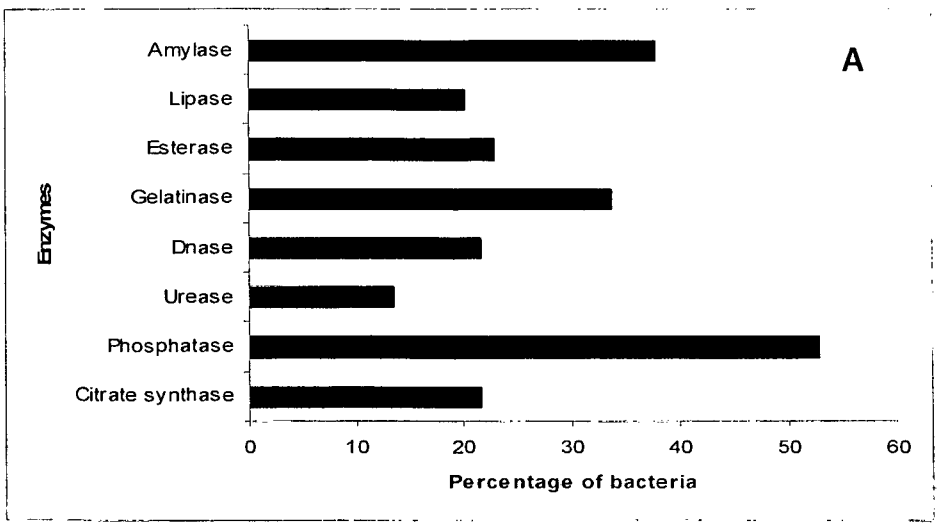


Fig. 70. Variation in the activity of phosphatase with depth (m)

(vi) Comparison of enzymatic activities in OMZ and non-OMZ regions

Among the hydrolytic enzymes the OMZ isolates had higher activity of urease (14%), DNase (22%) and lipase (20%) while in the non-OMZ region the highest activity was shown for the enzymes gelatinse, esterase and amylase (74%, 42% and 71%). However, the phospatase activity was almost similar with 54% for both the OMZ and non-OMZ regions (Fig.71A&B).



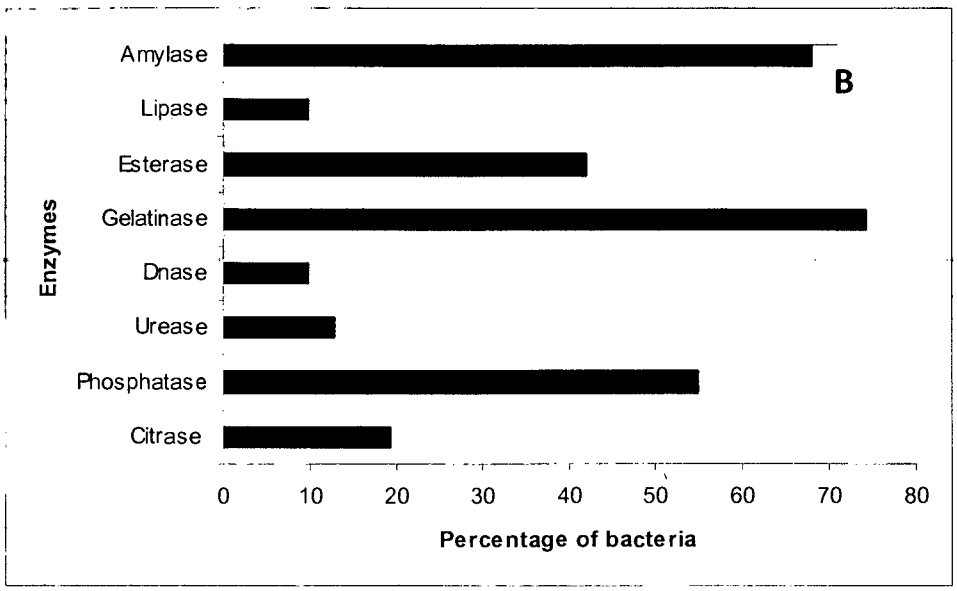
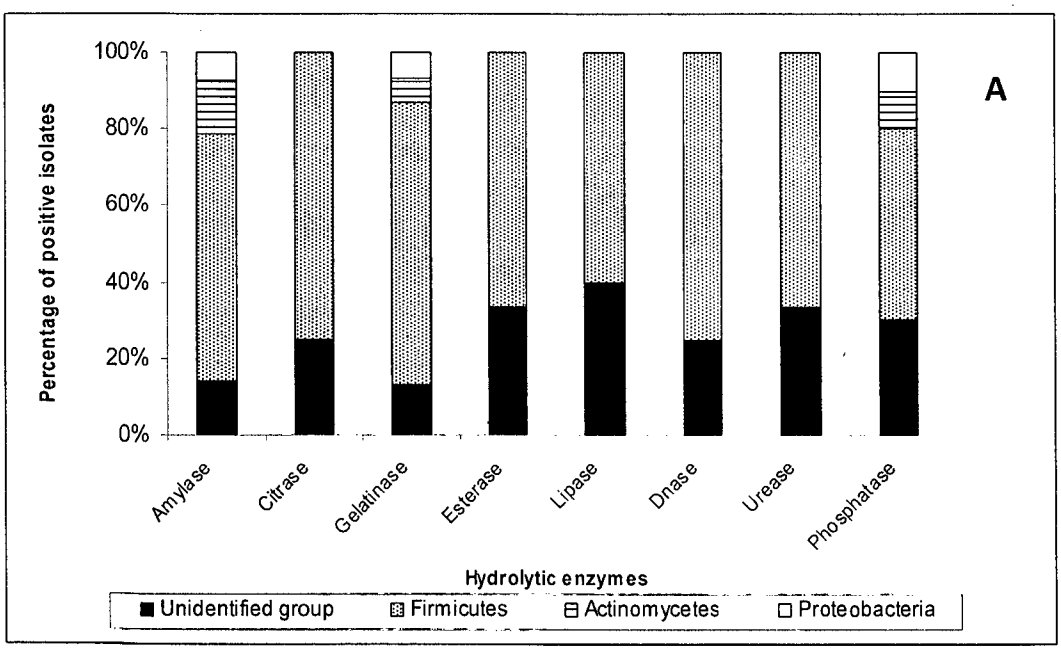


Fig. 71. Comparison of hydrolytic enzyme activities in (A) OMZ and (B) non-OMZ

Overall phylotype-wise comparison of enzymatic activities of OMZ and non-OMZ isolates showed that in OMZ the different phylotypes exhibited enzymatic activities whereas in the non-OMZ region it was restricted to two groups (Fig. 72A &B)



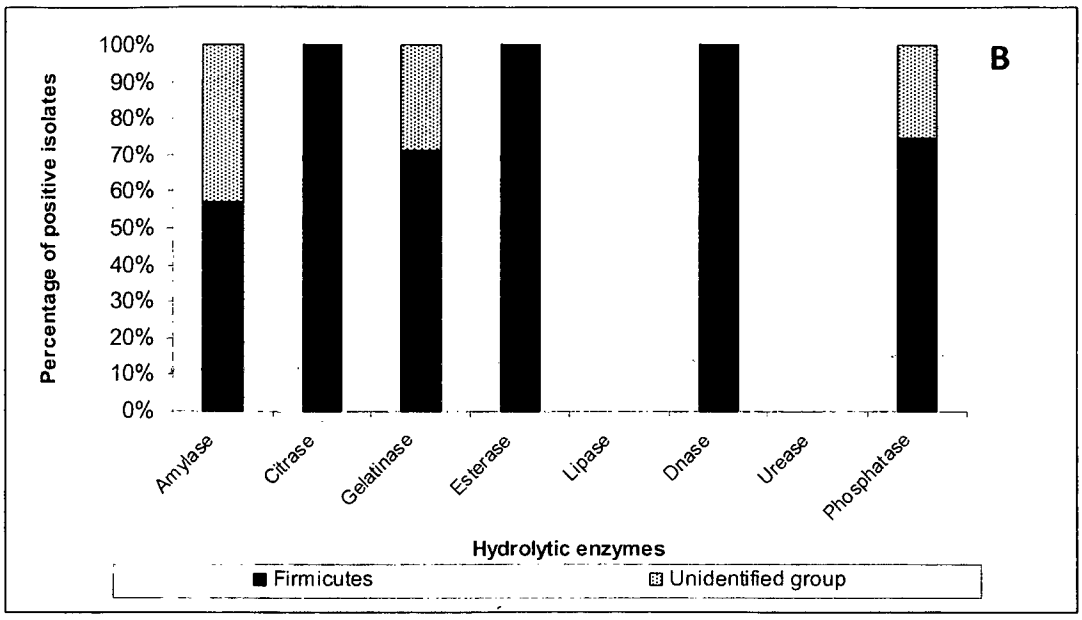


Fig. 72. Distribution of hydrolytic enzyme activities among phylotypes in the (A) OMZ and (B) non-OMZ

The present study showed that the OMZ bacterial community is phylogenetically distinct and metabolically active.

4.5.3.Statistical analysis

4.5.3.1. Statistical analysis of the 16S rRNA- DGGE band profiles

Herarchical cluster analysis was performed to gain an overview on the relatedness of the bands representing each sampling sites based on cluster analysis of the intensity matrix. The cluster analysis of DGGE band data of the three transects is presented in Figure 72. Each station seems to be independent at >80% similarity. At 80% similarity, 500 m stations of Goa and Ratnagiri grouped as one cluster. As the similarity decreased the clustering increased with deeper stations forming the major cluster, except 200 m station at Goa and 1000 m station at Karwar. The communities could be differentiated into two clusters as a function of depths. Thus the analysis showed the existence of distant bacterial phylotype in OMZ and non-OMZ of AS indicating that the spatial factor was important in determining the bacterial assemblage composition.

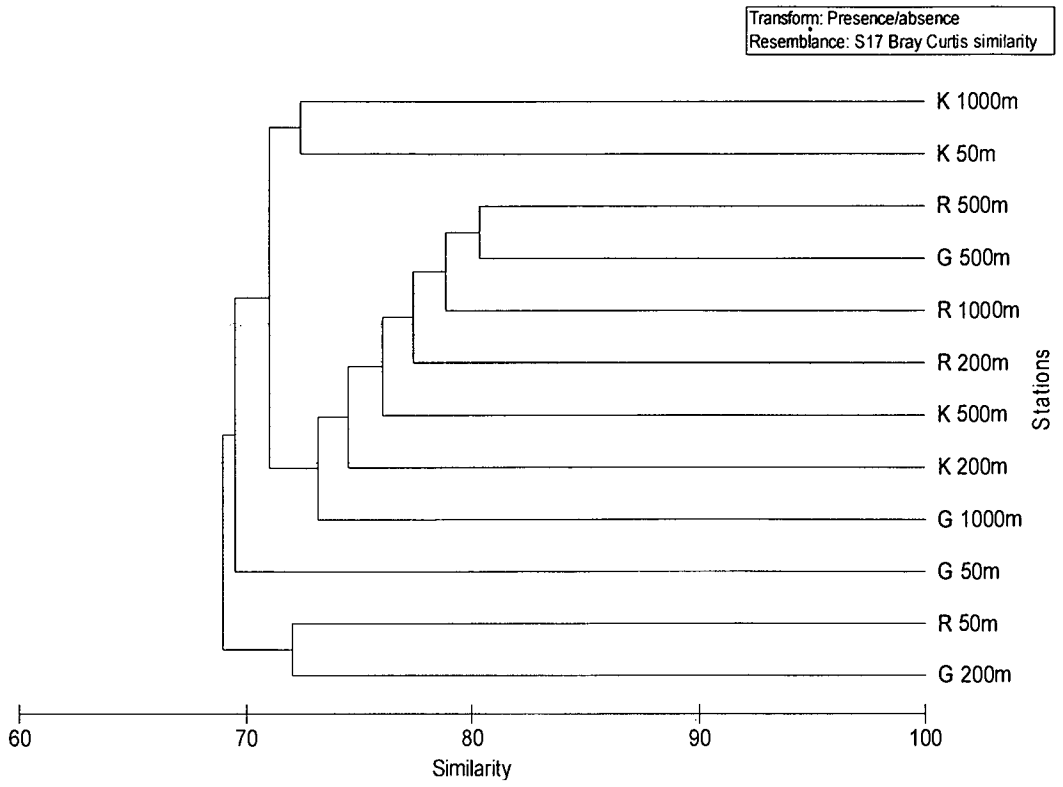


Fig. 72. Hierarchical cluster analysis of the 16S rRNA DGGE band profiles of three transects (G-Goa,R-Ratnagiri and K- Karwar) and depths

Correlation of 16S rRNA-DGGE band profiles to Environmental parameters

DGGE was used to study the spatial variation in bacterial community at various depths in three transects and CCA was applied to analyze the environmental factors on the community. The eigen values of the oordination analyses are presented in Table 22. Concerning the variance of the species data, the first axis explained 15% of the total variation, the first and the second axes explained 29.7%. The species environmental correlation was high with a value of 1 indicating a relationship between species and environmental variables. Biplot scaling of CCA is shown in Figure 73. The OMZ and non-OMZ regions were clearly demarcated forming two groups. Environmental variables such as nitrite, protein, temperature, sand and dissolved oxygen influenced the non-OMZ regions. Interestingly, clay, phosphate, nitrate, TC, TOC, TN, Eh and silicate influenced the OMZ.

Axes	Eigen values	Percentage	Cumulative percentage variance of species data	Species-environment correlations
Axis 1	0.093	15.051	15.051	1
Axis 2	0.091	14.678	29.73	1
Axis 3	0.076	12.321	42.05	1
Axis 4	0.065	10.501	52.552	1

	Envi. Axis 1	Envi. Axis 2	Envi. Axis 3	Envi. Axis 4
Temperature	-0.285	0.188	0.478	-0.045
Salinity	-0.45	0.009	0.264	-0.181
pH	-0.549	0.123	0.378	-0.075
DO	-0.668	0.089	0.248	-0.104
Nitrate	0.477	-0.082	-0.403	0.199
Nitrite	-0.391	0.443	0.12	-0.214
Phosphate	0.545	-0.142	-0.377	0.091
Silicate	0.173	0.083	-0.458	0.04
Eh	0.114	-0.21	-0.053	-0.087
Water Content	-0.001	-0.406	0.167	-0.138
TC	0.508	-0.33	-0.346	0.148
TN	0.264	-0.495	-0.12	-0.099
TH	0.077	0.022	0.321	-0.553
TS	-0.446	0.143	0.054	0.281
TOC	0.544	-0.33	-0.36	0.126
Carbohydrates	-0.247	0.01	0.068	-0.218
Lipids	-0.229	0.029	-0.361	-0.008
Protein	-0.491	0.311	0.247	-0.152
Sand	-0.253	0.165	0.154	0.224
Silt	-0.765	-0.021	-0.01	-0.208
Clay	0.787	-0.1	-0.101	0.007

Table 23. Eigen values and variance decomposition for CCA and interest correlation coefficients

In order to achieve a detailed analysis of bacterial community and factors influencing distinct phylotypes CCA of the bacterial phylotypes (sequenced bands) and environmental factors were performed (Fig. 74). CCA with interspecies distances was performed to calculate the influence of environmental variables on specific phylotypes. The first axis explained 23% of the total variation, the first and the second axes together explained 41.7% of the variation. The different excised bands could be grouped into three.

Group 1 was mainly influenced by silt, lipids, silicate, phosphate, nitrate, TN, TOC and water content. Group 2 was mainly influenced by temperature, sand, clay and Eh. Group 3 had two bands/phylotypes that did not group with the others.

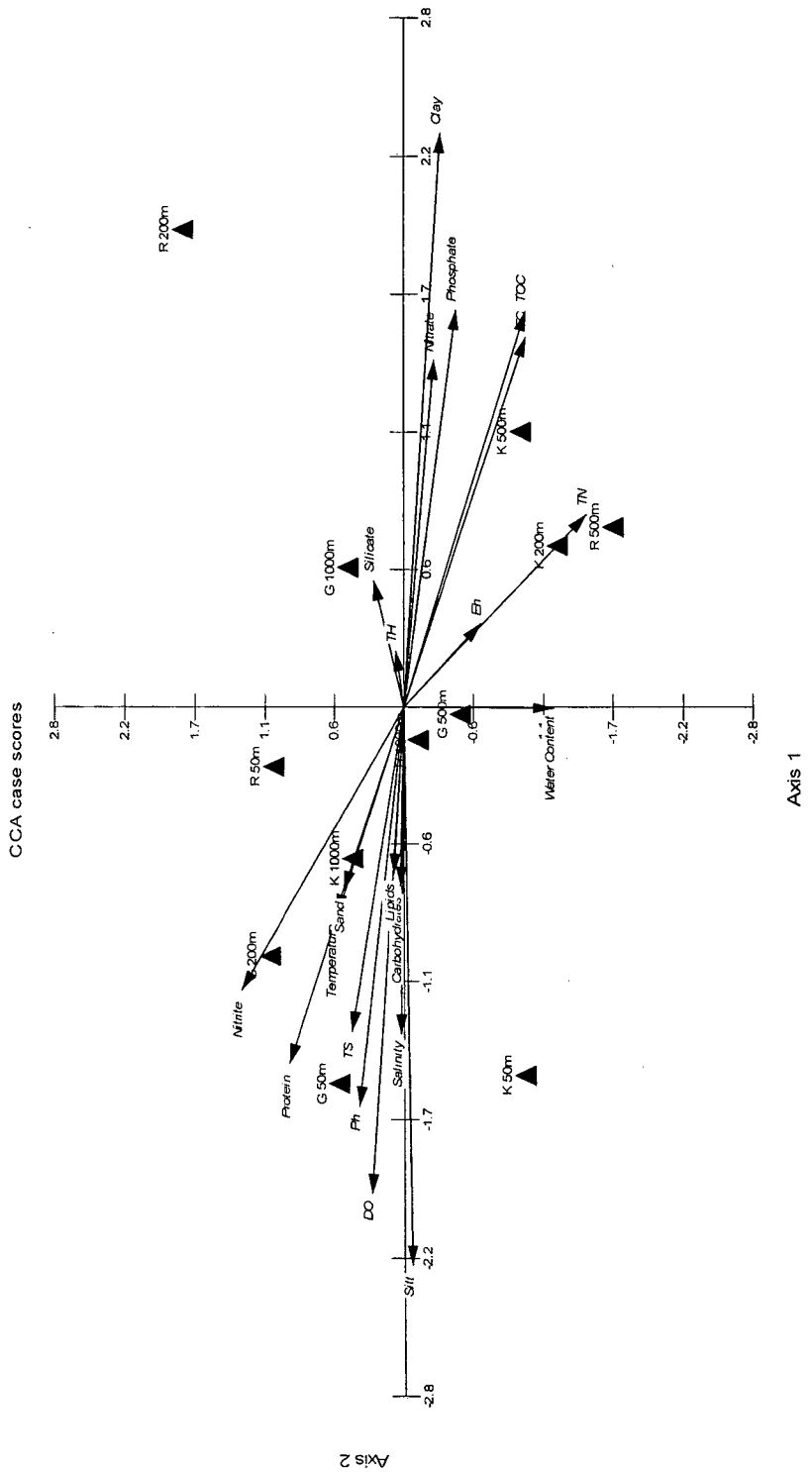


Fig. 73. CCA biplots of intersample distances of DGGE profiles of the whole community from all sites using environmental variables. Triangles indicate the sampling stations .

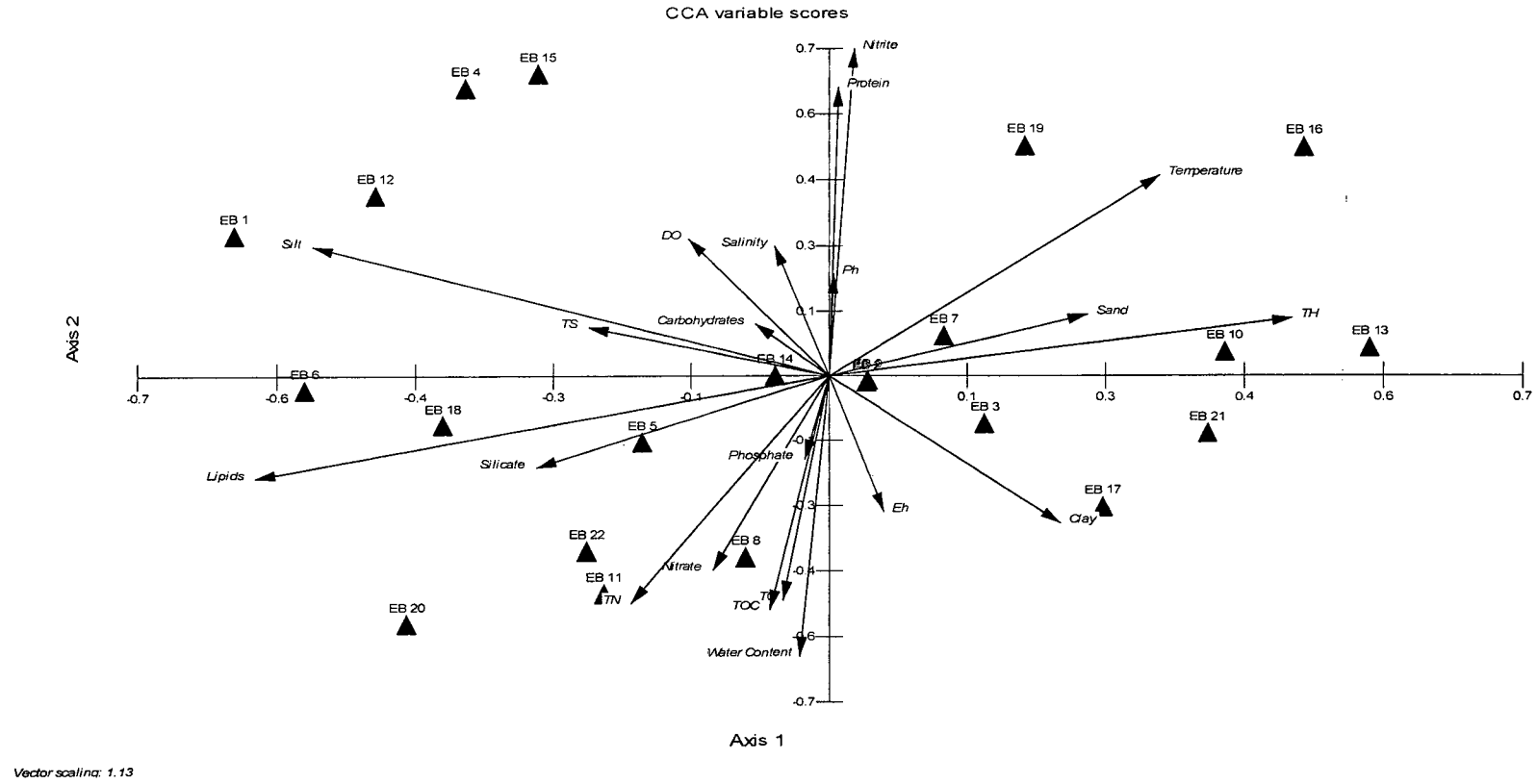


Fig. 74. Biplot of interspecies distances; CCA of DGGE fingerprints of the bacterial community using environmental variables. TRIANGLES with numbers indicate sequenced bands.

4.5.3.2.Statistical analysis of the *dsr*B-DGGE band profiles

At about 45% similarity, two clusters were formed; one with 1000 m sites off Karwar, Goa and Ratnagiri and the other with the remaining sites (Fig. 75). Further sub-division of the major cluster grouped the *dsr*B gene into two sub clusters with the Karwar bands grouping into one cluster except for K 500 m. There was clustering of OMZ and non-OMZ sites at higher similarity, but did not form discrete major clusters at lower similarities.

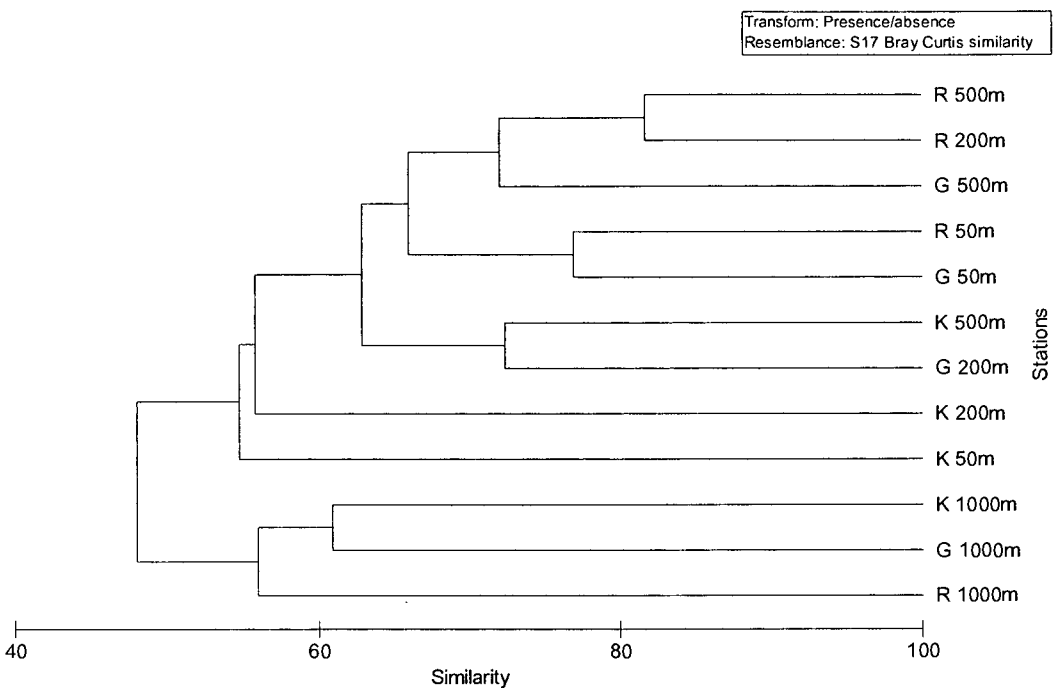


Fig. 75. Cluster analysis of the *dsr*B DGGE band profiles of three transects (Goa, Ratnagiri and Karwar) and four depths (50 m, 200 m, 500 m and 1000 m).

Correlation of *dsr*B-DGGE band profiles to Environmental parameters

CCA of *dsr*B DGGE profiles was carried out in order to understand the relationship between the sulphate reducing bacterial community and environmental parameters (Fig. 76 & 77). The eigen values of the ordination analysis are presented in Table 20. The first axis explained 21.4% of the variation and the first and second axis together explained 36.7% of the variation. Species- environmental correlation was high with a value of 1. The important variables in the first axis were temperature, nitrate, silicate, carbohydrates, lipids, sand and Eh. The important variables in the second axis bringing about change were nitrite, TH, TOC and water content.

	Eigenvalues	Percentage	Cumulative percentage variance of species data	Species-environment correlations
Axis 1	0.256	21.465	21.465	1
Axis 2	0.182	15.275	36.74	1
Axis 3	0.152	12.793	49.533	1
Axis 4	0.136	11.412	60.946	1

	Envi. Axis 1	Envi. Axis 2	Envi. Axis 3	Envi. Axis 4
Temperature	-0.424	0.09	0.727	0.255
Nitrate	0.319	0.079	-0.743	-0.38
Nitrite	0.114	-0.488	0.255	0.476
Phosphate	0.102	-0.073	-0.822	-0.195
Silicate	0.632	-0.117	-0.7	-0.054
TC	-0.093	0.207	-0.683	-0.468
TN	0.061	-0.009	-0.226	-0.105
TH	0.297	-0.473	0.044	0.183
TS	-0.315	-0.019	0.284	0.233
TOC	-0.018	0.219	-0.691	-0.46
Carbohydrates	0.264	-0.089	0.643	0.06
Lipids	0.532	-0.015	-0.218	0.251
Proteins	-0.187	-0.149	0.665	0.332
Sand	-0.335	0.021	0.005	-0.067
Silt	0.117	-0.093	0.6	0.422
Clay	0.146	0.061	-0.493	-0.296
Eh	0.339	-0.022	0.049	-0.309
Water Content	0.3	-0.327	0.002	-0.089
DO	-0.007	-0.214	0.66	0.462
Salinity	-0.25	-0.2	0.698	0.247
pH	-0.05	-0.135	0.68	0.39

Table 24. Eigen values and variance decomposition for CCA and interset correlation coefficients

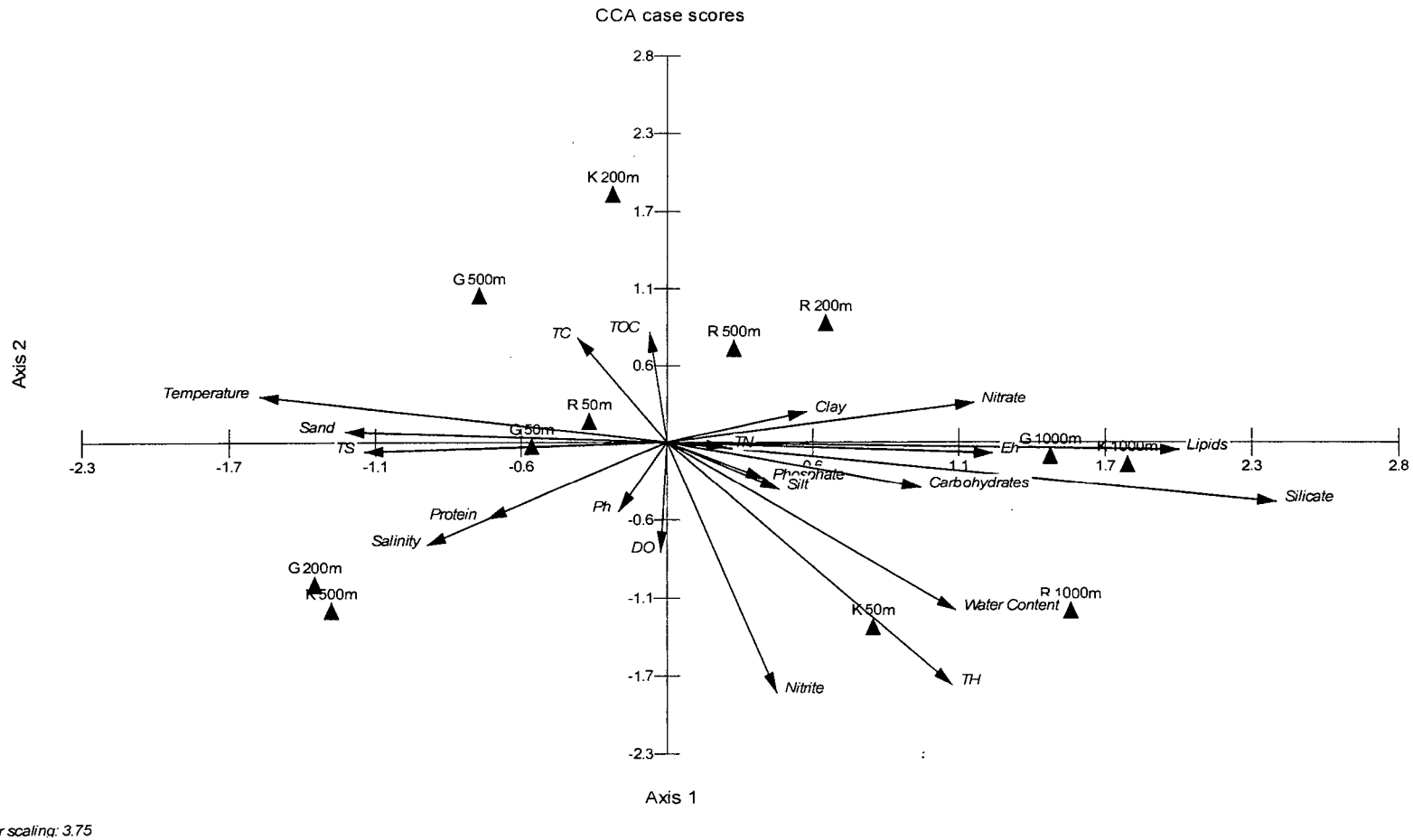
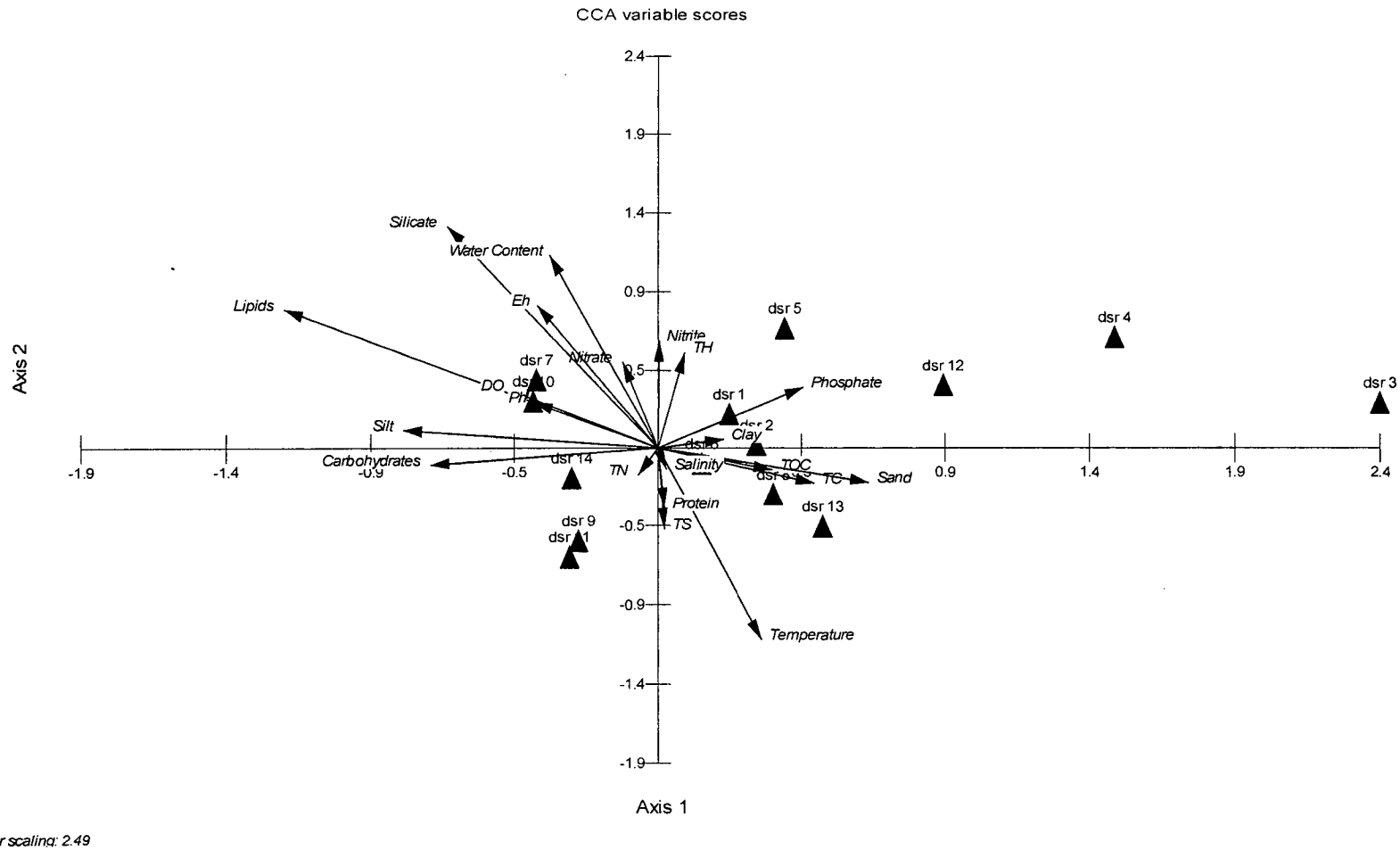


Fig. 76. CCA biplots of intersample distances of DGGE profiles of the whole community from all sites using environmental variables. Triangles indicate the sampling stations .



Vector scaling: 2.49

Fig. 77. Biplot of interspecies distances; CCA of DGGE fingerprints of the bacterial community using environmental variables. Triangles with numbers indicate sequenced bands

CHAPTER 5

DISCUSSION

Deep sea sediments represent one of the largest ecosystems on earth. Knowing the phylogeny, occurrence of different benthic bacterial population and factors governing it is the first step towards understanding the ecosystem functioning. The results are discussed under three sub-headings.

HYDROGRAPHY

The Arabian Sea (AS) is considered as one of the world's most productive systems characterized by an overall nutrient rich environment (Ryther *et al.,* 1996; Qasim, 1977) and therefore the most sought after system by oceanographers. Due to the high productive waters, large amount of organic matter reaches the sea floor (Paropkari *et al.,* 1992, 1993). Sedimentary organic carbon which peaks in the mid-slope region, substantially below the core of the OMZ is the most striking feature of the AS sediment. The geographical and temporal coverage on benthic studies, in general, has been inadequate compared to its pelagic counterpart.

In this study, the hydrographical and geochemical parameters of the three transects covering the coastal non-OMZ and the deep sea OMZ showed regional variability influenced by location, quality and quantity of the abiotic variables. The hierarchical cluster analysis separated the area into two zones at Euclidean distance 8 with non-OMZ clustering at a distance of 7 while OMZ stations clustered at 5.5 euclidean distances. The main common attributes for both the zones were temperature, DO, nitrate and nitrite of the overlying waters. The exclusive variables for the OMZ were temperature, nitrate and silicate whereas for non-OMZ, it was temperature, nitrate and nitrite (Table 6). Despite its origin from land runoff with sufficient nutrient load, low nitrate values observed in subsurface waters is attributed to efficient algal uptake (Naqvi *et al.,* 2000). In addition it has been reported by Krishnan *et al.,* (2008) that increase of nitrate concentration below 20 m depth indicates that nitrification is more pronounced in the deeper depths than in the surface. Thus it was clearly showed that a reducing condition is observed in the OMZ with high nitrate and silicate and low oxygen.

The sediment variables were also equally responsible in differentiating the two zones. Among the sediment variables, the driving forces for OMZ were protein, lipid and clay whereas in the non–OMZ, the quantity of carbon (TC), TOC and its associated variables such as silt were the exclusively decisive factors. Sediment grain size has been ascribed to absorb substrate and protect the habitat (De Flaun and Mayer, 1983). TOC in the top layer of AS sediment was high and such regional high values and variability have been reported by Sirocko and Ittekkot (1992), which was due to the high primary production and particle flux from the water column. TC and TOC were inter dependent variables for both the zones, but their influence was not seen in the OMZ as the co-variables for these parameters were different reflecting the differences in the nature or proportion of the elements. TOC of the non-OMZ region (50 m water depth) was limited by proteins, carbohydrates and lipids whereas in the OMZ lipids and proteins were correlated. Such difference in the nature of organic matter has been reported earlier (Jacob *et al.,* 2008). The contribution of hydrolysable amino acids and carbohydrates to total organic carbon in the surface sediments was found to be low (~10%) along the Oman margin, while organic matter in Peru margin sediments was mostly labile (Alagarsamy, 2003). The OMZ labile organic matter was limited by silicate, reflecting partial decomposed diatom due to difference in transport (particle settling) leading to availability of particulate and dissolved substances. Similar correlation between total organic matter with labile matter and grain size has been reported for the west coast sediments of AS (Jacob *et al.*, 2008). In the ASOMZ, much of the organic matter reaches the sea floor in decomposed state due to slow sedimentation and the O_2 depleted environment close to the bottom constrain further mineralization (Schuffert *et al.*, 1994; Middleburg and Levin, 2009). This was shown by the concentration of lipids highest in the 1000 m depth in all the transects showing slow degradation or recalcitrant nature. O_2 penetration depth in slope sediment is variable, depending on bottom-water concentration as well as on particle flux to the sediments (Soetaert *et al.*, 1996). Further it has been reported that the hydrological conditions of the overlying water were favourable for the accumulation of organic matter in the surface sediments of

the west coast (Jacob *et al.*, 2008). The results of the present study are in good agreement with other similar studies in the upwelling systems of Peru and Namibia which demonstrated that the sediment below the OMZ has distinct characteristics (Liu *et al.*, 2003a, 2003b; Schafer *et al.*, 2007). Thus the biogeochemical properties examined in this study showed differences among the OMZ and non-OMZ with high overall values of nutrients and organic matters.

ABUNDANCE

Bacteria in the deep sea sediments constitute the largest global fraction of total benthic bacteria, i.e. around 76% (ca. 3.8 x 10^{30}) of global bacteria (ca 5 x 10^{30}) (Deming and Yager, 1992; Whitman *et al.*, 1998). About 75% of the integrated bacterial biomass is found in the top 10 cm of the sediments (Lochte, 1992). Despite, their recognized relevance in carbon cycling and nutrient regeneration on global scale, comprehension of the benthic ecosystem functioning is still constrained by the lack of adequate information on metabolism (Turley, 2000), the fraction of active and dormant cells (Deming and Baross, 1993; Luna and Danovaro, 2008) and on benthic bacterial diversity. Bottom sediment, particularly upper few centimeters thick is where heterotrophic bacteria occur in great numbers and are the place in which the processes of mineralization of organic matter take place most intensively (Pfannkuche, 1992). Thus an accurate measure of total microbial biomass is important for understanding the organic carbon turnover in the deep sea. Understanding benthic bacterial abundance and its controls have eluded researchers for decades due in part to methodological and conceptual difficulties (Kemp, 1990). However, inspite of uncommon topography of AS, it must be pointed out that the geographical and temporal coverage of benthic microbiology in general has been inadequate compared to its pelagic counterpart. Until now, only a few attempts have been made to describe the microbial diversity and composition of eastern AS sediment (Jebaraj *et al.*, 2010; Divya *et al.*, 2010).

Total bacteria number (TDC) and viable numbers (TVC) in the studied area were 10^9 and 10^8 g^{-1} DW, respectively. Similar range of 10^8 g^{-1} DW has been reported in other areas (Cammem, 1982; Danovaro *et al.,* 2002; Montagna, 1982; Schmidt *et al.,* 1998). Exceptionally low TDC and TVC were recorded at 200 m depth station especially at Goa and Ratnagiri transects which may be due to the heterogeneity observed in sediments. This heterogeneity may be due to large range of spatial scales of variability in microbial activities between 10 and 100 m in seafloor sediments (Lavigne *et al.,* 1997). Moreover, 200 m stations at Goa and Ratnagiri had higher sand content compared to the other depth stations. In the present study, the percentage of viable bacteria was almost half of the total bacteria. These values were higher than that reported in the deep Mediterranean sediments (Luna *et al.*, 2004). There a lack of adequate information on the relative number of bacterial assemblages (Cole, 1999; Smith and del Giorgio, 2003), especially in sediments. However, it is comparable to the highly productive pelagic environment (Zweifel and Hagstrom, 1995; del Giorgio *et al.,* 1997; Raghukumar, 2005). Interestingly, the OMZ viable counts were higher than that of the coastal sediments, where bacterial assemblages are presumably very active (Luna *et al.*, 2002).

The variation in aerobic CFU ranged from 10^2-10^4 per gram dry wt. Such values have been reported in the OMZ waters of the Arabian Sea (Goltekar *et al.,* 2006). The abundance depended on the media concentrations which may reflect the nutrient status of the sampling location (Brock *et al.,* 1993; 1994). In the OMZ stations the abundance of aerobic bacteria were one order less in full strength and low concentration of NA (100% &10%) compared to 25% NA. Though the load of organic carbon is very high in the OMZ sediments the fraction of bioavailable carbon is very less (Moodley *et al.,* 2011). This may be the reason for the lesser retrievability of bacteria in the higher nutrient concentration. Thus the bacteria thriving in the OMZ sediments require an optimum concentration of nutrients. However, in the non-OMZ, the abundance of bacteria was independent on the media composition. The coastal waters are very dynamic compared to the deeper OMZ region where the circulation of water

is very sluggish. The capability of the non-OMZ bacteria to be retrievable in all the concentrations of nutrients indicates these organisms are organically more resilient than their OMZ counterparts.

Factors responsible for the distribution of bacteria are limited as studies mainly addressed understanding of phylogenetic relationship rather than identifying patterns and constraints. The horizontal distribution of bacteria is to a significant degree conditioned by the nature of the bed and the type of bottom sediment as clay being rich in organic matter usually contains considerably more bacteria than sandy sediments. The detritus or the particulate organic matter (POM) raining from the richer productive waters of the ocean often forms a layer in the sediment-water interphase and is the nutritional basis of life (Turley, 2000). The sediment-water interphase fluxes materials carried through the water column and incorporates it within the sediment. The respiration in the sediment-water interphase is dominated by bacteria (Pfannkuche, 1993) and they play an important role in the decomposition of material on the deep sea bed (Lochte and Turley, 1988; Turley and Lochte, 1990).The high biomass reflects high organic inputs to the deep sea sediments, which is substantially higher for AS than other regions and also the regional variability to the TOC flux contributing to 30-70% of the turnover (Boetius *et al.,* 2000). Correlation analysis of the environmental factors showed that most of the studied parameters constrained abundance showing that the environmental variables dictate the abundance of bacterial assemblages. This constrain was more for the non-OMZ showing that the oxic coastal regions were more governed as previously reported by environmental variables and CCA clearly corroborated this as 62% of the variance was by the first canonical axis. The correlation and CCA confirm that the components mentioned in Tables 10 - 12 represented a potential source of substrates from diatom (nutrients and silicates were significantly correlated). Occurrences of high abundances of diatoms along the coastal waters of the AS have been reported by D'Costa and Anil (2010). These substrates were governed by abiotic factors including the horizontal shift of oxic to hypoxic sediment which stimulated the deep-sea bacteria. Thus the AS has a large standing stock of microbial biomass

compared to other benthic deep sea environment, likely reflecting the enhanced input through particle sedimentation. This can the reason for ATP was the most influencing biotic factor influencing both the regions. Distribution and abundance were influenced by the availability of environmental resources that are fundamental for growth and reproduction (Stepanauskas *et al.,* 2003; Horner-Devine *et al.,* 2004; Yokokawa and Nagata, 2005). In conclusion, the driving variable for the OMZ was aerobic bacteria isolated on low concentration of nutrients while for non-OMZ it was anaerobic bacteria isolated on high concentration of nutrients based on PCA of the bacterial assemblage. This was observed by the low retrievability of aerobic bacteria at very low nutrient concentration. The OMZ sediments forms a bed for the enriched organic matter (Paropkari *et al.,* 1992). Also the oxidation of organic matter in the anaerobic conditions is slower compared to aerobic conditions (Kristensen and Devol, 1995). The diversity of substrates in the deeper region due accumulation provide beneficial conditions for the bacteria to oxidize them using the electron acceptors like nitrate, sulphate and other organic molecules. The supply of organic material is a key factor determining the structure and activity of benthic microbial communities (Meyer–Reil, 1983). Sediments in deep waters are exclusively dependent upon the nutrient supply from the water column and the major transformations of organic material occur at the sediment-water interphase (Bender and Heggie, 1984). However, based on sparse deep sea literature, the downward flux, nature of organic carbon most clearly predicted bacterial abundance (Deming and Baross, 1993).

Comparison of a few of the environmental and bacterial abundance parameters did not show any particular trend. Spatial and temporal variability is frequently observed in marine system. This variability has been shown to be at times from micro scale to global scale (Amour et al., 2009). Multiple factors have been attributed for the variability. Sedimentary bacteria live in a physically and chemically complex environment (Nair et al., 2000) and the measured net concentration depends on number of variables and co-variables. Sediments may be characterized by an episodic supply of organic matter (Tsunogai and Noriki, 1987) or a constant supply of organic matter.

Thus the relationships between the parameters are variable and depend on the prevailing ecological conditions.

Abundance of anaerobic bacteria also showed an increased trend with depth, thus reflecting the prevalence of oxic zone at all the 50 m depth stations. Sulphate reducing bacteria (SRB) constitute a diverse group of prokaryotes that contribute to a variety of essential functions in many anaerobic environments. In addition to their obvious importance to the sulphur cycle, SRB are important regulators of a variety of processes in various ecosystems (Fauque, 1995; Barton and Tomei, 1995). Because of their importance to critical processes in ecosystem functioning, their microbiology and ecological significance in sulphur cycling in marine sediments have been intensively studied over the past 15 years. SRB in the ASOMZ and non-OMZ was of the order of 10^4 g^{-1}DW with higher concentration in OMZ sediment. In the other marine sediments, SRB numbers varied from 10^2 to 10^6 (Jorgensen, 1977, 1978; Laanbroek and Pfenning, 1981; Bak and Pfenning, 1991) whereas in the sediments from Gulf of Gdansk and Kiel Bight it ranged from 10^2 to 10^4 g^{-1}DW (Bussmann and Reichardt, 1991; Mudryk and Skorczewski, 2000). This number is constrained as there are still no optimum methods to determine the abundance of SRB with precision (Gibson, 1987). In this study, lactate and acetate utilizing SRB were enumerated with the former being more abundant. However, in surface marine sediments of Kattegat, Jorgensen and Bak (1991) reported that acetate oxidizing bacteria were dominant. These SRB utilize a large variety of electron donors, which allows them to colonize very diverse marine environments (Ravenschlag *et al.,* 2000; Purdy *et al.,* 2001; Liu *et al.,* 2003b; Mussmann *et al.,* 2005; Wagner *et al.,* 1998; Leloup *et al.,* 2006, 2007). The rich organic matter of the area would have lead to higher lactate production by fermenters which would lead to further degradation to acetate for SRBact. Lipid content of sediment was found to be one of the variables which showed high correlation with TOC. The abundance of SRB in surface sediments of the AS was not exceptionally high in spite of high organic matter. Interestingly, the TDLO was equally abundant reflecting a balance between the oxidative and reductive cycle of

Sulphur both in the OMZ and non-OMZ sediments. New discoveries which show that the pathways of the cycle and metabolic capacities of the bacteria are much more flexible than has been expected continue to appear. The sulphate reducing bacteria are not bound to reducing environments but may live under oxidizing conditions, in which they may respire with nitrate or even with oxygen. SRB may also use sulphite, thiosulphate or elemental sulphur, either as alternative electron acceptors or for disproportionation of sulphur compounds (a type of organic fermentation) in their anaerobic energy metabolism (Bak and Cypionka, 1987; Bak and Pfenning, 1987; Widdel and Pfenning, 1984). During the disproportionation of thiosulphate, the two sulphur atoms are transformed into sulphate and sulphide. Also experiments of Dilling and Cypionka, (1990) with washed cell suspensions have established that some SRB are even able to reverse their sulphur metabolism and oxidize reduced sulphur species such as sulphide or thiosulphate to sulphate in the presence of oxygen. Based on the results obtained in the present study it clearly indicates that further spatial and temporal studies have to be carried out to elucidate which and to what extent the ecological factors control the horizontal pattern of the bacterial assemblages.

DIVERSITY

Microbial community structure analysis gives an understanding of functional and biogeographical relationship and such data are vital for an improved understanding of the benthic ecosystem process of the OMZs. Studies have supported the contention that most benthic bacteria are autochthonous (Ravenschlag *et al.,* 1999), not merely accumulating from the pelagic zone. Benthic communities also contain many ubiquitous, broadly distributed prokaryotic groups since environmental conditions and processes can be generally similar over wide tracts of ocean beds.

Spatial Variation of 16S rRNA diversity

Sediment bacterial communities represent a reservoir of genetic variability similar to soil environments, showing approximately 10^4 species

per gram (Torsvik *et al.,* 2002). Systematic studies on the bacterial diversity in the sediments are very few. Among these studies only two reports are available on the diversity of OMZ sediments i.e. those of Pacific and Atlantic Ocean (Liu *et al.,* 2003a&b; Schafer *et al.,* 2007) based on clone library. These studies have been mainly conducted to decipher new phylotypes. In the present study, three transects covering OMZ and non-OMZ were analyzed to understand the difference in benthic bacterial community and the influence of environmental variables on the diversity. The non-OMZ at 50 m which is located in the continental shelf is well ventilated with dissolved oxygen concentrations of 3-5 ml L^{-1}, while OMZ (200, 500 and 1000 m water depth) are situated in the continental slope where there is a vertical decrease in the dissolved oxygen concentration from 0.5 mlL^{-1} to as low as 0 mlL^{-1}. DGGE analysis indicated that the community appeared to be relatively different in the two zones along the 3 transects. The number of the bands was higher in the OMZ compared to non-OMZ. Oxygen is a strong evolutionary force and the dominating factor determining functional interactions and spatial structure of many microbial communities (Fenchel and Finlay, 2008). DGGE analysis indicated that the community appeared to be relatively different both transect wise and zone wise. This high diversity may have arisen from active deposition and transformation of deposited organic matter (Soltwedel and Vopel, 2001). Though studies have been carried out on the diversity of bacteria in the OMZ water using cultivation-independent method (Riemann *et al.,* 1999, Fuchs *et al.,* 2005; Stevens and Ulloa, 2008; Molina and Farias, 2009) including the AS, it has not been attempted mainly because of difference in methodology. An attempt has been made to compare bacterial diversity of water and sediment in AS based on clone library studies of 1000 m depth station of Goa transect to differentiate the autochthous sediment bacteria in the latter part of the discussion.

Shannon-Weiner diversity index (H') describes the diversity of the dominant bacteria more accurately (Chong *et al.,* 2009a). Diversity index and species richness was comparably high in OMZ. One of the limitations in the interpretation of presence-absence binary data generated from DGGE using

diversity indices relates to the ability to differentiate between sites with same or similar number of bands (Gafan and Sparatt, 2005). However, although successful, the banding pattern represents mainly the major constituents of the analyzed community (Heuer and Smalla, 1997). Species that contribute less than 1% of the total population would not be readily detected by this molecular approach (Muyzer *et al.,* 1993).

The important phylogenetic groups obtained were Proteobacteria, Acidobacteria, Actinobacteria, Chloroflexi, Nitrospira and Bacteriodetes. The majority of the retrieved bacterial sequences were Deltaproteobacteria. Each of these phyla is discussed separately with the available data from marine sediments.

Phylum Proteobacteria

Proteobacteria was the dominant group observed at all stations and transects. Such dominance of Proteobacteria in the Cascadian Margin and mud volcano at San Biagio-Belpasso (Yakimov *et al.,* 2002; Knittel *et al.,* 2003) has been reported. Gupta (2000) in his review on the phylogeny of prokaryotes states that this phylum is of biological significance as it is the most dominant and diverse group of the microbial assemblage.

The Deltaproteobacteria, which includes the anaerobic sulphate reducing bacteria, were the most abundant class, suggesting the prevalence of sulphate reduction in this region. Sulphate reducing bacteria falling in genera *Desulfosarcina* and *Desulfofrigus* have been retrieved from the OMZ waters of Arabian Sea (Riemann *et al.,* 1999; Fuchs *et al.,* 2005). Deltaproteobacteria were also reported as dominant group in Antarctic and Arctic marine sediments (Powell *et al.,* 2003, Bowman *et al.,* 2000, Ravenschlag *et al.,* 1999). 73% of the sequences showed close affiliation to Syntrophobacterales and Desulfovibrionales. Sulphate concentrations varied from 39.8 to 12.7 mM in the pore waters of the eastern AS indicating sulphate reduction (Karisiddaiah *et al.,* 2006). Similar occurrence of high sulphate reducers has been reported in other marine sediments (Gray and Herwig, 1996). The dominance of this group was not restricted only to Mid

Atlantic Ridge (Lopez Garcia *et al.,* 2003) but also in Tokyo and Sagami bays (Urakawa *et al.,* 1999 and 2000). However, a few did not show any resemblance to cultured representatives but were closely related to clones detected at cold seeps, mud volcano and harbour sediments (Fang *et al.,* 2006; Losekann *et al.,* 2007; Zhang and Rock, 2008).

Gammaproteobacteria formed a major group after Deltaproteobacteria in the phylum Proteobacteria in the both the sites with higher occurrence in the OMZ. Occurrence of this has been reported among culturable and non-culturable bacteria of pelagic waters (Lopez Garcia *et al.,* 2001, Fuhrman and Davis, 1997) and sediments (Hugenholtz *et al.,* 1998; Cho and Giovannoni, 2004). There have also been reports of active diazotrophic planktonic Gammaproteobacteria in the Arabian Sea (Bird *et al.,* 2005). Thus the possibilities of the presence of sediment diazotrophic gammaproteobacterial communities cannot be ruled out and future studies should be conducted using functional probes. Also the largest bacteria which have been reported such as *Thioploca* sp. and *Thiomargarita* sp. falling in the *Gammaproteobacteria* in the OMZ sediments off Chile, off Namibia and off Oman (Schulz *et al.,* 1996; Gallardo *et al.,* 1998; Schmaljohann, 2001) have not been observed in OMZ sediments in the present study.

Phylum Actinobacteria

Actinobacteria have been found both in the photic zone and in deep water, and seem to be more frequently recovered in deep water (DeLong *et al.,* 2006). The presence of Actinobacteria in marine ecosystems has been attributed by some to run off from fringing terrestrial habitat (Piza *et al.,* 2004). Other studies have reported a widespread and persistent occurrence of indigenous actinobacterial populations in marine sediments due to their physiological adaptations for growth in the marine environments (Colquhoun *et al.,* 1998; Mincer *et al.,* 2002; Stach *et al.,* 2003). These results suggest that Actinobacteria are adapted to marine environment and may be a dominant group in this ecosystem as they are in soils.

Phylum Chloroflexi

In DGGE analysis this group was restricted to the deeper two OMZ sites. The presence of Chloroflexi related SAR 202 cluster, which forms a monophyletic subgroup in the phylum, has been reported in the oxic (Morris *et al.,* 2004) and the oxygen minimum zone waters (Stevens and Ulloa, 2008) although these were not found in the present study. The members of this phylum exhibit unusual metabolic diversity like anoxygenic photosynthesis, organotrophy and reduction of chlorinated hydrocarbons (Maymo-Gatell *et al.,* 1995). Schafer *et al.,* (2007) in their work on the microbial diversity of the sediment of Benguela upwelling system reported the presence of this phylum in sulphate-depleted depths. Their presence seems to be a common feature of many deep sub-seafloor sediments (Jorgensen and D'Hondt, 2006). Chloroflexi members were also reported in the 16S rRNA clone libraries of (Ocean Drilling Projects) ODP sites of Peru margin (Teske, 2006). Recent studies reported that Chloroflexi-like 16S rRNA sequences were found from such diverse environments as activated sludge, freshwater sediment, open ocean and high temperature thermal spring (Bradford *et al.,* 1996; Wise *et al.,* 1997; Giovannoni *et al.,* 1996; Hugenholtz *et al.,* 1998).

However, resolution of the DGGE profiles, in terms of band numbers, is not always sufficient to illustrate the considerable bacterial diversity in indigenous communities and some studies have shown that fragments of different sequences might migrate at the same position (Valley *et al.,* 1997). The data obtained from DGGE profiling is more accurately considered as the structure of dominant populations rather than a general measure of bacterial diversity (Kowalchuk *et al.,* (2006), due to the fear that only numerically abundant phylotypes will be detected (Nakatsu, 2007). The application of DGGE enables the identification of mostly the dominant populations. DGGE provided general insights into the composition of bacterial communities associated with the sediments of the three transects and also depth variation. Since clone library presents a detailed community composition to reveal the identities of bacterial associates which may likely to be involved in

the metabolic activities, analysis of 1000 m depth sediment off Goa transect has been compared with DGGE and discussed in this context.

Phylogenetic Analysis of 16S rRNA library of OMZ (Off Goa 1000 m)

Sedimentary microorganisms are poorly understood as majority is uncultivated and most are phylogenetically distinct from those in the terrestrial environments (D'Hondt *et al.,* 2002). Culture-independent molecular approaches revealed that sediment microbial communities are predominantly composed of phylotypes lacking closely related cultivable strains (Inagaki *et al.,* 2003; Parkes *et al.,* 2005). The phylogenetic analysis of the clones derived from the OMZ core sediment showed a wide representation of various taxonomic groups, including Proteobacteria, Planctomycetes, Nitrospira, Firmicutes, Bacteriodetes, Acidobacteria, Spirochetes, Chloroflexi and Verrucomicrobia. Proteobacteria formed the major group in the clone library that is in congruence with the results of DGGE analysis. Planctomycetes formed the next major group. Despite their significance in OMZ they were not obtained in the DGGE analysis. Around 8.8% of the sequences in the library were affiliated to the phylum Chloroflexi, forming the third dominant taxonomic group. Interestingly, uncultured candidate divisions formed a major part in the clone library. Two clusters could not be assigned to any phylum was also obtained. It was observed that DGGE analysis revealed the presence of only 6 phylogenetic groups while the clone library gave 14 different taxonomic groups. All the six groups obtained in DGGE were invariably seen in all the sites and transects, but their abundance in these sites varied. However, as in the clone libray Deltaproteobacteria was the major group, suggesting its importance in this region.

Phylum Planctomycetes

Planctomycetes formed the second dominant group with 12.7% representation in the library. Inspite of the recent success in isolating members of Planctomycetes (Schlesner, 1994; Schlesner *et al.,* 2004), the phylum remains one of those underrepresented in microbial culture

collections and represents a minor fraction of the sequences available in the Ribosomal Database Project database (Cole *et al.,* 2005). Though sparsely studied, the members of this phylum are significant because of their abundance in the marine environment (Glockner *et al.,* 2010) and their role in anaerobic ammonium oxidation (anammox) (Alldredge, 2000; Strous *et al.,* 1999; Kuypers *et al.,* 2005, Thamdrup *et al.,* 2006, Galan *et al.,* 2009). The OMZ waters of AS denitrification zones are the deepest and the thickest (Paulimer and Ruiz-Pino, 2009). However, interesting study conducted by Elshahed *et al.,* (2007) on the culturable Planctomycetes of Sulphide/Sulphur-rich in Zoodleton Spring showed that anaerobic sulphur reduction and carbohydrate fermentation are two possible metabolic processes that may enable to grow in anaerobic environments. This monophyletic and deeply branching bacterial phylum has also been reported in permanently anoxic basins such as Black Sea, Golfo Dulce (Kuypers *et al.,* 2003; Dalsgaard *et al.,* 2003), OMZ sediments of Peru (Hamersley *et al.,* 2007). Studies conducted by Woebken *et al.,* (2008) showed that the anammox bacterial sequences of the clones from the waters of the three major OMZs i.e. off Namibia, off Peru and Arabian Sea were all closely related to Candidatus *Scalindua* genus. But sequences showing similarity to this genus was not obtained from the present study.

Other Phylogenetic Groups

Various minor groups were represented in the library that included Firmicutes, Bacteroidetes, Acidobacteria, Nitrospira, Spirochetes and Verrucomicrobia. It is known that marine sediments and water harbour these groups of bacteria and their tight association with particles is thought to be helping them for the degradation of recalcitrant compounds (Ravenschlag *et al.,* 1999, Polymenakou *et al.,* 2005; Martin-Cuadrado *et al.,* 2007; Quaiser *et al.,* 2008). Among them Bacteriodetes is known to be taking part in organic material degradation and have been isolated from wide range of habitats (Chong *et al.,* 2009b). This group was represented by less than 2% in the OMZ sediments whereas in the waters their relative abundance was 2-10% (Kirchman, 2002; Abell and Bowman 2005; Delong *et al.,* 2006). This

group has a characteristic of having only one predominant family in an environment. In the sediment, Flavobacteriaceae family was represented. The clone showed similarity to Flavobacteriales and similar ones have also been reported in the hydrothermal sediments (Lopez-Garcia *et al.,* 2003).

Four sequences in the library belonged to the group Acidobacteria. Though this phylum with a few culturable representatives was well represented in soils, they were also encountered in marine habitats (Delong *et al.* 2006; Quaiser *et al.* 2008). Verrucomicrobia group, which are prosthecate, aerobic heterotrophs (Hedlund *et al.,* 1997) have also been found to be obligate anaerobic heterotrophs (Chin *et al.,* 2001). Hence occurrence of the anaerobic members of this group in the sub-oxic sediments of the present study is not unusual.

Candidate Divisions

Candidate divisions which involve groups that have no cultivated representatives in environment are assigned as OP series (Hugenholtz *et al.,* 1998). In the OMZ sediments, phylogenetic candidate divisions were spread in OP3, OP8 and OP11 and uncultured group I and II which were not assigned to any phylotype formed a major part of the library. The OP11 candidate divisions are widespread and phylogenetically diverse (Harris *et al.,* 2004) and have been detected primarily in the anaerobic ecosystems (Barns *et al.,* 1994; Ludwig *et al.,* 1997; Dojka *et al.,* 2000). Though sediments may seem unlikely to support ubiquitous dispersal of microorganisms compared to water, some bacterial taxa have shown wide spread distribution in marine sediments. This may be due to environmental selection.

From the clone library analysis it is difficult to say how representative they are of these sediments, the sequences obtained are only a fraction of those present in the sediment (Powell *et al.,* 2003). Sediment microbial communities are extremely diverse and the amount of effort necessary to sample most phylotypes is not practically achievable, especially with any statistically valid level of replication (Kemp and Aller, 2004). Despite this

clone libraries provide a means to sample a large number of microbial phylotypes that would otherwise be unknowable, and provide a starting point for developing more targeted analyses (Bisset *et al.,* 2006). The phylogenetic analysis of the clones derived from OMZ sediments of AS showed a wide representation of various taxonomic groups and few were also reported in other OMZs such as the eastern Pacific and the Benguela system (Schafer *et al.,* 2007; Stevens and Ulloa, 2008). The relative abundance of organic matter in the AS sediment may alter the microbial community (Coolen *et al.,* 2002). Eastern AS sediments underlying the OMZ consist of laminated mud, rich in organic matter (Paropkari *et al.,* 1993), which forms an excellent habitat for the microorganisms to flourish. Overall a few phylogenetic distribution of the bacterial assemblage resembled with those found in marine environment using culture independent methods. Interestingly, four groups reported in the OMZ water column of the AS (Fuchs *et al.,* 2005) were also found in the sediment. Similar assemblages may be due to transportation of pelagic population to the seabed via constant detritus rain, followed by the genetic exchange between pelagic and benthic population.

Bacterial composition obtained using both the clone library and PCR-DGGE analysis showed that the Proteobacteria was the dominant phylum in the OMZ sediments. The bacterial communities associated with the mineralization of macro-molecular compounds present in theses regions are more likely to dominate the patterns of diversity. However, former yielded 14 groups while the latter only 6 groups suggesting that clone library analysis gave a better picture of bacterial community structure.

The richness (number of 16S rRNA gene fragment from a sample) and relative abundance (structure or evenness) of the community reflect selective pressure that shape diversity within communities (Dunbar *et al.,* 2000). This information was quantified from PCR-amplified 16S rRNA gene diversity. Comparative analysis of diversity of the clone library of the present study with sediments of Gulf of Mexico, North Sea and South China Sea showed that the OTUs were the highest for the study suggesting the

enormous diversity in the OMZ sediments of the eastern Arabian Sea. This is further supported by the higher values of the diversity indices such as Shannon- Wiener and Simpson's diversity indices which are indicative of OTU richness and common OTU abundance in this region. In addition, the Abundance Based Estimator (ACE) and Chao1 richness estimators were also higher in OMZ sediment library, though South China Sea sediment clone library had the highest ACE of 757 at a distance of $\geq$ 0.03. The diversity analysis showed that though the coverage of the Arabian Sea clone library was only 22%, the diversity indices and estimates were high suggesting the enormous diversity of bacteria in this unique environment. Sediments representing a greater length of time may harbour a greater number and diversity of species may be because species had a greater time to accumulate and/or a greater concentration of nutrients available for bacteria. The OMZ sediments, which represent a greater diversity of species of bacteria may be due to the prolonged accumulation of organic matter which may be less recalcitrant. Paired reciprocal comparisons between the OMZ library and with each of other three libraries of Gulf of Mexico (Orcutt *et al.,* 2010), North Sea (Wegener *et al.,* 2008) and South China Sea (Dai *et al.,* 2002) using LIBSHUFF statistics indicated that these libraries differed significantly with each other. This finding is in agreement because of the presence of two unidentified clades. Since the homologous coverage of this library with respect to evolutionary distance was high at D<0.2, it suggested that the library contained the representatives of the original community. The paired comparisons between the clone libraries showed that all the libraries were significantly different from each other. Thus it can be noted that though sediments belonging to Gulf of Mexico, North Sea and South China Sea are hypoxic, the bacterial communities thriving in these regions may be different from those found in the Arabian Sea OMZ sediments. In the present study, the coverage was comparatively higher than the three areas hence it is reasonable to suggest that the data was representative of the *in situ* bacterial community inspite of the drawbacks of clone library method (Sogin *et al.,* 2006). Moreover higher diversity was found in this sediment compared to the other suboxic sediments. Though the

biogeochemical implications of the phylogenetic diversity observed in marine microbial communities is not yet well understood, bacterial species composition is significant in controlling the rates and patterns of organic matter hydrolysis (Riemann *et al.,* 1999). Assessing the functional significance remains a challenge as sequences of OMZ separated from closely related sequences are not available in GenBank. However, at this juncture this diversity observed cannot be related to geochemical properties. In summary, bacterial community composition of OMZ were diverse with a few unclassified groups. In addition to being diverse and widespread most of the OTUs were found to be sulphate reducing bacteria in the OMZ by employing both the approaches.

Culture-Dependant Taxonomic Diversity

Though 16S rRNA based culture-independent approach is widely used and provide taxonomic classification (Woese *et al.,* 1985; Pace *et al.,* 1986; Amann *et al.,* 1995) they do not elaborate the metabolic activities in biogeochemical cycles and therefore pure cultures are necessary. ARDRA method adopted in this study showed that the OMZ region had higher number of phylotypes compared to the non-OMZ regions. This was true in the case of unique phylotypes suggesting the microheterogenity. Not many studies have been carried out on the diversity of culturable bacteria in the OMZ sediments except those on the diversity of the culturable bacteria in the sediments (Divya *et al.,* 2010). The culturable population retrieved by 16S rRNA sequencing belonged to Firmicutes, Proteobacteria, Actinobacteria and unaffiliated bacteria. Firmicutes was the dominant group retrieved compared to clone library and DGGE analysis where Proteobacteria was the major group. These species isolated from the OMZ sediments are not only invaluable part of microbial diversity, but also good candidates for elucidating their adaptation mechanism for surviving in low-oxygen environments, participation in biogeochemical cycles and for exploring their potential biotechnological applications.

Functional Diversity

Spatial variation of dsrB diversity

From the present study, it was clear that the taxonomical bacterial composition of OMZ were diverse with a few unclassified groups. In addition to being diverse and widespread, it was found that most of the OTUs were sulphate reducing bacteria in the OMZ in both the approaches. Carbon cycling in OMZ sediments is coupled with the reduction of a variety of different electron acceptors, including oxygen, nitrate, sulphate, manganese and iron. To date patterns of microbial functional diversity have not been systematically evaluated in the OMZ sediments. Studies have been focused almost exclusively on bacterial denitrification and on this functional guild (Braker *et al.,* 2000; Scala and Kerkhof, 1999; 2000). Studies on the diversity of sulphate reducing bacteria in the OMZ sediments especially using the functional probes are meager except for the studies of Liu *et al.,* (2003b) in Pacific OMZ sediments. Sulphate reduction is a dominant anaerobic carbon oxidation pathway along the margins especially in the OMZ sediments (Canfield *et al.,* 1993; Devol *et al.,* 1997; Hartnett and Devol, 2003; Jorgensen, 1983; Naqvi *et al.,* 2000), accounting for the oxidation of > 50% of the total organic carbon (Hartnett and Devol, 2003; Thamdrup and Canfield, 1996). As a dominant terminal accepting process, sulphate reducing bacteria should be particularly sensitive to organic carbon dynamics in the OMZ sediments. The present study examined the natural diversity and distributions of sulphate-reducing bacteria along a depth gradient extending down the shelf-slope transition zone of the eastern Arabian Sea. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified DNA fragments is another molecular tool that has been used to determine the presence and distribution of SRB in natural and engineered environments (Santegoeds *et al.,* 1999). However, although successful, the banding pattern represents mainly the major constituents of the analyzed community (Heuer and Smalla, 1997). Species that contribute to less than 1% of the total population would not be readily detected by this molecular approach (Muyzer *et al.,* 1993). The DGGE with eubacterial primers mainly

detects the major constitutents of the analyzed community overlooking the less abundant but potentially very important species (Muyzer *et al.,* 1993). Extensive new genetic diversity was recovered at all depths and sites, and comparative sequence analysis showed the predominance of novel/uncultured sulphate reducers that have not been described to date. The majority of the retrieved bacterial sequences were uncultured sulphate reducing bacteria. Liu *et al.,* (2003b) studied the molecular diversity of sulphate reducing bacteia in carbon gradient off Pacific Ocean and found the presence of mainly the taxa belonging to the Deltaproteobacteria (*Desulfobulbus propionicus*, *Desulfosarcina variabilis*) and Bacillus-Clostridium (*Desulfotomaculum putei*). In the present study, the diversity of the sequences was not very high as most of the sequences had no close relatives and formed a previously unrecognized line of descent seemingly affiliated with the delta subdivision of class Proteobacteria. Such instances have been reported in the Pacific Ocean margin sediments (Liu *et al.,* 2003b). These results suggest that these continental margin sedimentary habitats could harbour novel sulphate reducing bacteria. The continental margins are the major depositional environments for organic carbon on a global scale (Premuzic *et al.,* 1982; Walsh, 1991), but the ultimate fate of this carbon is largely determined by the activities of the native biota. *Desulfobulbus* and *Desulfotomaculum* have also been reported in the continental margin sediments of Pacific Ocean (Liu *et al.,* 2003b). *Desulfobulbus*-like sequences have also been reported to dominate in the fresh water and vegetated marine sediments (Li *et al.,* 1999; Hines *et al.,* 1999). Strains of this group are capable of oxidizing a great variety of different electron donors completely to carbon dioxide (Widdel and Bak, 1992) which would clearly provide a competitive advantage in environments where a broad range of carbon compounds are readily available. Sulphate reduction rates in the margin sediments of the Arabian Sea are modest (20-190 $nmolcm^{-3}d^{-1}$) and the porewater sulphate concentration are not greatly depleted (Schmaljohann *et al.,* 2001; Cowie *et al.,* 1999). In the present study, the species abundance of *dsr*B genes increased from 50 m to 500 m, but decreased rapidly at 1000 m in all transects. This was true although the

deeper sampling sites on each transect had higher carbon concentration. These results reflect the refractory nature of organic matter or an inhibition of its post-oxic decay (Cowie et al., 1999). Such reduction in the sulphate reduction rates have been detected in the continental margin of eastern Pacific (Liu *et al.,* 2003). Several previous studies have shown that as organic carbon descends through the water column labile compounds are preferentially oxidized leaving behind recalcitrant carbon compounds that are less susceptible to enzymatic degradation (Devol and Christensen, 1993, Skoog and Benner, 1997; Wakeham *et al.,* 1997). The sulphate reducing Firmicutes formed the third major group in the OMZ. These bacteria are capable of forming endospores and thus persist under unfavourable (eg. Sulphate limiting) conditions. SRBs are capable of switching to an energy conserving metabolism that requires less and/or no sulphate, such as syntrophic fermentation. Effectively, the syntrophic function was observed in a wide range of phylogenetically diverse SRB, including members of *Syntrophobacter* genera (Wallrabenstein *et al.,* 1994; Schink, 1997), *Desulfovibrio* sp. (Guyot *et al.,* 1986) and *Desulfotomaculum* sp. (Pluddge *et al.,* 2002). In the Black Sea sediment, the sulphate and methane zones showed a similar predominance of novel sequence types which cluster with gram positive SRBs. Although physiological features cannot be directly concluded form phylogenetic relationship, the present comparative study of the *dsr*B gene sequences suggest some interesting features of SRB communities. Based on the present study it is tempting to conclude that sulphate availability plays a significant role for the phylogenetic composition of the SRB community.

The detection limit of the DSRp2060F/DSR4R DGGE-primer set without GC-clamp was 33.1 $fgul^{-1}$, but the attachment of a 40-bp GC-clamp for DGGE analysis resulted in a 100-fold decrease of this detection limit. Vanbroekhoven *et al.,* (2004) found that the attached GC-clamp plays a crucial role in processes controlling PCR-sensitivities of *Acinetobacter* specific PCR systems. DGGE analysis of amplified *dsr*B gene fragments could differentiate between SRB at the genus and species level and shows that the *dsr*B based DGGE will likely be effective for diversity analysis of

SRB populations (Geets *et al.,* 2006). Although the *dsr*-based phylogenetic tree is largely consistent with conventional 16S rDNA-based phylogenetic tree topologies, seven *Desulfotomaculum* sp. and two *Thermodesulfobacterium* sp. have been reported to possess non-orthologous *dsr*-genes (Klien *et al.,* 2001; Zverlove *et al.,* 2005).

Phylogenetic Analysis of *dsr*B library of OMZ (Off Goa 1000 m)

Sulphate Reducing Bacteria is of importance to the critical processes in ecosystem functioning and environmental remediation, thus increasing interest in SRB has been shown over the last decade. Different culture-independent methods have been used to study SRB populations in various ecosystems, resulting in an increased knowledge of their diversity. An approach involving 16S rDNA does not appear suitable to investigate communities of sulphate reducing bacteria, as sulphate reduction is widespread among phylogenetically unrelated groups. The enzyme *dsr* only expresses in sulphate reducing prokaryotes, catalyses the final step of sulphate reduction, and reduction of sulphite to sulphide. Sulphate reduction has shown to be an important process in the deeply buried sediments of the Peru continental margin due to the interstitial water depth profiles of sulphate (D'Hondt *et al.,* 2003). The populations of sulphate reducing bacteria are known to be a major component of bacterial communities in the marine sediments. They are found in various anoxic environments such as Northern Everglades wetland, estuarine and coastal marine sediments, Antarctic marine sediments, saline lake sediments and rice root (Castro *et al.,* 2002; Bowman and McCuaig, 2003; Purdy *et al.,* 2002; Kozumi *et al.,* 2004; Ikenaga *et al.,* 2003). The so-called functional genes are more directly correlated with the physiology and habitat range of an organism. Comparative analysis of the *dsr* genes recovered from the environment offered a foundation for identifying novel sulphate reducing bacterium within a phylogenetic framework. The diversity of sulphate respiring organisms is as yet poorly delineated, because 16S rRNA sequences alone cannot be used to infer physiology of novel lineages. Sulphate-reducing bacteria play a key role in the sulphur cycle and organic matter degradation in the marine

and estuarine ecosystem (Kohler *et al.,* 1984; Fukui and Takii, 1990). Marine bottom sediments provide an optimum environment for these microorganisms (Mudryk *et al.,* 2000). It has been reported that it is the organic matter availability and not the sulphate concentration that principally limits SRB numbes and sulphate reduction rates in marine bottom sediments (Jorgensen, 1982; Ward and Winefry, 1985; Fukui and Takii, 1990). Sulphate is the second most abundant anion in sea water and sulphate reduction thus accounts for the majority of anaerobic mineralization in coastal sediments (Jorgensen 1977, 1982, 2006; Soetaert *et al.,* 1996).

Culture-Dependant Functional Diversity

Bacteria produce hydrolytic enzymes prior to POM decomposition so that POM is cleaved into smaller molecules, which can support bacterial metabolism (Lochte *et al.,* 2000). Bacteria can respond rapidly to the arrival of material, by producing enzymes that break it down to smaller fraction which they can incorporate to fuel their metabolism. This process dominates the biogeochemistry of the sediment-water interphase and the rate and nature of what gets laid down in the sediments (Turley, 2000). Studies have revealed that the oxic and anoxic bacteria are able to degrade organic matter at similar rates (Kristensen *et al.,* 1995; Hall *et al.*, 1998). However, aerobic heterotrophic bacteria are capable of producing certain hydrolytic end products of their metabolism that are effective in initiating degradative pathways of old, refractory and structurally complex organic matter (Mayer *et al.,*1994; Hedges and Keil, 1995; Kristensen *et al.,* 1995; Hall *et al.,* 1998) compared to anaerobic bacteria and thus play an important role in nutrient cycling. The metabolic versatility of deep-sea bacteria may, therefore, enable the breakdown of compounds that are unavailable to other organisms (Deming and Baross, 1993). The seasonal flux of POC in the Arabian Sea and its microbial consumption are thought to be responsible for the presence of intense permanent oxygen minimum zone (OMZ) which occurs in the water depths ranging from 150-1500 m (Wyrtki, 1971; Schnetger *et al.,* 2000). The OMZ isolates had higher activity of Urease, DNase and Lipase compared to the non-OMZ isolates. Dell'Anno and Corinaldesi (2004)

demonstrated that the extracellular DNA concentration as well as the degradation of this DNA in sediments was higher than that in water. Also they have suggested that the preservation of DNA in deeper sediment layers may be favoured in the benthic systems with higher sedimentation rates. Phosphatase activity was the highest in the deepest site suggesting the presence of the substrate in the region. Studies on the production of phosphate in the pore waters of suboxic continental margins show that increase in phosphate concentration is due to various factors such as microbial degradation of organic matter (Froelich *et al.,* 1988; Ruttenburg and Berner 1993), desorption from iron oxides (Sundby *et al.,* 1992; Slomp *et al.,* 1998) and dissolution of fish debris (Suess, 1981). Benthic phosphorous regeneration and consequent dissolution of phosphate in the sediment water interphase is more extensive under oxygen-depleted conditions (Ingall and Jahnke, 1994). These enzyme activities signify the role of culturable bacteria in the degradation and minerialization of organic matter present in these sediments and the production and activity depend primarily on the availability, distribution, type and concentration of the organic substrates (Boetius, 1995). This was observed in the case of amylase, gelatinase and esterase activities and the low activities could be attributed to the recalcitrant nature of organic matter. Studies have shown that organic matter in the Peru margin sediments were labile while in the Oman margin sediments, most of the organic material reaching the sea bottom was already degraded (Emeis *et al.,* 1991; Smallwood and Wolf, 2000). This may be due to the long residing time of sinking particles in the water column (Rao and Lamboy, 1995). Furthermore, Alagarsamy (2003) reported that the contribution of hydrolysable carbohydrates and aminoacids to the total organic carbon in Oman margin sediments is only 10%. This supports the hypothesis that the organic matter in these sediments is recalcitrant in nature (Suthof *et al.,* 2000). Among the various groups, Firmicutes was the major enzyme producing group expressing all the enzymes tested and was found to be metabolically versatile The most frequently obtained cultured strains were members of the spore-forming genus *Bacillus* within the Firmicutes, isolated from the different ODP sites and Peru (D'Hondt *et al.,* 2002).

Actinobacteria were found in Pacific Ocean and Peru site (D'Hondt *et al.,* 2002).

The significance of metabolically active aerobic culturable bacteria in cycling of organic matter has been reiterated in this study. The OMZ sediments of the AS harbour diverse bacteria which have enormous catabolic efficiencies. The supply of organic material to the OMZ sediment is a key factor in stimulating the production of extracellular hydrolytic enzymes. These hydrolytic enzymes are responsible for organic matter mineralization and play important role *in situ* biogeochemical processes.

Bacterial community structure is generally regulated by the ability of bacteria to cope with various environmental conditions. Canonical Correspondance Analysis (CCA) is proven to be sensitive in detecting the relationship between bacterial community composition and environmental parameters (Iwamoto *et al.,* 2000; Mouser *et al.,* 2005 and Jiang *et al.,* 2007) was employed by embedding the environmental data within the biotic analysis. Kowalchuk *et al.,* (2006) suggested that the data obtained from DGGE profiling is more accurately considered as the structure of the dominant populations rather than a general measure of bacterial diversity, due to the fact that only numerically abundant phylotypes will be detected (Nakatsu, 2007). One of the limitations of interpretation of the binary data generated from DGGE using diversity indices relies to the inability to differentiate between sites with same or similar number of bands (Gafan *et al.,* 2005). The CCA of the DGGE profiles showed that the non-OMZ regions such as 50 m stations of Goa, Karwar and Ratnagiri were highly influenced by DO, proteins, nitrate, salinity, silt and TS. Interestingly G-200 also was influenced by these factors. This suggests that these parameters do play an important role in the composition and variation in these parameters would bring about a change in the bacterial community structure in these sediments. All the remaining sites clustered together and the important factors influencing the diversity were TOC, TC, phosphate and nitrate. The continental margins are major depositional environments for organic carbon on a global scale (Premuzic *et al.,* 1982; Walsh, 1991). Oxygen is a key factor in controlling the community composition in the present study and also has been reported in other sediments especially from the OMZ regions (Liu

et al., 2003). Thus environment had a role in the distribution of bacterial diversity in the OMZ and non-OMZ sediments of the Arabian Sea. The analysis based on the interspecies distance revealed the influence of different factors on specific phylotypes. Three groups could be demarcated from the CCA plots. The group 1 phylotypes was influenced lipids, TS, DO and TN. It has also been reported that bacterial community was found to change with the dominant source of organic matter (Bisset *et al.,* 2006). Most of the Deltaproteobacteria belonged to this group in addition to Gammaproteobacteria and Actinobacteria. Deltaproteobacteria are mainly reported to be present in the benthic environments than other region (Bowman and McCuaig, 2003). The group II included the other groups. The important environmental factors that influenced these phylotypes were temperature, salinity, TH and sand. A third group included two phylotypes that were not influenced by any of the environmental factors. Latitudinal gradients in the bacterial community composition may be related to the difference in dissolved oxygen of the overlying water and type of organic matter or even the texture of the sediment and thus a combination of DO, LOM and grain size showed the highest explanatory value for the bacterial community structure across the study locations. On the whole the results of this study supports the idea that bacterial community structure and function is determined by the environment.

CHAPTER 6

SUMMARY AND CONCLUSION

Oxygen minimum zones (OMZ) are large volumes of oxygen-deprived waters with dissolved oxygen concentration as low as 0.5 mlL^{-1} at intermediate depths (10 -1300 m on an average) in the eastern tropical oceans (Stramma *et al.,* 2008). This phenomenon is widespread in the world oceans and occurs as permanent, seasonal or episodic feature (Kamykowski and Zentara, 1990). Of the total OMZ area, 59% occurs in Indian Ocean (Arabian Sea and Bay of Bengal), 31% in the Eastern Pacific Ocean and 10% in the Southeastern Atlantic Ocean (Helly and Levin, 2004). The open ocean deep water OMZ in the Arabian Sea occurs permanently between 150-1500 m (Wyrtki, 1971; von Stackelberg, 1972). The zone is >750 m thick and extends to 25,00,000 km^2 (Paulmier and Ruiz-Pino, 2009). The rain of detritus from the euphotic layers gets accumulated on the western continental margin of India, resulting in the deposition and consequent enrichment of organic carbon (>4%) in the sediments (Paropkari *et al.*, 1992, 1993). This region where OMZ impinges on the sea floor creates a strong gradient in dissolved oxygen concentrations and serves as a specialized habitat for the organisms.

The benthic bacterial population thriving in OMZ sediment would make major contributions to the biogeochemical cycles in the Arabian Sea where significant amount of carbon is preserved. Study of bacterial diversity in marine environment is important for understanding their distribution, community structure and thereby the functioning of ecosystem. The ability to measure bacterial diversity is a prerequisite for the systematic study of bacterial biogeography and community assembly. However, microbiological studies in the OMZ region have been sparse. Therefore, a systematic study was carried out in the Arabian Sea OMZ with the following objectives:

1. To assess the diversity of culturable and unculturable benthic bacteria in different areas of OMZ of AS using cultivation-dependant and cultivation-independent techniques.
2. To characterize the spatial abundance and diversity of the components of bacterial community to environmental parameters.

3. To elucidate the metabolic/functional diversity of the bacterial community.

Approach

In order to meet the above objectives a spatial gradient along 3 transects (Karwar, Goa, Ratnagiri) in the AS were selected, in the continental margin, viz. 50, 200, 500 and 1000 m. The site at 50 m is located in the continental shelf and is oxic while the sites at 200, 500 and 1000 m are situated in the continental slope which falls in the OMZ and therefore suboxic. Water and sediment samples were collected from the above stations during the cruise no. 254 onboard FORV *Sagar Sampada* (May 2007).

The physico-chemical parameters such as temperature, salinity, pH, dissolved oxygen and nutrients (nitrate, nitrite, phosphate and silicate) of overlying water column from all stations in the three transects were estimated using standard techniques. The geochemical parameters of sediment samples such as Eh, total organic carbon and labile organic carbon were also estimated using standard protocols.

Bacterial abundance (both direct total and viable) was estimated using DAPI method (Porter and Feig, 1980; Kogure *et al.,* 1979). Retrievable counts of bacteria were estimated by culturing on varying concentrations of nutrients (100%, 25%, 10% and 0%) in Nutrient Agar medium. A polyphasic approach was adopted to study the bacterial diversity of the sediments. Cultivation-independent approach such as Denaturing Gradient Gel Electrophoresis (DGGE) and 16S rRNA clone library analysis were used to study the spatial diversity and phylogenetic prediction of bacteria. Cultivation-dependant approach comprised the biochemical characterization and ARDRA analysis of the bacterial isolates. The functional diversity of the culturable fraction was assessed by the ability of the bacterial isolates to utilize various substrates. DGGE and clone library analysis of the *dsr*B gene was carried out in order to study the cultivation-independent functional diversity.

In order to understand the interrelationships among the environmental variables, and their relation to abundance and diversity, the data sets were also subjected to statistical analyses (Spearman's rank correlation, Principal Component analyses and Canonical Correspondence Analysis using the software Primer 6 and MVSP 3.13.).

Results

The highlights of the results are listed below:

- Based on the hydrographical variables and geochemistry of the eastern continental margin of the AS, it can be separated into the oxic and hypoxic regions. At 50 m depth stations the dissolved oxygen concentration was >3 mlL^{-1} whereas in the deeper stations it was <0.5 mlL^{-1}. The outer shelf and slope are affected by the flow of west India undercurrent that is slightly better oxygenated than the water it mixes with, thus preventing bottom waters from turning anoxic at least to the south of Goa. These regions are exposed to long-term natural upwelling and high production. The important common attributes for the two zones were temperature, dissolved oxygen and nutrients of the overlying waters. Oxygen depletion is exacerbated in AS as it is a land –enclosed water body where there is a long residence time and little exchange with sources of oxygenated waters. The nitrate concentrations at shallow stations were very low (0.2±0.04 to 1.36±0.01 µM). The concentrations at the deeper station were high (0.21±0.04 to 44±0.5 µM). This trend was seen with all the studied nutrients of the overlying water and geochemistry of the sediments. Transect-wise, Goa recorded high concentration of nutrients. Total organic carbon and phosphate were the footprints of the oxic-50 m depth stations which forms the non-OMZ region. In the deeper, hypoxic regions the factors responsible for the variation of the OMZ was silicate and nature of the organic matter. The hydrographical characteristics of the overlying water and geochemistry were the drivers influencing the quantity and nature of the organic matter of the OMZ sediments.

- Bacterial abundance in the AS sediment was 10^{8-9} g^{-1}DW. It was higher in the OMZ sediments compared to that of non-OMZ. High bacterial abundance in the deeper stations suggests that OMZ region forms an environment, conducive for the survival of bacteria due to high carbon content, ample supply of electron acceptors and higher proportion of clay. The aerobic and anaerobic viability was an order lesser in these regions. The retrievability, however ranged from 10^{3-4} g^{-1}DW. The bacterial abundance of different assemblages in these two zones showed variation, but the extent of variation was different.

- Sulphate reducing bacterial (SRB) numbers were 10^{3-4} g^{-1}DW in the sediment. These bacteria are involved in the mineralization of organic matter, particularly in organic-rich marine sediments. A significant proportion of organic molecules (fatty acid) and polymer hydrolysis are ultimately oxidized through sulphate respiration and correlation analysis showed significant relationship with the lipid and protein contents of the sediment.

- High abundance of Thiobacillus denitrificans like organism (TDLO) (10^3 g^{-1}DW) may detoxify sediments and this may facilitate habitation of benthic communities. The density of SRB and TDLO was almost equal. This balance may be a factor for the constancy observed in sulphate concentration in these sediments.

- DGGE analysis of the 16S rRNA gene showed that the number of phylotypes was higher in the OMZ (54±2.3) compared to non-OMZ (49±2). The groups obtained belonged to Proteobacteria, Actinobacteria, Acidobacteria, Nitrospira and Bacteriodetes. In the Phylum Proteobacteria, Deltaproteobacteria was the most dominant class followed by Gammaproteobacteria. Proteobacteria was the major phylum observed at all sampled depths (Range=42-61%) followed by the Actinobacteria (Range=17-30%). The abundance of Deltaproteobacteria which mainly includes the sulphate reducing bacteria suggests that sulphate reduction may be an important biogeochemical process taking place in the OMZ sediments. In the

OMZ, the density of the phyla such as Proteobacteria, Nitrospira and Chloroflexi were higher than the non-OMZ and were 44%, 9% and 4%, respectively. The study showed that there was difference in the benthic bacterial diversity between the OMZ and non-OMZ.

- The Shannon-Weiner diversity index based on DGGE bands of all the transects, was also high in the OMZ, ranging from 3.5-3.8 compared to non-OMZ with the highest (3.8) observed at 500 m depth station off Goa which forms the core of the OMZ.
- Fourteen taxonomic groups were obtained from the 16S rRNA clone library analysis and the major phylum encountered was the Proteobacteria (52%) followed by Planctomycetes (12.7%) and Chloroflexi (8.8%). Among the Proteobacteria, Deltaproteobacteria formed the major class (62.5%).The other phyla included Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Firmicutes, Bacteriodetes, Verrucomicrobia, Acidobacteria, Spirochetes and Nitrospira.
- An interesting observation was the presence of three uncultured candidate divisions OP3, OP8 and OP11, accounting for 3.9%, 2.9% and 0.9% of the sequences, respectively. Fifteen clones were not assigned to any phylum and were placed in two separate clusters.
- The high values of diversity indices of 16S rRNA clone library reflect the enormous diversity in the OMZ sediments compared to other suboxic sediments and ASOMZ water. Primary difference among dominant genera was their ability to function in the presence and gradients of different electron acceptors.
- Culturable fraction of the bacteria obtained included Firmicutes, Proteobacteria, Actinobacteria and unaffiliated bacteria. Firmicutes was the predominant group in the OMZ and non-OMZ regions with more than 50% of the culturable isolates. The presence of such low number of groups may be due to limitation of the method for isolation

and characterizations and/or the possibility of limited labile substrates that reach the bottom of the sea from the overlying euphotic zone.

- High number of ARDRA phylotypes (8-22) was obtained in the sediments, reflecting heterogeneity in diversity even among the retrieved bacterial isolates. Total number of unique phylotypes (12) was higher in the OMZ sediments.
- Functional diversity based DGGE analysis of *dsr*B gene showed that the majority of the sequences belonged to the uncultured sulphate reducing bacteria (43%), followed by Proteobacteria (36%) and Firmicutes (21%). Uncultured SRB (48%) and Firmicutes (30%) dominated the OMZ region. Further estimation of the bacterial clone library of dsrB gene showed that the major group was the uncultured sulphate reducing bacteria followed by Proteobacteria and Firmicutes and was in congruence with the DGGE results.
- In the sediment, 29% percentage of the 16S rRNA clone libraries were associated with sulphate reducing Deltaproteobacteria, supporting the importance of this group in terminal remineralization processes within carbon rich seafloor habitats.
- Culture-dependant enzyme profile showed that OMZ isolates had higher activity of urease, DNase and lipase. Bacterial diversity was higher in the deeper depths ie., OMZ region, suggesting that the environmental conditions prevailing in this region was favourable for the survival of bacteria. There are different metabolic capabilities related to metabolic adjustment due to biotic variables. Hydrolytic and fermentative bacteria are key players in the organic carbon breakdown and are expected to respond quickly to increased carbon loading. Among the heterotrophic bacteria species, Firmicutes (75%) produced protease used in the breakdown of exogenous insoluble proteins.
- Bacterial responses to oxygen gradient are intimately tied to geochemistry, in both sediments and water column. Canonical

Correspondence Analysis (CCA) was employed for extracting latent environmental gradients from ecological datasets as it defines the link between species and environment by embedding the environmental data within the biotic analysis. The CCA of the DGGE profiles showed that the non-OMZ regions such as 50 m stations off Goa, Karwar and Ratnagiri were highly influenced by DO, protein, nitrate, salinity, silt and total sulphur. Rest of the stations clustered together and the important factors influencing the diversity were total carbon, total organic carbon, phosphate and nitrate. Apart from the sole factors acting to shape communities and ecosystems, other factors also have individual and synergistic effects. Thus, in this study environmental variables dictated the distribution of bacterial diversity in the OMZ and non-OMZ sediments of the AS. The concept ***"Everything is everywhere, the environment selects"*** is underscored in this work. The primary goal of characterizing the community and environmental factors driving sediment bacterial structure was achieved in this study. It was also seen that bacteria play an important role in nutrient cycling in both regions. Although the reasons for bacterial variation is not fully understood, the abundance and phylogenetic analysis described in this study would supplement the current understanding of biogeographic distribution of benthic bacteria in the OMZ and would also provide important basic data for future studies on bacterial function.

Conclusions

The following conclusions could be drawn from the study:

- The study, first of its kind, elucidated the phylogenetic and functional diversity of bacterial community and also the driving forces in the OMZ of the Arabian Sea.
- In depth study of bacterial diversity in the core of the OMZ involving both culture-dependant and independent methods showed that the OMZ bacterial community is phylogenetically distinct and metabolically active. It also implied the existence of active metabolic processes, especially the sulphur cycle that have remained unstudied in OMZs and deserve greater attention.
- The diversity of SRB is poorly delineated and paucity of available sequences related to SRB renders its phylogenetic placement tenuous. The data generated in this study will increase the available sequence for future comparative studies of the sediments of OMZ.
- Polyphasic approach showed that by following the cultivation-independent method, a better picture of bacterial taxonomic and functional diversities could be drawn as compared to the cultivation-dependant methods in the Arabian Sea OMZ sediments. Application of ARDRA was very effective in getting a better picture of taxonomic information and resolution within culturable taxa.

Scope for Future Work

Several phylotype groups have been found to be broadly distributed in marine sediments, both in shallow and deep waters. The paucity of data means that many groups have been detected only in a few sediment samples and more samples need to be analyzed in greater detail to establish the true ubiquity and distribution of specific prokaryotic groups in the OMZ.

As this study was restricted to small area of the OMZ of AS, it is at present hard to affirmatively conclude the strong coupling between environment and bacterial diversity. The spatial and temporal coverage of benthic biogeochemical studies and the functional role of bacteria in the Arabian Sea are severely inadequate and should be the focus for future investigations. Application of new emerging molecular techniques like FISH, MS and isotopic substrates would indeed help in quantifying the bacterial groups and their target specific functions.

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Appendix

APPENDIX

Composition of media, reagents and buffers

A1- Composition of Media

- **Nutrient Agar**

Peptone	- 10.0g
Beef extract	- 10 g
Sodium chloride	- 5 g
Agar	- 12.0 g
Sea water	-1000 ml
pH	-7.2

- **Hugh And Leifson's Agar**

Sodium chloride	- 5.0 g
Glucose	- 10.0 g
Dipotassium hydrogen phosphate	- 0.30 g
Bromothymol blue	- 0.05 g
Sea water	- 1000 ml

- **Methyl Red – Voges Proskauer Medium**

Buffered peptone	-7.0 g
Dextrose	- 5.0 g
Dipotassium phosphate	- 5.0 g
Sea water	-1000 ml
pH	- 6.9 +/- 0.2

- **Mannitol Motility Agar**

Peptone	- 20g
Mannitol	- 2.0 g
Potassium nitrate	-1.0 g
Phenol red	- 0.04 g
Agar	- 3.0 g
pH	-7.6 +/-0.2

- **Simmons Citrate Agar**

Magnesium sulphate	- 0.2 g
Ammonium dihydrogen phosphate	- 1.0 g
Dipotassium phosphate	- 1.0 g
Sodium citrate	- 2.0 g
Sodium chloride	- 5.0 g
Bromothymol blue	- 0.08 g
Sea water	- 1000 ml
Agar	- 15.0 g

- **Triple sugar Iron Agar**

Peptone	- 10.0 g
Casein enzyme hydrolysate	- 10.0 g
Yeast extract	- 3.0 g
Beef extract	- 3.0 g
Lactose	- 10.0 g
Sucrose	- 10.0 g
Dextrose	- 1.0 g
Sodium chloride	- 5.0 g
Ferrous sulphate	- 0.20 g
Sodium thiosulphate	- 0.30 g
Phenol red	- 0.024 g
Agar	- 12.0 g
Sea water	- 1000 ml
pH	-7.4 + /- 0.2

- **Nitrate Broth**

Peptone	-10 g
Potassium nitrate	- 3 g
Sea water	-1000 ml
pH	-7.2

- **Peptone Broth**

Peptone	- 10.0 g
Potassium nitrate	- 3.0 g
Sea water	- 1000 ml
pH	- 7.0

- **Moller Decarboxylase Broth W/Arginine**

Peptone	- 5.0 g
Beef extract	- 5.0 g
Dextrose	- 0.50 g
Bromocresol purple	- 0.01 g
Cresol red	- 0.005 g
Pyridoxal	- 0.005 g
L-Arginine hydrochloride	- 10 g
pH at 25°C	- 6.0+/-0.2

- **Moller Decarboxylase Broth W/ Ornithine Hydrochloride**

Peptone	- 5.0 g
Beef extract	- 5 .0 g
Dextrose	- 0.50 g
Bromocresol purple	- 0.01 g
Cresol red	- 0.005 g
Pyridoxal	- 0.005 g
L-Ornithine hydrochloride	- 10.0 g
Seawater	- 1000 ml
pH	- 6.0 +/- 0.2

- **Moller Decarboxylase Broth W/ Lysine Hydrochloride**

Peptone	- 5.0 g
Beef extract	- 5.0 g
Dextrose	- 0.50 g
Bromocresol purple	- 0.01 g
Cresol red	- 0.005 g
Pyridoxal	- 0.005 g
L-Lysine hydrochloride	- 10.0 g
Sea water	-1000 ml
pH	- 6.0 +/- 0.2

- **Starch Agar**

Peptone	- 5.0 g
Beef extract	- 10.0 g
Sodium chloride	- 5.0 g
Soluble starch	- 2.0 g
Agar	- 12 .0 g
Sea water	- 1000 ml
pH	- 7.2 – 7.4

- **Lipid Hydrolysis Medium**

Peptone	- 10 g
Beef extract	- 10 g
Sodium chloride	- 5.0 g
Calcium chloride	- 0.1g
Tween	- 1%
Agar	- 12.0 g
Sea water	- 1000 ml

- **Gelatin Medium**

Peptone	- 10.0 g
Beef extract	- 10.0 g
Sodium chloride	- 5.0 g
Gelatinase	- 4.0 g
Sea water	- 1000 ml
pH	- 7.2 -7.4

- **Phenolphthalein Phosphate Agar**

Peptone	- 5.0 g
Beef extract	- 3.0 g
Sodium chloride	- 5.0 g
Sodium phenolphthalein phosphate	- 0.012 g
Agar	- 15.0 g

- **DNase Agar W/ Toludine Blue**

Tryptose	- 20g
Deoxyribonucleic acid	- 2.0 g
Sodium chloride	- 5.0 g
Toludine blue	- 0.10 g
Agar	- 15.0 g
pH at 25°C	-7.3 +/- 0.2

- **Luria Bertani Agar**

Sodium Chloride	– 10 g
Tryptone	– 10 g
Yeast Extract	– 5 g
Agar	– 20 g
Distilled water	– 1000 ml
pH	– 7

- **Sulphate Reducing Bacteria (SRB) Medium**

Yeast extract	- 1 g
Ammonium chloride	- 2 g
Potassium hydrogen phosphate	- 0.2 g
Sodium acetate	- 1 g
Sodium lactate	- 8 ml
Trace element solution	- 1 ml
Agar	- 8 g
Sea water	- 1000 ml
pH	7.8-8

Autoclave separately:

Ferrous sulphate	- 1 g/10 ml
Sodium thioglycolate	- 0.6 g/10 ml
Sodium sulphide	- 5 g/100 ml

To above media add:

Ferrous sulphate	- 5ml
Sodium thioglycolate	- 5 ml
Sodium sulphide	- 2.5 ml

- **Thiobacillus Denitrificans Like Organisms (TDLO) Medium**

Sodium thiosulphate	– 5 g
Potassium nitrate	– 1g
Dipotassium hydrogen phosphate	– 0.2 g
Magnesium chloride	– 0.1 g
Calcium chloride	– 0.01 g
Ferric chloride	– 0.01 g
Phenol red	– 0.01g
Agar	– 10 g
Distilled water	– 1000 ml

Autoclave Sodium bicarbonate (1 g/10 ml) separately. Add 10 ml/L to the above medium.

Readymade culture media (Hi-Media ,Mumbai) were also used.

A2- Composition of reagents

Biochemical

- **Carbohydrates**

5% TCA solution
Trichloroacetic acid5g
DW100ml

5% phenol solution
Phenol crystals5g
DW100ml
H_2SO_4 (95.5%, specific gravity 1.82)

- **Proteins**

Reagent A (2% Na_2CO_3 in 0.1 N NaOH)
Na_2CO_3 2g
0.1N NaOH 100ml

Reagent B (0.5% $CuSO_4$ in 1% sodium potassium tartarate solution)
$CuSO_4$ 0.5g
DW 50ml
Na-K tartarate 1g
DW 50ml
Mix a and b together

Reagent C
Reagent A 50ml
Reagent B (Prepare fresh) 1ml

Folin's reagent (1:1 dilution)
Folin ciocalteau phenol reagent 5 ml
DW 5ml

1N NaOH
NaOH 4g
DW 100ml

Distilled water

- **Lipids**

0.5% dichromate in conc. H_2SO_4
$K_2Cr_2O_4$ 0.75g
DW 10ml
H_2SO_4 (95.5%, specific gravity 1.82) 500ml

Organic solvent

$CHCl_3$	200ml
CH_3OH	400 ml
DW	160ml

Analytical grade chloroform $CHCl_3$

- **Biomass- ATP concentration**

Tris buffer

Tris (hydroxymethyl) amino methane	0.75g
DW	200 ml

pH7.7-7.8
pH was adjusted with 20% HCl

Firefly extract preparation

Luciferin-luciferase enzyme (Sigma, FLE50)	50mg
Autoclaved DW	5ml

Age the enzyme at 4(±2)°C for 3h

Bacteriological

- **Gram staining**

Crystal violet

Crystal violet	- 10.0 mg
Absolute alcohol	- 100.0 ml
Distilled water	- 900.0 ml

Grams iodine

Iodine	- 6.0 g
Potassium iodide	- 9.0 g
Distilled water	- 900.0 ml

Alcohol - 95%

Safranin

Safranin	- 10.0 ml
Distilled water	- 100.0 ml

- **Oxidase Reagent**

N'N'N'N'- tera dimethyl paraphenylenediamine	- 1.0 g
Distilled water	- 100 ml

Dissolved N'N'N'N'- tetra dimethyl paraphenylenediamine dihydrochloride in 100 ml distilled water and kept in amber coloured bottle to prevent oxidation.

- **Catalase Reagent**

Hydrogen peroxide	- 0.3 ml
Distilled water	- 10.0 ml

- **Kovac's Reagent**

p-dimethylaminobenzaldehyde	- 5.0 g
Amyl alcohol	- 75.0 ml
Hydrochloric acid	-25.0 ml

Dissolve p-dimethylaminobenzaldehyde in amyl alcohol, then add HCl and store in a Refrigerator.

- **Gelatin Precipitating Reagent**

15% mercuric chloride in 20 %(vol/ vol) concentrated HCl

- **Baritts reagent A**

Alpha – naphthol	- 0.5 g
Absolute ethanol	- 95 ml

- **Barrits reagent B**

Potassium hydroxide	- 40.0 g
Creatine	- 0.3 g

- **Methyl Red Solution**

Methyl Red (pH indicator)	- 0.05 gm
Ethanol	- 28ml
Distilled water	-22 ml

- **Potassium hydroxide solution**

Potassium hydroxide	- 3.0 g
Distilled water	- 100 ml

- **Nitrate Reagent 1**

Alpha-naphthylamine	- 0.5 g
Acetic acid 5N	-100 ml

- **Nitrate Reagent 2**

Sulphanilic acid - 0.8 g
Acetic acid 5N - 100 ml
Dissolve by heating

Molecular

- **1M EDTA**
  EDTA - 37.24 g
  MIlliQ Water - 100 ml
  pH - 8.0

- **5M NaCl**
  NaCl - 14.60 g
  Milli Q water - 100 ml
  pH - 8.0

- **1M NaOH**
  NaOH - 4 g
  MilliQ water - 100 ml
  pH - 8.0

- **1% Tween 20**
  Tween20 - 1 ml
  Milli Q Water - 100 ml

- **CTAB/NaCl**
  CTAB - 10 g
  0.7M NaCl - 4.1 g
  MilliQ Water -100 ml

- **10% CTAB**
  CTAB - 10.0 g
  MilliQ Water - 100 ml

- **20%SDS**
  SDS - 20.0 g
  MilliQ Water - 100 ml
  pH - 8.0

- **70% Ethanol**
  Absolute Ethanol - 70 ml
  MilliQ Water - 30 ml

- **2.6M Ammonium Acetate**
  Ammonium acetate - 20.027 g
  MilliQ Water -100 ml
  pH - 8.0

- **5M Potassium Acetate**
  Potassium Acetate - 49.0 g
  MilliQ Water - 100 ml
  pH - 8.0

- **30% Polyethylene glycol (PEG)/ 1.6M NaCl**
  PEG - 30.0 g
  1.6 M NaCl - 4.66 g
  MilliQ water – 100 ml
  pH - 8

- **Chloroform / Isoamylalcohol (24:1)**
  Chloroform - 24 .0 ml
  Isoamyl alcohol - 1 ml

- **Proteinase K(20mg/ml)**
  Proteinase K - 20.0 g
  MilliQ water - 1ml

- **Ethidium Bromide(10mg/ml)**
  Ethidium Bromide -10 mg
  MilliQ Water -1ml

- **Gel Loading Buffer**
  Glycerine – 50.09%
  Bromophenol blue – 0.25%
  EDTA - 50mM

Reagents for Denaturing Gradient Gel Electrophoresis

Running Buffer (1X TAE Buffer)

50X TAE Buffer: 140 ml
Make up the final volume to 7 litres by adding MilliQ Water

10% Ammonium PerSulphate Solution:

To be used fresh. Should not be more than 6-7 days old.
Ammonium Persulphate: 0.3 ml

Double Distilled Water: 3 ml.

Acrylamide Solutions:

Required: 40% Acrylamide/Bis Solutions
First Prepare:
100 ml of stock 40% Acrylamide/Bis(37.5:1)
Acrylamide: 38.93 gm
Bis-Acrylamide: 1.07 gm
Make up the final volume to 100 ml by adding Double Distilled Water

Working Solution:
Using this stock, prepare the working solutions as under:

0% Denaturing Solution:

Reagent	10% Gel (For 100-300 bp DNA
40% Acrylamide/Bis	25 ml
50X TAE	2 ml
Double Distilled Water	73 ml

100% Denaturing Solution:

Reagent	10% Gel (For 100-300 bp DNA
40% Acrylamide/Bis	25 ml
50X TAE	2 ml
Formamide(Deionized)	40 ml
Urea	42 gm

To the above, add Double Distilled water to make up the final volume to 100 ml.

Before casting the gel, add following volumes of TEMED and 10% Ammonium Persulphate in the above working solution:

TEMED: 0.09% (v/v) of the gel solution.
10% Ammonium Persulphate: 0.09%(v/v) of the gel solution..

DCode Dye Solution:
To be added in the 100% Denaturing solution while casting gel. Its purpose is to track the formation of gradient, when pouring a gradient gel.

Bromophenol blue	0.05 gm
Xylene Cyanol	0.05 gm
1X TAE Buffer	10 ml

2X Gel Loading Dye:

Add equal volume of 2X Gel loading dye to the sample.

2% Bromophenol (Prepared in DD water	250 ul
2% Xylene Cyanol (Prepared in DD Water)	250 ul
100% Glycerol	7 ml
Double Distilled water	2.5 ml
FINAL VOLUME	**10 ml**

A3- Composition of buffers

- **1M Tris**
  Tris HCl - 12.14 g
  Milli Q Water - 100 ml
  pH - 8.0

- **100mM Tris EDTA**
  10mM Tris HCl - 0.12 g
  1mM EDTA - 0.037 g
  Milli Q Water - 100 ml
  pH - 8.0

- **0.1M Sodium Phosphate Buffer**
  Na_2HPO_4 - 1.374 g
  NaH_2PO_4 - 0.01146 g

MilliQ Water - 100 ml
pH - 4.5

- **Gray and Herwig Extraction Buffer**
  500mM NaCl - 1.46 g
  50mMEDTA - 1.862
  4%SDS - 4g
  50mM Tris - 0.607g
  MilliQ water - 100ml
  pH - 8.0

- **Lovell and Piceno Extraction Buffer**
  1mM Sodium phosphate buffer
  Na_2HPO_4 - 57.7 ml
  NaH_2PO_4 - 42.3ml
  Milli Q Water -100ml
  pH - 7.0
  Solid SDS - 0.3 g

- **Yeates Extraction Buffer**
  100mM Tris HCl - 1.214g
  100mM Sodium EDTA - 3.72g
  1.5M NaCl - 4.383 g
  MilliQ Water - 100 ml
  pH - 8.0

- **Zhou Buffer**
  100mM Tris HCl -1.214 g
  100mM Sodium phosphate
  NaH_2PO_4 - 6.8 g
  Na_2HPO_4 - 9.32 g
  1.5M NaCl - 4.6g
  100mM Sodium EDTA - 3.72 g
  MilliQ water -100 ml
  pH - 8.0

- **Chrombatch Buffer**
  0.33M Tris HCl - 3.99g
  0.001M EDTA -0.037224 g
  MilliQ water -100 ml
  pH - 8.0

- **Wash Buffer**

10mM Tris HCl	- 0.12 g
0.1mM Sodium EDTA	- 0.0037 g
1% Triton –X 100	- 1ml
Guanidine thiocyanate(5M)	- 59.08g
MilliQ Water	- 100 ml
pH	- 8.0

- **Extraction Buffer**

100mM Tris HCl	- 1.214 g
1.5M NaCl	- 4.383g
100mM Sodium phosphate	
NaH_2PO_4	- 6.8 g
Na_2HPO_4	- 9.32 g
100mM EDTA	- 3.72g
Guanidine thiocyanate (5M)	- 59.08g
MIlliQ Water	- 100ml
pH	- 8.0

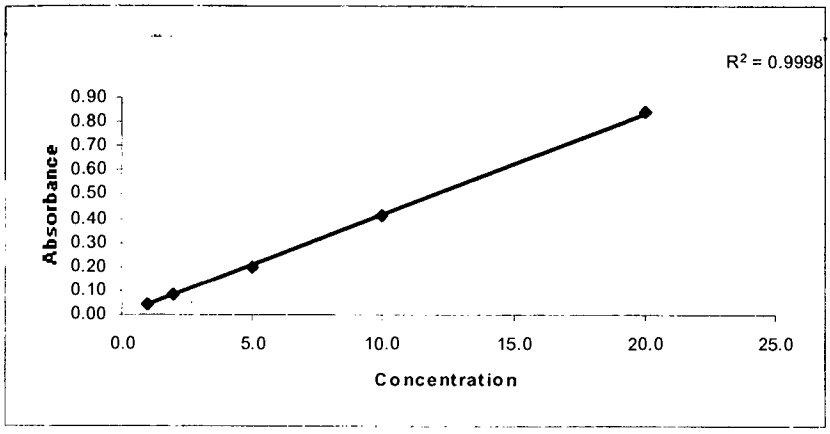
- **50X TAE**

Tris	- 121g
0.5M EDTA	- 18.62 g
Acetic acid	- 28.55ml
MilliQ water	- 100ml
pH	- 8.0

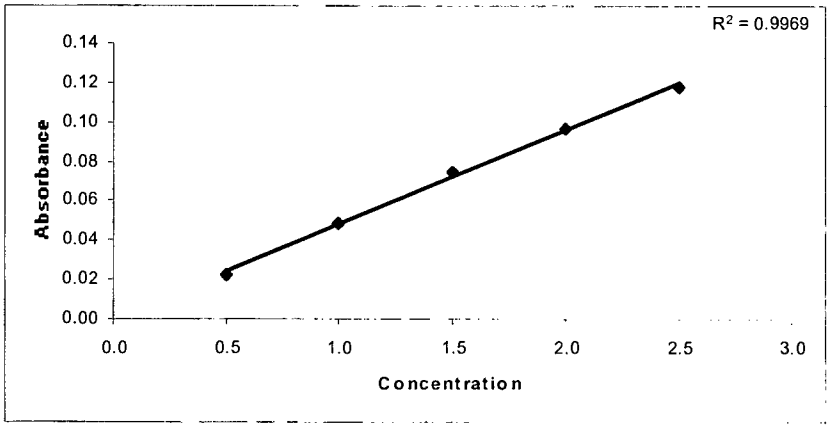
Supplementary Methods and Data

A4- Standard Graphs

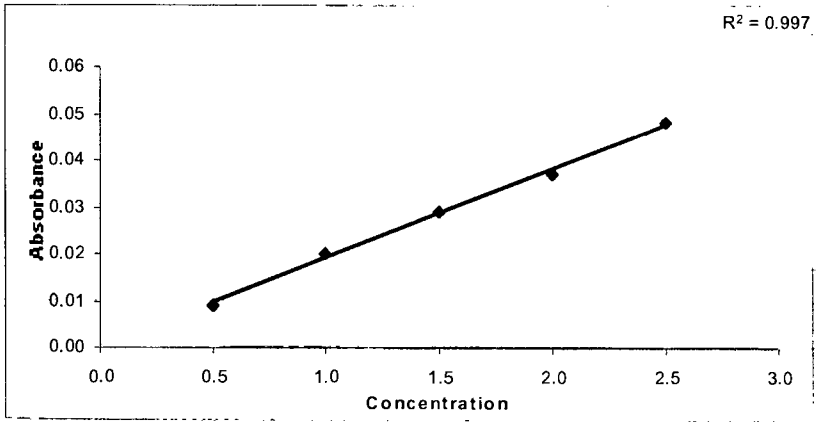
1. Nitrate



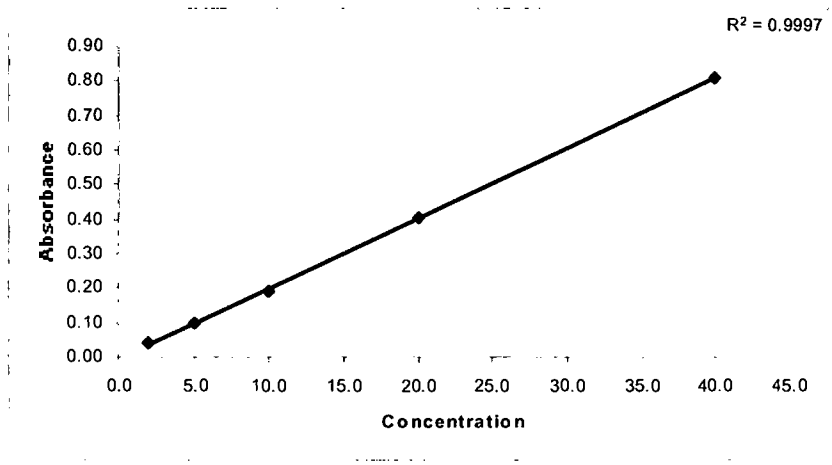
2. Nitrite



3. Phosphate

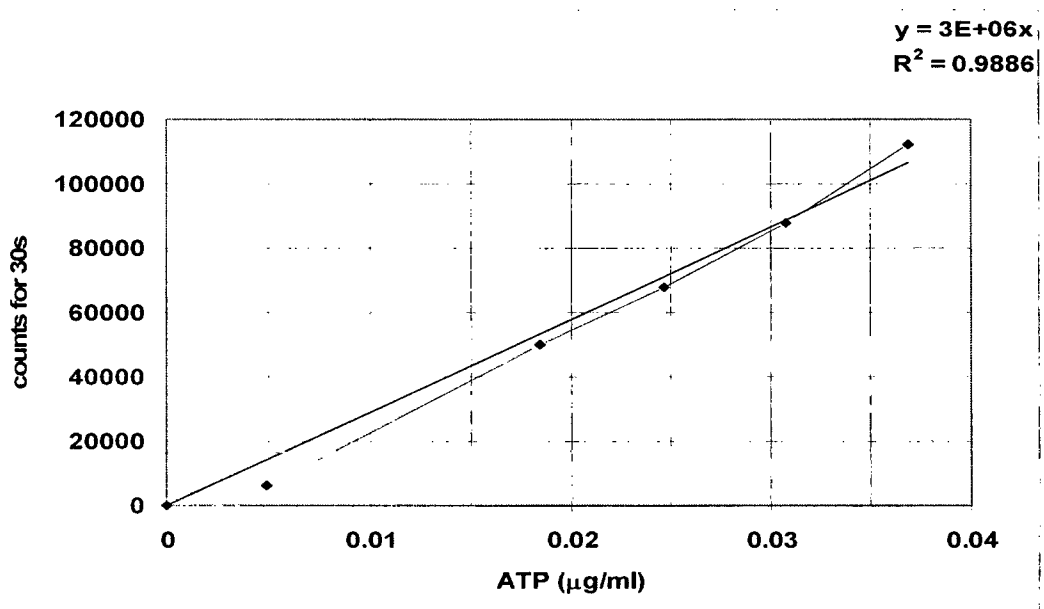


4. Silicate



5. Adenosine Triphosphate

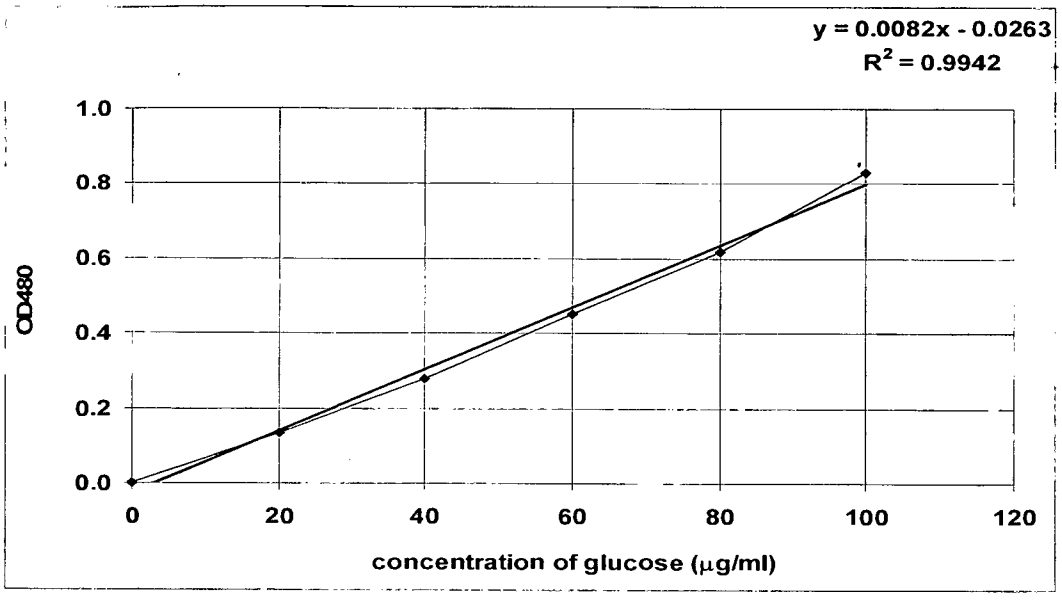
Standard Curve for carbohydrate estimation using ATP as standard



6. Labile Organic Matter (LOM)

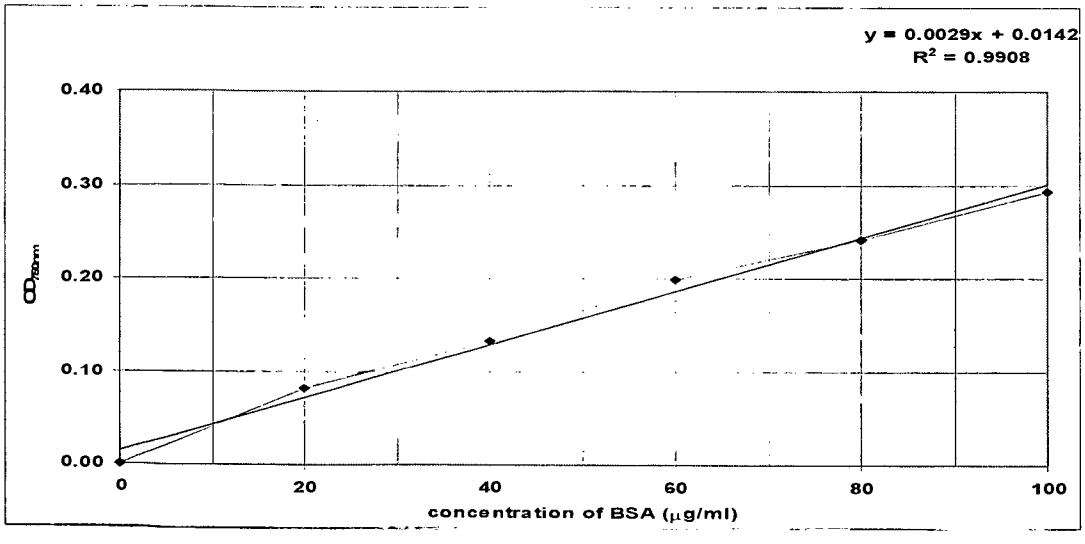
i) Estimation of Carbohydrates

Standard Curve for carbohydrate estimation using glucose as standard



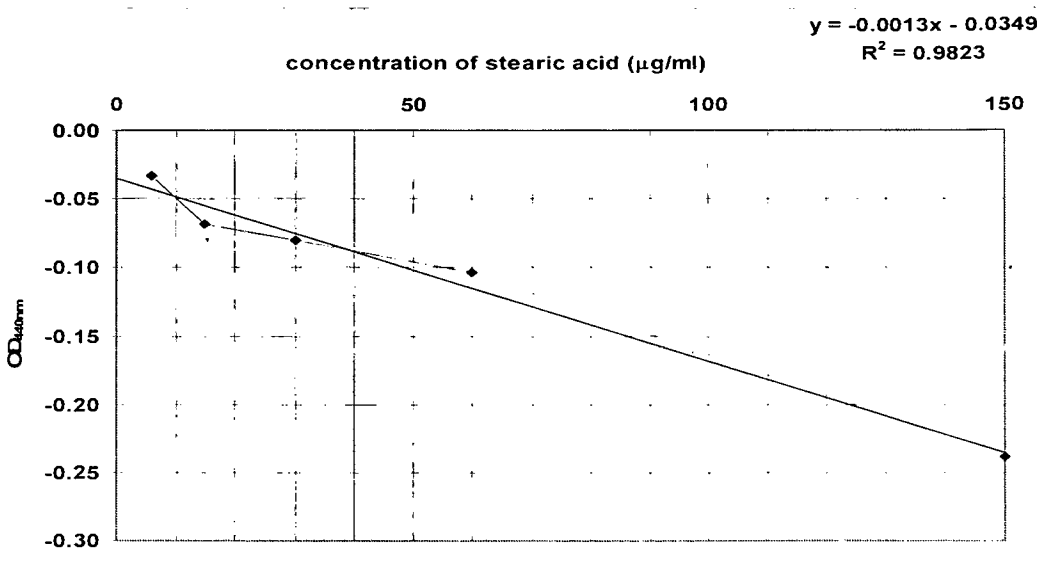
ii) Estimation of Proteins

Standard Curve for protein estimation using bovine serum albumin (BSA) as standard



iii) Estimation of Lipids

Standard Curve for lipid estimation using stearic acid as standard



A5- Methods of sediment DNA extraction

a) Cell Extraction method

i. Steffan *et al.*, (1988) Method

1. About 200 mg of sediment was taken in a fresh tube and suspended in 500 µl of 0.1 M sodium phosphate buffer (pH 4.5).
2. This was homogenized by horizontal vortexing for 3 minutes, with occassional cooling for 1 minute and added 50 µl of 20% SDS added and blended again for 5 minutes.
3. Cooled and centrifuged at 1000 rpm for 10 minutes, supernatants taken and maintained in ice.
4. Washing was performed with 500 µl of 0.1 M sodium phosphate buffer without the addition of SDS and centrifuged at 1000 rpm for 10 minutes.
5. Supernatants were recovered and combined followed by centrifugation at 10000 rpm for 30 minutes.

6. The above were suspended in 200 µl of 0.1% sodium hexaphosphate and centrifuged at 10,000 rpm for 30 minutes.
7. Supernatants were discarded and procedure was repeated again.
8. Pellets were suspended in chrombatch buffer (0.33 M Tris HCl, 0.001 M EDTA, pH 8) and centrifuged at 10000 rpm for 15 minutes.
9. Pellet recovered and suspended in 500 µl of chrombatch buffer.
10. Added 10 µl of lysozyme (5 mg/ml) and incubated for 2 hours at 37°C.
11. The suspension was heated to 60°C and 50 µl of 20% SDS added to a final concentration of 1%.
12. Incubation at 55°C for 10 minutes and cooled in ice for 2 hours.
13. Centrifugation for 20 minutes at 12000 rpm at 5°C and supernatants taken (first lysate).
14. Washed and centrifuged again at same rpm, supernatant taken and pooled with the first lysate.
15. Solid ammonium acetate was added to a final concentration of 2.5 M.
16. Centrifugation for 30 minutes at 12000 rpm followed by 12 hours incubation after adding 2.5 volumes of ice cold 95% ethanol.
17. Centrifugation at 12000 rpm for 30 minutes, followed by 70% ethanol wash.
18. Centrifugation at 10000 rpm for 10 minutes and pellets were air dried.
19. Pellet suspended in 10 mM TE buffer (pH 8).

b) Direct lysis method

Five different protocols were followed for direct lysis method.

i. Purohit *et al.*, (2003) Method

1. Sediment samples (200 mg) were taken in 1.5 microcentrifuge tubes.
2. The pellet was washed three times with sterile distilled water.

3. To the pellet 900 µl of 50 mM Tris (pH 9) was added followed by incubation at 50°C for 15 minutes and spin at 10,000 rpm for 10 minutes.
4. This was followed by addition of100 µl of 1% Tween 20, mixed thoroughly, incubated at 55°C for 15 minutes and spinned at 10,000 rpm for 10 minutes.
5. The supernatant was discarded and pellet was washed with 1000 µl of distilled water.
6. A mixture of 0.01% Tween 20 (200 µl) and 300 µl acetone were added, mixed well by inversion and spinned for 10 minutes. The supernatant was discarded.
7. The pellet was washed with 1000 µl distilled water and incubated at 50°C for 15 minutes.
8. Dissolved the pellet in 25 µl of NaOH (0.05 M), followed by incubation at room temperature for 30 minutes.
9. To the solution 25 µl of 1 M Tris (pH 7.5) and 3 µl of proteinase K (20 mg/ml) were added and incubated at 55°C for 30 minutes.
10. The total volume was made up to 500 µl with distilled water.
11. Equal volumes of tris saturated phenol and chloroform were added, mixed for 15 minutes and centrifuged at 11,000 rpm for 10 minutes.
12. Aqueous layer was taken and transferred in to a new tube, added equal volume of chloroform, mixed well and spinned for 10 minutes.
13. The upper layer was taken in a fresh tube and added 1/10 the volume of 3 M sodium acetate and 0.7 volume of isopropanol followed by incubation for 12 hours. Spinned at 11000 rpm for 30 minutes and discarded the supernatant.
14. The pellet was washed with 70% ethanol, spinned for 10 minutes and air dried.
15. The pellet was redissolved in 30 µl of 10 mM Tris EDTA (TE) buffer (pH 8).

ii. Gray and Herwig (1996) Method

1. Sediment (200 mg) was homogenized by horizontal vortexing with 0.2 g of 0.1 mm silica beads , 20 mg of polyvinylpolypyrolidone (PVPP) and 500 µl of pre-heated lysis buffer pH 8.
2. Tubes were incubated at 70°C for 1 hour followed by spinning at 16000 rpm for 10 minutes.
3. The supernatants were incubated on ice for 15 minutes.
4. Two volumes (1000 µl) of 100% ethanol were added, mixed by inversion for 15 minutes and incubated at -20°C for 15 minutes followed by centrifugation at 16000 rpm for 10 minutes.
5. Pellets were washed with 70% ethanol and spinned at 10000 rpm for 10 minutes.
6. The pellet was air dried and resuspended in 30 µl 10 mM TE buffer (pH 8).

iii. Lovell and Piceno (1994) Method

1. In a 1.5 ml microcentrifuge tube,100 mg of sediment was taken and added 500 µl of 1 mM sodium phosphate buffer (pH 7) and 0.3 g solid SDS followed by incubation at 70°C for 30 minutes with occassional shaking.
2. The above tubes were spinned at 10000 rpm for 15 minutes and supernatants transferred to a fresh tube.
3. The pellets were washed again with 1 mM sodium phosphate buffer (pH 7) and 1 % SDS and the supernatants from the above and earlier were pooled and incubated on ice for 1-2 hours.
4. Centrifuged at 5000 rpm for 5 minutes and supernatants recovered.
5. To the supernatant 0.5 M solid potassium acetate was added and again incubated on ice for 2 hours. Spinned at 10000 rpm for 30 minutes.

6. Supernatant mixed with equal volume of isopropanol and centrifuged at 10000 rpm for 30 minutes.
7. The pellets were washed with 70% ethanol.
8. The pellets redissolved in 30 µl of TE buffer (pH 8).
9. Added 10 µl of ethidium bromide (0.6 mg/ml) and 1000 µl of ammonium acetate (2.6 M), incubated in the dark for 5minutes.
10. The above was extracted with equal volume of phenol, mixed by inverting and recovered aqueous phase.
11. Added equal volume of isopropanol and centrifuged at 10000 rpm for 30 minutes.
12. Washed the pellet with 70% ethanol and spinned at 10000 rpm for 10 minutes.
13. Resuspended the pellet in 30 µl of 10 mM TE buffer (pH 8).

iv. Yeates *et al.*, (1997) Method

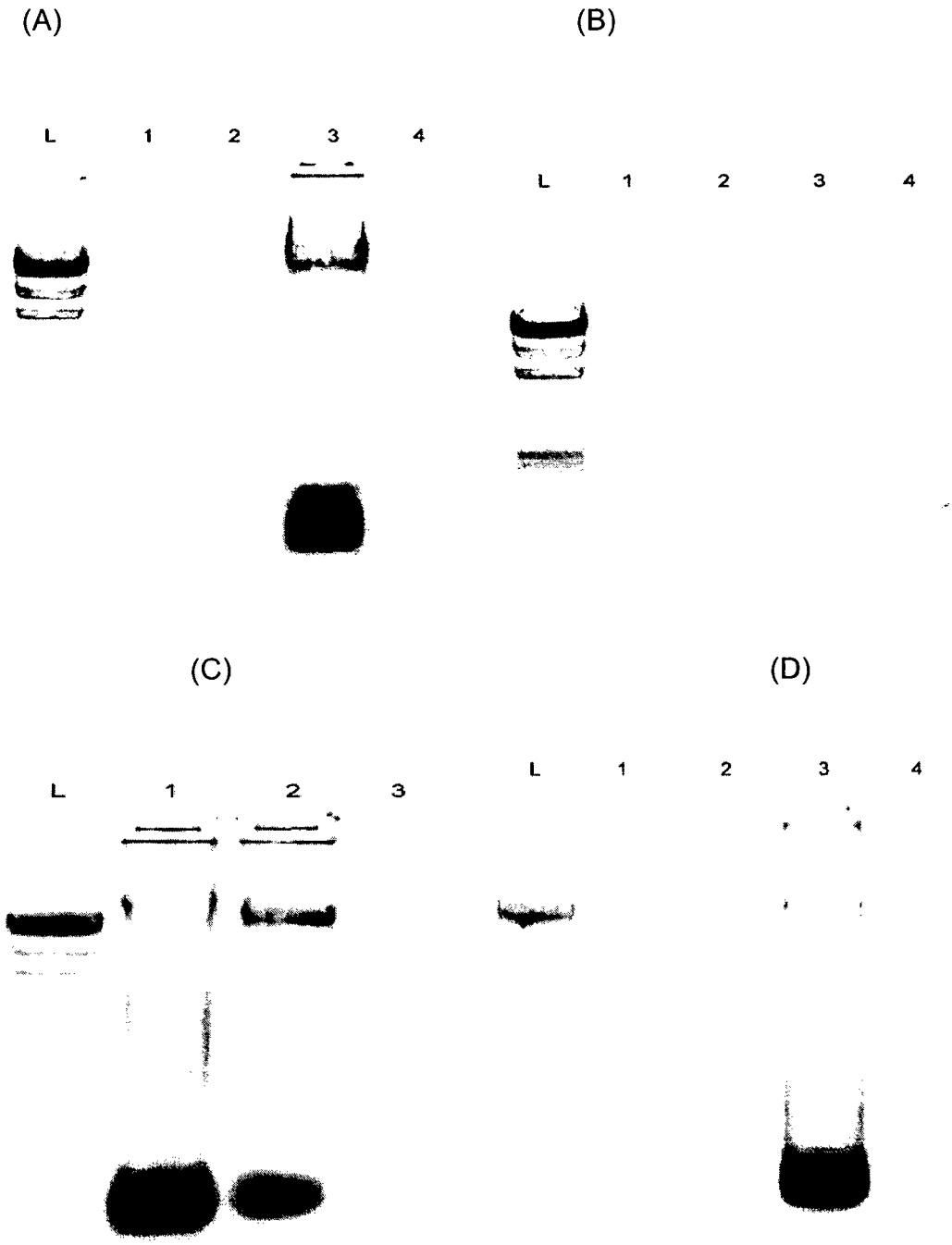
1. About 100 mg of sediment was taken in a fresh tube containing 2 g of silica beads, added 1000 µl of extraction buffer (pH 8) and blended horizontal vortexing.
2. To the homogenized solution, 50 µl of 20% SDS was added and vortexed again. Then centrifuged at 6000 rpm for 10 minutes, supernatant recovered.
3. Added 500 µl of 30% polyethylene glycol (PEG) in 1.6 M NaCl.
4. The tube was incubated at room temperature for 2 hours followed by centrifugation at 10000 rpm for 20 minutes.
5. Pellet was washed with 10 mM TE buffer and 1 mM EDTA, and added 1000 µl of 0.5 M potassium acetate.
6. Incubated in ice for 15 minutes and centrifuged at 16,000 rpm for 15 minutes.

7. Aqueous phase was extracted with phenol/chloroform (1:1) and chloroform /isoamyl (24:1).
8. DNA was precipitated by adding 600 µl isopropanol followed by overnight incubation. This was centrifuged at 16000 rpm for 15 minutes.
9. The pellet was washed with 70% ethanol and spinned at 10000 rpm for 10 minutes.
10. The pellet was air dried and resuspended in 30 µl of TE buffer.

v. Zhou *et al.*, (1996) Method

1. To 100 mg of the sediment 500 µl extraction buffer (pH 8), 50 µl 10% CTAB, 50 µl of 20 % SDS, and 10 µl of proteinase K (20 mg /ml) were added.
2. Vortexed and incubated at 65 ^{o}C for 2 hours and centrifuged at 10000 rpm for 10 minutes.
3. To the supernatant equal volume of chloroform/ isoamyl alcohol (24:1) and added).
4. Mixed thoroughly by inversion and spinned at 10000 rpm for 10 minutes.
5. The aqueous layer was transferred to a fresh tube, added 0.6 volumes of isopropanol and incubated for 12 hours at room temperature.
6. Spinned at 10,000 rpm for 30 minutes and washed the pellet with 70% ethanol.
7. The pellet was air dried and resuspended in 30 µl of 10mM TE (pH 8).

Results



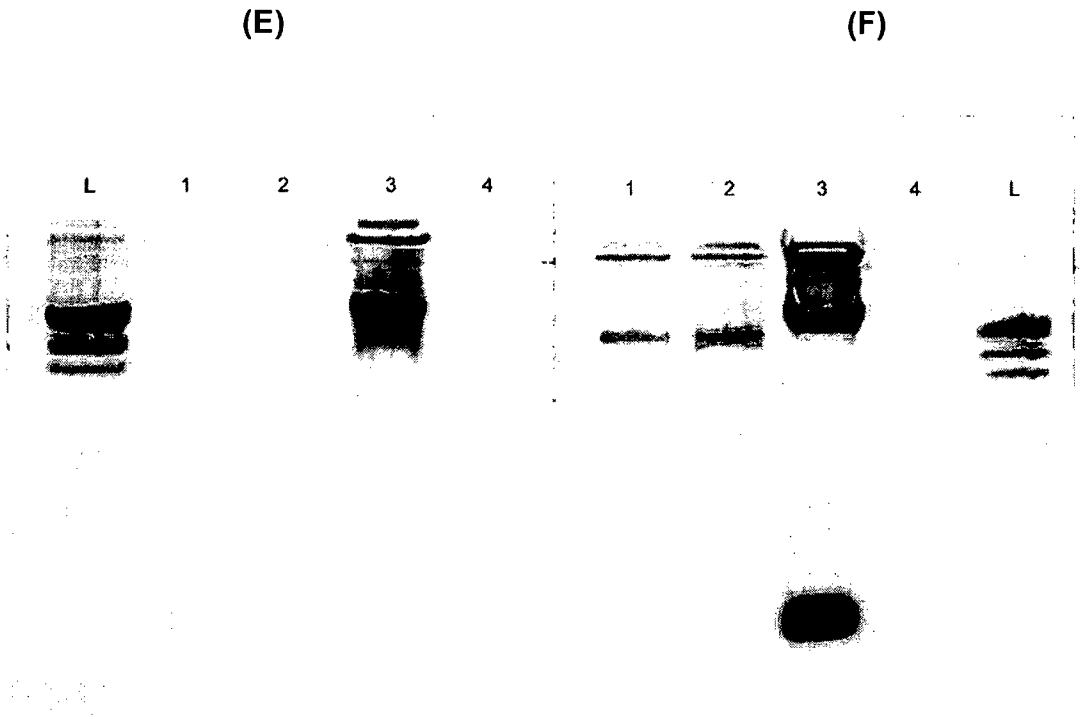


Fig. S1. Gel pictures showing genomic DNA extracted from sediments using (A) Purohit method (B) Gray method (C) Lovell method (D) Yeates method (E) Zhou method (F) Steffan method (L-*Hae* III digest Lambda DNA ladder, 1-Sample 1, 2-Sample 2, 3-Positive control (MTCC 434*Stenotrophomonas maltophila*), 4-Negative control)

A6-HYDROGRAPHY-Multiple Correlation Analysis

	Temperature	Nitrate	Nitrite	Phosphate	Silicate	TC	TN	TH	TS	TOC	Carbohydrates	Lipids	Protein	Sand	Silt	Clay	Eh	Water Content	DO	Salinity	pH
Temperature	1																				
Nitrate	-0.89	1																			
Nitrite	-0.39	0.40	1																		
Phosphate	-0.57	0.39	0.70	1																	
Silicate	-0.90	0.90	0.57	0.45	1																
TC	-0.03	-0.26	-0.62	-0.12	-0.33	1															
TN	-0.48	0.32	0.18	0.48	0.20	0.13	1														
TH	-0.38	0.21	0.52	0.60	0.34	-0.16	0.57	1													
TS	0.01	0.17	0.16	-0.14	-0.03	-0.19	0.39	-0.07	1												
TOC	-0.27	-0.04	-0.58	0.04	-0.10	0.93	0.23	-0.04	-0.36	1											
Carbohydrates	-0.25	0.47	-0.18	-0.25	0.26	-0.20	0.24	0.18	0.08	-0.07	1										
Lipids	-0.81	0.81	0.43	0.26	0.85	-0.29	0.50	0.35	0.36	-0.11	0.34	1									
Protein	0.90	-0.72	-0.20	-0.58	-0.72	-0.18	-0.55	-0.54	0.18	-0.45	-0.23	-0.66	1								
Sand	0.67	-0.56	-0.23	-0.61	-0.46	0.07	-0.86	-0.50	-0.09	-0.19	-0.32	-0.58	0.75	1							
Silt	-0.39	0.29	0.06	-0.19	0.42	-0.04	0.29	-0.03	0.20	0.01	0.26	0.64	-0.15	-0.23	1						
Clay	-0.35	0.31	0.17	0.68	0.14	-0.03	0.60	0.48	-0.05	0.17	0.12	0.10	-0.58	-0.76	-0.46	1					
Eh	0.15	-0.14	0.26	0.01	-0.16	-0.17	0.45	0.09	0.85	-0.35	-0.29	0.26	0.22	-0.12	0.22	-0.03	1				
Water Content	-0.80	0.67	0.28	0.61	0.54	0.13	0.88	0.60	0.23	0.32	0.36	0.66	-0.83	-0.88	0.25	0.64	0.14	1			
DO	-0.72	0.62	0.74	0.58	0.87	-0.40	0.16	0.60	-0.25	-0.20	0.12	0.66	-0.59	-0.36	0.36	0.09	-0.19	0.43	1		
Salinity	0.13	-0.26	-0.44	0.10	-0.53	0.66	0.54	0.10	0.21	0.58	0.00	-0.33	-0.10	-0.28	-0.34	0.49	0.19	0.37	-0.59	1	
Ph	0.33	-0.35	-0.25	-0.08	-0.23	0.00	-0.51	-0.36	-0.39	0.04	-0.56	-0.39	0.12	0.26	-0.50	0.09	-0.18	-0.52	-0.27	-0.14	1

Supplement Table 1. Correlation matrix of the environmental variables of OMZ

	Temperature	Nitrate	Nitrite	Phosphate	Silicate	TC	TN	TH	TS	TOC	Carbo hydrates	Lipids	Protein	Sand	Silt	Clay	Eh	Water Content	DO	Salinity	Ph
Temperature	1																				
Nitrate	0.99	1																			
Nitrite	0.98	0.98	1																		
Phosphate	0.59	0.60	0.75	1																	
Silicate	0.49	0.50	0.66	0.99	1																
TC	-0.96	-0.96	-1.00	-0.79	-0.71	1															
TN	0.92	0.92	0.98	0.87	0.80	-0.99	1														
TH	0.49	0.49	0.66	0.99	1.00	-0.71	0.80	1													
TS	0.91	0.91	0.81	0.22	0.10	-0.77	0.68	0.09	1												
TOC	-0.97	-0.98	-1.00	-0.76	-0.67	1.00	-0.98	-0.67	-0.80	1											
Carbohydrates	0.99	0.99	0.95	0.49	0.38	-0.92	0.86	0.38	0.96	-0.94	1										
Lipids	0.83	0.83	0.70	0.05	-0.07	-0.65	0.54	-0.08	0.99	-0.69	0.90	1									
Protein	0.99	0.99	0.94	0.49	0.38	-0.92	0.86	0.37	0.96	-0.94	1.00	0.90	1								
Sand	-0.98	-0.98	-1.00	-0.76	-0.67	1.00	-0.98	-0.67	-0.80	1.00	-0.94	-0.69	-0.94	1							
Silt	0.56	0.55	0.37	-0.34	-0.45	-0.31	0.18	-0.45	0.85	-0.36	0.66	0.92	0.66	-0.36	1						
Clay	0.70	0.71	0.84	0.99	0.96	-0.87	0.93	0.96	0.36	-0.84	0.61	0.19	0.61	-0.84	-0.20	1					
Eh	1.00	1.00	0.96	0.53	0.42	-0.94	0.88	0.41	0.94	-0.95	1.00	0.88	1.00	-0.95	0.62	0.64	1				
Water Content	-0.21	-0.20	0.00	0.66	0.75	-0.06	0.20	0.75	-0.59	-0.01	-0.33	-0.72	-0.33	-0.01	-0.93	0.55	-0.29	1			
DO	-0.52	-0.53	-0.69	-1.00	-1.00	0.74	-0.82	-1.00	-0.13	0.70	-0.41	0.04	-0.41	0.70	0.42	-0.97	-0.45	-0.72	1		
Salinity	0.99	0.99	0.95	0.50	0.39	-0.93	0.87	0.39	0.95	-0.94	1.00	0.89	1.00	-0.94	0.65	0.62	1.00	-0.32	-0.42	1	
Ph	-0.95	-0.95	-0.87	-0.33	-0.21	0.84	-0.76	-0.20	-0.99	0.86	-0.98	-0.96	-0.98	0.87	-0.78	-0.46	-0.98	0.49	0.24	-0.98	1

Supplement Table 2. Correlation matrix of the environmental variables of non-OMZ

A7- Aerobic CFU (colony counts) in different dilution and nutrient concentrations

Table 1: Colony counts in different serial dilutions in 100% nutrient medium concentration

Transect	Depth	Number of colonies				Average	CFU/ml	CFU/gdrywt
		10^{-2}		10^{-3}				
Goa	50 m	32	30	4	2	31	3100	2.8×10^3
	200 m	4	1	0	0	2.5	250	1.5×10^2
	500 m	6	10	1	0	8	800	1.5×10^3
	1000 m	15	10	2	3	12.5	1250	2.7×10^3
Ratnagiri	50 m	43	33	5	6	38	3800	4.6×10^3
	200 m	43	33	2	4	38	3800	2.3×10^3
	500 m	6	6	0	0	6	600	9.2×10^2
	1000 m	8	5	0	1	6.5	650	7.6×10^2
Karwar	50 m	9	18	2	1	13.5	1350	2.04×10^3
	200 m	8	9	1	0	8.5	850	1.3×10^3
	500 m	7	5	0	0	6	600	9.6×10^2
	1000 m	5	8	0	0	6.5	650	1.5×10^3

Table 2: Colony counts in different serial dilutions in 25% nutrient medium concentration

Transect	Depth	Number of colonies				Average	CFU/ml	CFU/gdrywt
		10^{-2}		10^{-3}				
Goa	50 m	37	38	5	2	37.5	3750	3.4×10^3
	200 m	21	20	3	2	20.5	2050	1.2×10^3
	500 m	16	11	2	1	13.5	1350	2.5×10^3
	1000 m	78	75	9	7	76.5	7650	1.6×10^4
Ratnagiri	50 m	31	20	3	1	25.5	2550	3.1×10^3
	200 m	31	24	4	2	27.5	2750	1.7×10^3
	500 m	35	29	5	2	32	3200	4.9×10^3
	1000 m	33	32	3	4	32.5	3250	3.8×10^3
Karwar	50 m	32	36	4	3	34	3400	5.1×10^3
	200 m	31	35	1	4	33	3300	5.2×10^3
	500 m	31	27	4	2	29	2900	4.6×10^3
	1000 m	31	28	2	3	29.5	2950	7.02×10^3

Table 3: Colony counts in different serial dilutions in 10% nutrient medium concentration

Transect	Depth	Number of colonies				Average	CFU/ml	CFU/gdrywt
		10^{-2}		10^{-3}				
Goa	50 m	44	60	1	3	52	5200	4.7×10^3
	200 m	6	2	0	0	4	400	2.4×10^2
	500 m	18	30	2	3	24	2400	4.6×10^3
	1000 m	154	120	34	32	137	13700	2.9×10^4
Ratnagiri	50 m	36	48	4	5	42	4200	5.1×10^3
	200 m	7	8	0	1	7.5	750	4.6×10^2
	500 m	8	7	0	0	7.5	750	1.1×10^3
	1000 m	65	68	8	6	66.5	6650	7.8×10^3
Karwar	50 m	37	66	4	2	51.5	5150	7.8×10^3
	200 m	6	8	1	0	7	700	1.1×10^3
	500 m	6	5	0	0	5.5	550	8.8×10^2
	1000 m	48	56	2	6	52	5200	1.2×10^4

Table 4: Colony counts in different serial dilutions in 0% nutrient concentration

Transect	Depth	Number of colonies				Average	CFU/ml	CFU/gdrywt
		10^{-2}		10^{-3}				
Goa	50 m	65	60	5	7	62.5	6250	5.6×10^3
	200 m	1	0	0	0	0.5	50	3.1×10^1
	500 m	0	0	0	0	0	0	nd
	1000 m	0	0	0	0	0	0	nd
Ratnagiri	50 m	22	12	1	2	17	1700	2.09×10^3
	200 m	4	2	0	0	3	300	1.8×10^2
	500 m	2	1	0	0	1.5	150	2.3×10^2
	1000 m	1	1	0	0	1	100	1.1×10^2
Karwar	50 m	15	20	1	1	17.5	1750	2.6×10^3
	200 m	2	3	0	0	2.5	250	3.9×10^2
	500 m	1	1	0	0	1	100	1.6×10^2
	1000 m	1	2	0	0	1.5	150	3.5×10^2

A8- Colony characteristics of isolates

Table 1: Colony characteristics of isolates from 50 m depth

SL No.	Isolate	Shape	Margin	Colour	Transparency	Elevation	Diameter (cm)
1	G3-1A	circular	entire	cream	opaque	raised	0.5
2	G3-1B	circular	entire	cream	opaque	convex	0.2
3	G3-2	circular	entire	cream	opaque	convex	0.3
4	G3-3A	circular	entire	cream	opaque	convex	0.4
5	G3-4A	circular	entire	cream	opaque	convex	0.3
6	G3-5A	circular	entire	cream	opaque	convex	0.4
7	G3-6A	circular	undulate	cream	opaque	raised	0.5
8	G3-6B	circular	entire	cream	opaque	convex	0.3
9	G3-7A	circular	entire	cream	opaque	raised	0.5
10	G3-8B	circular	entire	cream	opaque	raised	0.7
11	G3-9B	circular	entire	cream	opaque	raised	0.2
12	G3-10	circular	entire	cream	opaque	raised	0.4
13	G3-11B	circular	entire	brownish cream	opaque	raised	1.2
14	G3-12	circular	entire	cream	opaque	convex	0.5
15	G3-13A	circular	entire	cream	opaque	convex	0.4
16	G3-14A	circular	entire	cream	opaque	convex	0.2
17	G3-14B	circular	entire	brownish cream	opaque	raised	1
18	G3-15B	circular	entire	cream	opaque	raised	0.6
19	G3-16A	circular	entire	cream	opaque	convex	0.6
20	G3-16B	circular	entire	cream	opaque	raised	0.3
21	G3-17	circular	entire	cream	opaque	convex	0.5
22	G3-18B	circular	entire	cream	opaque	convex	0.3
23	G3-19A	circular	entire	cream	opaque	convex	0.5
24	G3-20A	circular	undulate	cream	opaque	raised	0.4
25	G3-21	circular	entire	cream	opaque	raised	0.3
26	G3-22B	circular	entire	cream	opaque	raised	0.5
27	G3-23B	circular	entire	cream	opaque	raised	0.4
28	G3-24B	circular	entire	cream	opaque	raised	1.2
29	G3-25	circular	entire	cream	opaque	convex	0.2
30	G3-26A	circular	entire	cream	opaque	convex	0.1
31	G3-27A	circular	entire	cream	opaque	raised	0.2

Table 2: Colony characteristics of isolates from 200 m depth

SL No.	Isolate	Shape	Margin	Colour	Transparency	Elevation	Diameter (cm)
1	G6-1	irregular	undulate	cream	opaque	raised	0.5
2	G6-2A	circular	entire	white	opaque	raised	0.3
3	G6-3B	circular	entire	cream	opaque	convex	0.2
4	G6-4	irregular	undulate	cream	opaque	raised	0.6
5	G6-5A	circular	entire	cream	opaque	convex	0.2
6	G6-6	circular	entire	cream	opaque	raised	0.6
7	G6-7A	circular	entire	cream	opaque	convex	0.1
8	G6-8	circular	entire	cream	opaque	convex	0.1
9	G6-9	irregular	lobate	cream	opaque	raised	0.8
10	G6-10	circular	entire	cream	opaque	raised	0.75
11	G6-11	irregular	lobate	cream	opaque	raised	0.7
12	G6-12B	irregular	lobate	cream	opaque	raised	1.2
13	G6-13A	circular	entire	cream	opaque	convex	0.3
14	G6-14	circular	entire	cream	opaque	convex	0.2
15	G6-15	circular	entire	white	opaque	raised	0.2
16	G6-16	irregular	lobate	cream	opaque	raised	0.6
17	G6-17	irregular	undulate	cream	opaque	raised	0.7
18	G6-18A	circular	entire	white	opaque	raised	0.1
19	G6-19A	circular	entire	cream	opaque	convex	0.4
20	G6-20	circular	entire	cream	opaque	convex	0.2
21	G6-21A	circular	entire	cream	opaque	raised	0.6
22	G6-22B	circular	entire	cream	opaque	convex	0.3
23	G6-23	irregular	lobate	cream	opaque	raised	0.5

Table 3: Colony characteristics of isolates from 500 m depth

SL No.	Isolate	Shape	Margin	Colour	Transparency	Elevation	Diameter (cm)
1	G9-1A	irregular	lobate	cream	opaque	raised	0.8
2	G9-1B	irregular	lobate	light brown	opaque	raised	0.7
3	G9-2	circular	entire	cream	opaque	raised	0.6
4	G9-3 A	circular	entire	cream	opaque	raised	0.7
5	G9-4	circular	entire	orange	opaque	raised	0.8
6	G9-5	circular	entire	brownish cream	opaque	raised	1.2
7	G9-6	circular	entire	orange	opaque	raised	0.8
8	G9-7A	circular	entire	cream	opaque	convex	0.3
9	G9-8B	circular	entire	cream	opaque	convex	0.1
10	G9-9B	circular	entire	cream	opaque	convex	0.4
11	G9-10A	circular	entire	brownish cream	opaque	raised	1.3
12	G9-11A	circular	undulate	cream	opaque	raised	0.5
13	G9-12	circular	undulate	cream	opaque	raised	0.4
14	G9-13A	irregular	lobate	cream	opaque	raised	0.6
15	G9-13B	circular	entire	orange	opaque	raised	0.9
16	G9-15A	circular	entire	cream	opaque	convex	0.2
17	G9-15B	circular	entire	cream	opaque	convex	0.2
18	G9-18A	circular	entire	flesh coloured	opaque	raised	0.4
19	G9-18B	circular	entire	cream	opaque	convex	0.2

Table 4: Colony characteristics of isolates from 1000 m depth

Sl No.	Isolate	Shape	Margin	Colour	Transparency	Elevation	Diameter (cm)
1	G12-1A	irregular	lobate	off white	opaque	flat	2
2	G12-1B	circular	entire	cream	opaque	raised	0.5
3	G12-2A	circular	entire	cream	opaque	flat	1.2
4	G12-3A	circular	entire	dark yellow	opaque	convex	0.3
5	G12-3B	circular	entire	cream	opaque	convex	0.3
6	G12-5A	circular	undulate	cream	opaque	raised	0.6
7	G12-5B	circular	entire	cream	opaque	raised	0.2
8	G12-6A	circular	entire	cream	opaque	raised	0.4
9	G12-6B	circular	entire	cream	opaque	raised	0.2
10	G12-7A	circular	entire	cream	opaque	raised	0.5
11	G12-7B	circular	entire	cream	opaque	raised	0.3
12	G12-8A	irregular	lobate	off white	opaque	flat	0.9
13	G12-8B	circular	entire	cream	opaque	raised	0.2
14	G12-9A	circular	entire	orangish yellow	opaque	convex	0.4
15	G12-10A	circular	undulate	cream	opaque	raised	0.5
16	G12-10B	circular	entire	cream	opaque	raised	0.4
17	G12-11A	circular	entire	cream	opaque	raised	0.5
18	G12-11B	circular	entire	cream	opaque	raised	0.3
19	G12-12A	rhizoid	erose	off white	opaque	flat	1.5
20	G12-12B	circular	entire	off white	opaque	flat	1.4
21	G12-13B	circular	entire	cream	opaque	raised	0.3
22	G12-14A	circular	entire	cream	opaque	raised	0.3
23	G12-14B	rhizoid	erose	off white	opaque	flat	1.2
24	G12-15A	circular	entire	off white	opaque	flat	1.5
25	G12-18A	circular	entire	orangish yellow	opaque	convex	0.2
26	G12-20A	circular	entire	cream	opaque	convex	0.1
27	G12-20B	circular	undulate	cream	opaque	raised	0.4
28	G12-21A	irregular	undulate	dirty cream	opaque	raised	0.3
29	G12-21B	irregular	undulate	dirty cream	opaque	raised	0.4
30	G12-22A	circular	entire	yellow	opaque	convex	0.2
31	G12-22B	circular	entire	yellow	opaque	convex	0.2
32	G12-23A	circular	entire	dark yellow	opaque	convex	0.2

A9- Principal Component Analysis of bacterial abundance variables of the three transect

Principal Component Analysis

Eigenvalues

PC	Eigenvalues	%Variation	Cum.%Variation
1	4.87E3	91.0	91.0
2	323	6.0	97.0
3	135	2.5	99.5
4	10.8	0.2	99.7
5	6.85	0.1	99.9

Eigenvectors

(Coefficients in the linear combinations of variables making up PC's)

Variable	PC1	PC2	PC3	PC4	PC5
TC	-0.815	-0.181	0.540	-0.002	0.092
TVCa	-0.437	-0.426	-0.770	-0.015	-0.132
TVCan	-0.377	0.875	-0.280	-0.048	0.016
CFUa (0%)	0.015	-0.087	-0.094	-0.064	0.234
CFUa (10%)	-0.014	-0.033	-0.057	0.696	0.168
CFUa (25%)	-0.010	0.013	-0.042	0.200	0.145
CFUa (100%)	-0.006	-0.026	-0.061	0.229	-0.004
CFUan (0%)	-0.004	0.013	0.006	0.156	-0.235
CFUan (100%)	-0.030	0.012	0.067	-0.091	-0.773
TDLO	-0.010	0.064	0.110	0.516	-0.433
SRBace	-0.019	0.055	-0.048	0.228	0.119
SRBlac	-0.022	0.051	-0.037	0.208	0.089
ATP	-0.004	-0.006	-0.002	0.149	0.083

Supplement Table 3. Eigen values and coefficient of all variables in the three transects

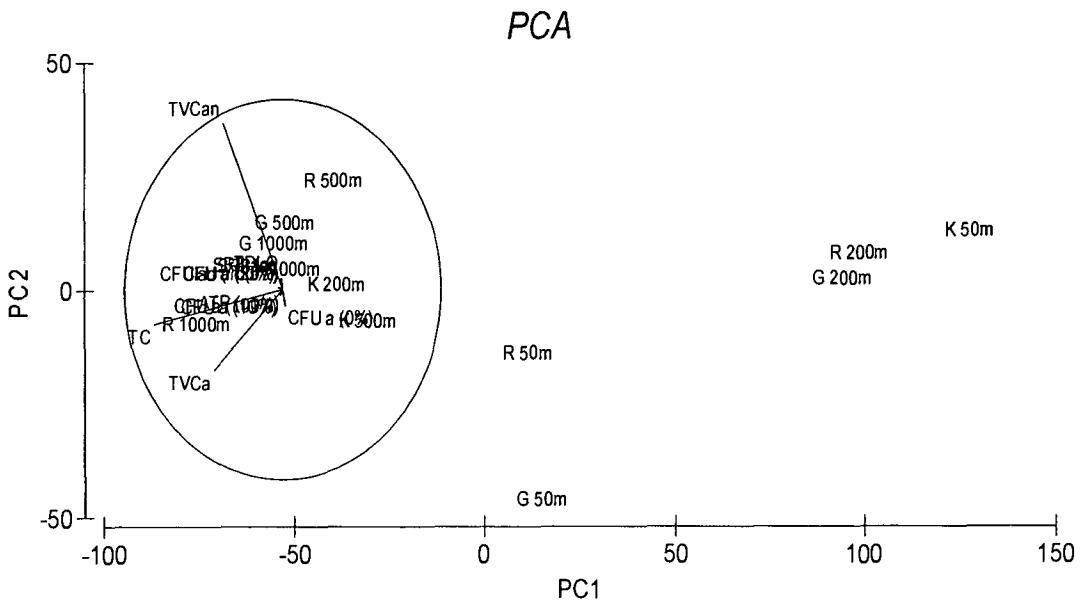


Fig. S2. Principal Component Analysis of variables of the three transects.

A10- DGGE Analysis

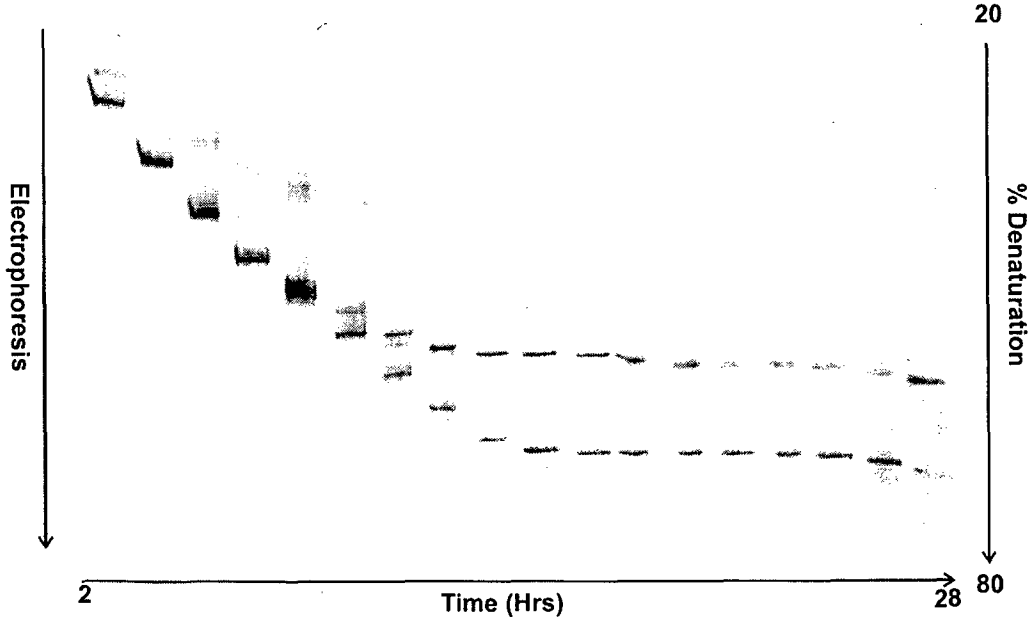


Fig. S3. Time Travel experiment of PCR amplification of two bacterial strains

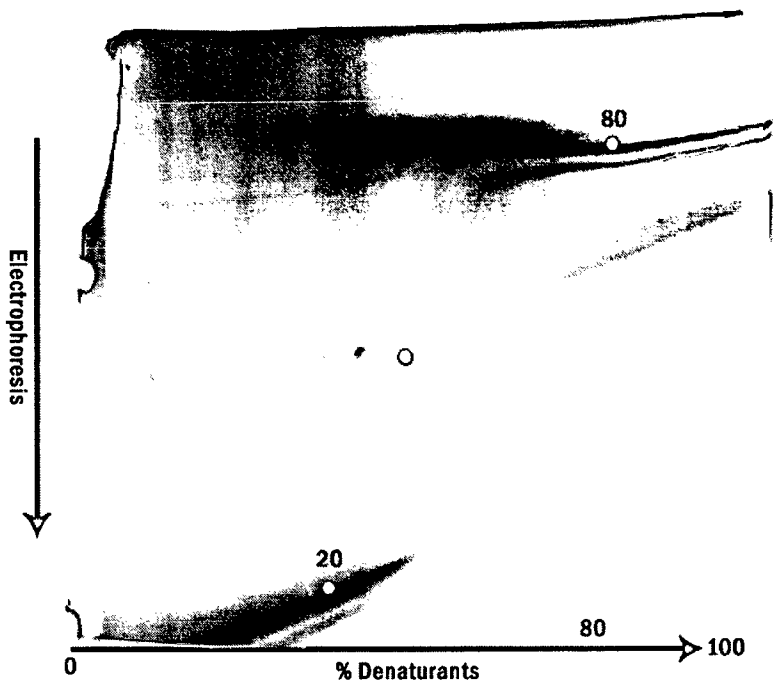


Fig. S4. Perpendicular gel image of 16S rRNA gene

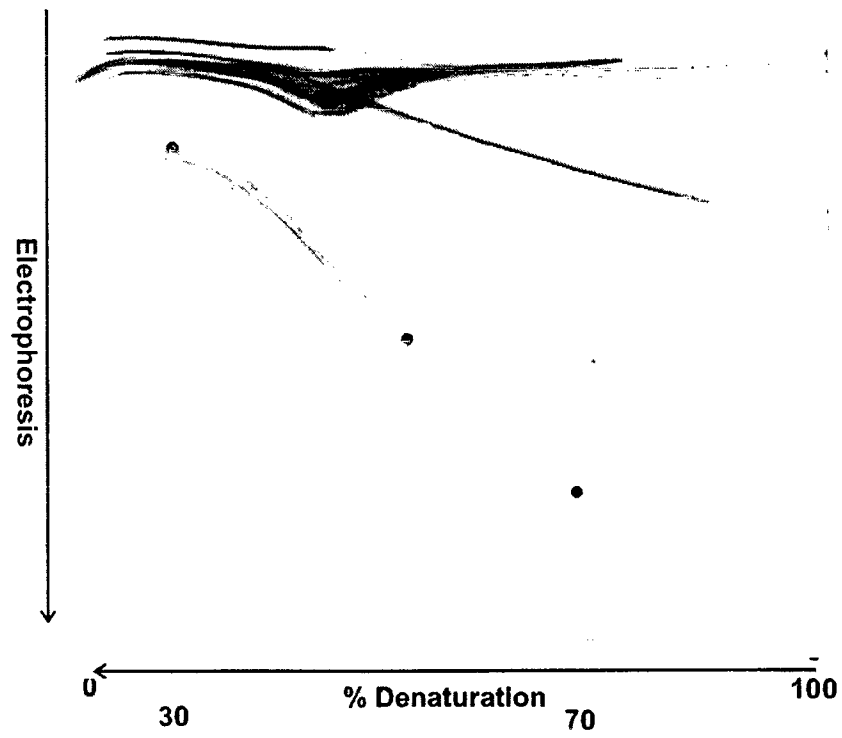


Fig. S5. Perpendicular gel image of *dsr*B gene

A11- Sequence data of the present study

1. dsrB gene sequences of DGGE analysis (dDSR) and length of sequence -bp in parenthesis

>dDSR 1 (341)
AACATCGTCCACACCCAGGGTTGGGTGCACTGCCACTCGTCGGCGTCGGACGCCTCCGGCGTTGTG
AAGGCTGTGATGGACGAGCTCTACCCTTATTTCACGGGTGAGAAGTCGCTGCCGGCAAAGATGCGG
GTGGCCTACGCGTGCTGCCTCAACATGTGCGGTGCGGTGCACTGCTCCGACATCGCGATTCTCGGC
GTGCACACCTCGGTGCCGCTCATCAACCACGCGGATCTCCCCAAGATGTGCGAGATCCCGACGTTG
GTGGCCTCGTGTCCGACGGGTGCGATCCGTCCGGCCACCGTCGACGGCCAGGCGTCCGGAGAAAAT
CGAGGCGCCCC

>dDSR 2 (344)
CTGGATCTACTGTCACACGCCGACCACCGACGCGTCTGGACCGGTAAAGGCCACCATGGATGTACT
TTTTGAAGATTTCACAGACATGAGGTTGCCGGCACATCTTCGCGTGTCACTGGCATGTTGTTTAAAT
ATGTGCGGTGCGGTGCATTGCTCGGATATCGCCATTTTAGGCTATCACCGCAAGGTTCCGATGCTG
GACCATGAATACCTGGATAAATTTTGCGAAATTCCGCTGGCTATCTCCGCCTGTCCGACATATGCCA
TCAAGCCGGCCAAAGTGACCCTGGAGGACGGCACCAAGGTCAACAGTGTCGCGCTGAACGTGCCC
CGATGTATGTTCT

>dDSR 4 (328)
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AAGGCGGTCCTGGACGATCTCTATGATGACTTCTGCTCCATGCGGATGCCGGCTCAGGTGCGAATT
GCCCTGGCCTGCTGTCTCAACATGTGCGGCGCTGTGCATTGCTCCGACATCGCCATCCTCGGGGTGC
ACCGCAAGCCGCCTTTCATCGAACACGAGCGTGTCAGCTCGGTCTGCGAGGTCCCGCTGGCCATCG
CCGCTTGCCCCACTGCGGCCATCAAGCCGGCCAAGGTGGATGACATGAAAAACCCCGTGGGCA

>dDSR 5 (323)
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AGCGCCCTATTTCTCGTCGAAGAGGCTGCCCGGCAAGACGCGCATCGCCATGGCCTGCTGCCTGAA
CATGTGCGGCGCCGTTCACTGCTCAGACATCGCGCTGGTGGGCGTGCACCGCAGGGTGCCGGTGAT
CGACCACGAACGTGTGTCCAAGGTTTGTGAGGTGCCGACCTTGGTCGCCTCCTGCCCCACGTTGGC
GATAAGGCCGAACCCCAAGGAGAAGAGCGTTACCATTACCGACGAGCGGTGCATGTACT

>dDSR 6 (332)
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CAACATGTGCGGCGCGGTCCACTGCTCCGACATTGCGATCCTCGGCGTTCACACCCGTGCGCCGGT
CATCAACCATGACGACCTGCCCAAAATGTGCGAGATCCCGACCCTGGTCGCCTCGTGCCCGACGGG
CGCCATTCGACCGGCGACGGTCGACGGCGTGCAGTCGGTGGAGATCGTCGACGAGCAGTGCATGT
ACT

>dDSR 7 (329)
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ATGTGTGGCGCCGTGCACTGCTCCGACATCGCCATCCTCGGGGTGCACCGCAAGCCGCCCTTCATC
GAGCACGAGCGGGTGAGCAAAGTCTGCGAGGTCCCGTTGGCCATCGCCGCTTGCCCCACCGCGGCC
ATCAAGCCGGCCAAGGTGGATGACATGAAGACCGTGGCGGTGGGAAACGAGCGCTGCATGTTCT

>dDSR 8 (328)
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CTATGACGACTTCTGTGGTATGCGGATGCCGGCTCAGGTACGAATTGCCCTGGCCTGCTGCCTCAA
CATGTGTGGCGCCGTGCACTGCTCCGACATTGCCATCCTCGGGGTGCACCGCAAGCCGCCTTTCATC
GAGCACGAGCGGGTCAGCTCGGTCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACTGCGGCC
ATCAAACCGGCCAAAGTGGATGACATGAAAACCGTGGCCGTGAAAAACGAACGCTGCATGTTC

>dDSR 9 (323)
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GGCGCCCTACTTCTCGTCGAAGAGGCTGCCCGGCAAGACGCGCATCGCCATGGCCTGCTGCCTGAA
CATGTGTGGTGCCGTTCACTGCTCAGACATCGCGCTGGTGGGCGTGCACCGCAAGGTGCCGGTGAT
CGACCATGAGCGAGTGGCCAAGCAATGTGAGGTGCCGACCTTGATCGCCTCCTGCCCCACGTTGGC
GATAAGGCCGAACCCCAAGGAGAAGAGCATTACCATTACCGACGAGCGGTGCATGTACT

>dDSR 10 (338)
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GCCCTGGCCTGCTGCCTCAACATGTGCGGCGCCGTGCACTGCTCCGACATCGCCATCCTCGGCGTG
CACCGCAAGCCGCCTTTCATTGAGCATGAGCGGGTGAGCAAGGTCTGCGAGGTTCCGCTCGCCATT
GCCGCTTGCCCCACCGCGGCCATCAAGCCGGCCAAGGTCGACGACATGAAAAGTTGGGCCCGGAA
AAACAAGGT

>dDSR 11 (341)
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ATTTGAATACTTCACCTCCATGACCCTGCCGGCTCAGGTACGTATTGCCCTGGCCAGCTGCCTGAAC
ATGTGCGGTGCGGTACATGCGTCCGATATCGCTCTGTTTGGTATCCATCGCAAGCCGCCGATGATC
GATCACAAGCGCATCACCGGTGTCTGCGAACTGCCGCTGGCAGTTGCTTCCTGTCCGCTCGGTGCG
GTCAAGCCCACCAAGGCGGTCGTAGACGGAGAAGAAATCAAGTCCGTAAAGGTCACGGTAGAACG
CTGTATGTTCT

>dDSR 12 (324)
CTGGGTGCACTGCCACAGCGCGGCCACCGACGCCTCGGGGCTGGTCAAGTCCATCATGGATGAGCT
GGTGCCCTACTTCTCGTCGAAGAGGCTGCCCGGCAAGACGCGCATCGCCATGGCCTGCTGCCTGAA
CATGTGTGGTGCCGTTCACTGCTCAGACATCGCGCTGGTGGGCGTGCACCGCAGGGTGCCGGTGAT
CGACCATGAGCGAGTGGCCAAGCAGTGTGAGGTGCCGACCTTGATCGCCTCCTGCCCCACGTTGGC
GATAAGGCCGAACCCCAAGGAGAAGAGCGTCACCATTACCGACGAGCGGTGCATGTACTG

>dDSR 13 (298)
AACATCGTCCACACCCAGGGATGGCTCCACTGCCACACCCCGGCCATCGATGCCTCCGGTGTCGTC
AAGGCCGTGATGGACGAGCTTTACGACTATTTCACCTCCATGACCCTGCCTGCCCAGGTGAGAATT
TCCCTGGCCTGCTGCCTGAACATGTGCGGCGCGGTGCACTGCTCGGACATCTCCATCCTCGGGATCC
ATCGTAAACCGCCTTTGATCGAGCACGATCGCGTAAGATGCGGCGTGCGAGGTTCCTCTGGCCATC
TCTGCCTTGCAACGCGAGGGCCCGGCGGAATTA

>dDSR 14 (340)
ATGGCTCCACTGCCACACCCCGGCCATCGACGCCTCCGGCGTCGTCAAGGCCGTGATGGACGAGCT
TTACGACTATTCCACCTCCATGACCCTGCCTGCACAGGTGAGAATTTCCCTGGCCTGCTGCCTGAAC
ATGTGCGGTGCGGTGCACTGCTCGGACATCTCCATCCTCGGGATCCATCGTAAACCGCCTTTGATCG
AGCACGATCGCGTGGATGCTGCGTGCGAGGTTCCTCTGGCCATCTCTGCCTGCCCCACGGCCGCGA
TCAAGCCGAAGAAGATCAAGGTGGGCGACAAAGAGGTGAAGAGCGTAGAGGTGAACGAGCCACG
GTGCATGTTC

2. Clone library analysis of dsrB gene sequences is referred as DSR and length of sequence -bp in parenthesis

>DSR3 (341)
CGGCCCCTCGTTCGGCGTTCGGACGCCTCCGGCGTTGTGAAGGCTGTGATGGACGAGCTCTACCCT
TATTTCACGGGTGAGAAGTCGCTGCCGGCAAAGATGCGGGTGGCCTACGCGTGCTGCCTCAACATG
TGCGGTGCGGTGCACTGCTCCGACATCGCGATTCTCGGCGTGCACACCTCGGTGCCGCTCATCAAC
CACGCGGATCTCCCCAAGATGTGCGAGATCCCGACGTTGGTGGCCTCGTGTCCGACGGGTGCGATC
CGTCCGGCCACCGTCGACGGCCAGGCGTCCGTAGAAATCGAGGACGCTCAGTGCATGTTCTGCGGT
AACTGCTACAC

>DSR4 (350)
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TGCGGTGCGGTCCACTGCTCGGATATTGCTATCCTCGGGTACCACCGCAAGCCGCCCATCCTGGAC
CATGAATATCTGGACAAGATGTGCGAGATTCCTCTTGCCATAGCGGCCTGTCCCACGGCAGCCATC
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CATGTACTGCGGTAACTGCTACAC

> DSR5 (341)
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CACGACGATCTTCCCAAGATGTGCGAGATCCCGACCCTCGTCGCCTCGTGCCCGACCGGCGCCATC
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TAACTGCTACAC

>DSR7 (329)
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GTTCCGACGTGGCCCTGCTGGGTTATCACAGAAAACCGCCGATCATTGACAATGAAGTCCTTGATC
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>DSR9 (326)
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TGCTCCGATATCGCCATCCTCGGGGTGCACCGCAAGCCGCCTTTAAAAGAGCACGGGCGTGTCAGC
TCGGTCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACTGCGGCCATCAAGCCGGCCAAGATG
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>DSR28 (335)
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TCCGACATCGCTATCCTGGGCTACCATCGCAAACCGCCCATGCTGGATCATGAATATCTGGACAAG
ATGTGCGAGATCCCGCTGGCCATTGCCGCCTGCCCGACGGCAGCGATTAAGCCGGCCAAGATTGGG
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TACAC

>DSR34 (323)
ATTGGCCCCCTCCGGGGTATCGTCAAGGCGGTCCTGGACGATCTCTATGATGACTTCTGCTCCATGC
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CCGACATCGCCATCCTCGGGGTGCACCGCAAGCCGCCTTTCATCGAACACGAGCGTGTCAGCTCGG
TCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACTGCGGCCATCAAGCCGGCCAAGGTGGATG
ACATGAAAACCGTGGCGGTGAGAAACGAGCGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR35 (321)
AACATCGTCCACACCCAGGGCTGGATTCACTGTCACACGCCGGCCACCGACGCGTCTGGACCGGTA
AAGGCCACCATGGATGTACTTTTTGAAGATTTCACAGACATGAGGTTGCCGGCACATGTTCGCGTG
TCACTGGCATGTTGTTTAAATATGTGCGGTGCGGTGCATTGCTCGGATATCGCCATTTTAGGCTATC
ACCGCAAGGTGCCGATGCTGGACCATGAATACCTGGATAAATTTTGCGAAATTCCGCTGGCTATCT
CCGCCTGTCCGACATATGCCATCAAGCCGGCCAAAGTGACCCTGGAGGACGGCACC

>DSR36 (329)
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GGCCTGCTGCTTGAATATGTGCGGCGCGGTGCACTGCTCGGATATTGCTATCCTGGGCTACCATCGC
AAGCCGCCCTTGATGGACCATGAATACCTGGACAAATTGTGCGAAATTCCGCTGGCCATCGCGTCC
TGTCCCACGGCAGCGCTCAAGCCGGGGAAAGTGGAACTGGAAGACGGCACCTCGGTTAAAAGC

>DSR38 (340)
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GCTGTTCACTATTCTGACATCGCGGTGGTTGCCGTGCACAGAAAAGTACCGACAGTAGACGAAGAA
AATGTAGGGAAAATGTGTGAAATTCCGACAACTGTTGCTTCAAGACCGACTTCGGCTATCAGACCG
AATCCAAAGACAAAATCAATTACTATTAATGATGAAAAGTGCATGTACTGCGGTAACTGCTACACA
CCACTACGCA

>DSR39 (319)
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CGGTTACCACCGCAAGCCGCCCATACTGGACCATGAATATCTGGACAAGATGTGCGAGGTGCCTCT
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>DSR42 (329)
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CACTGCTCCGACATCGCGGTGCTCGGTGTGCACCGCCGCGTACCGCTCATAAAGCACGAGGAGCTT
GCCAGGGCCTGCGAGGTGCCCACGACCGTGGCCTCCTGTCCAACCGGTGCCATCCGGCCGGCCACC
GTGGATGGCAAGCAGTCGGTGGAGGTGATCGAGGAGCAGTGTATGTTCTGCGGTAACTGCTACAC

>DSR43 (338)
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CGCTGTGCATTGCTCCGACATCGCCATCCTCGGCGTGCACCGCAAGCCGCCTTTCATCGAACACGA
GCGTGTCAGCTCGGTCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACTGCGGCCATCAAGCC
GGCCAAGGTGGATGACATGAAAACCGTGGCCGTGAGGAACGAGCGCTGCATGTTCTGCGGTAACT
GCTACAC

>DSR44 (321)
AACATCGTCCACACCCAGGGCTGGATCCACTGCCACACCCCGGCTATCGATGCCTCGGGTATCGTC
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GCTCTGGCCTGCTGTCTCAACATGTGCGGCGCTGTGCATTGCTCCGACATCGCCATCCTCGGCGTGC
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>DSR45 (290)
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GAAAAGTACCGACTTTAGACGAAGAAAATGTAGGGAAAATGTGTGAAATTCCGACAACTGTTGCT
TCATGTCCGACTTCGGCTATCAGACCGAATCCAAAGACAAAATCAATTACTATTAATGATGAAAAG
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>DSR46 (320)
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ATGCCGGCTCAGGTGCGAATTGCCCTGGCCTGCTGCCTCAACATGTGCGGCGCCGTGCACTGCTCC
GACATCGCCATCCTCGGCGTGCACCGCAAGCCGCCTTTCATTGAGCATGAGCGGGTGAGCAAGGTC
TGCGAGGTTCCGCTCGCCATTGCCGCTTGCCCCACCGCGGCCATCAAGCCGGCCAAGGTCGACGAC
ATGAAAAGTGTGGCCGTGAAGAACGAGCGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR47 (350)
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ATCATGAATATCTGGACAAGATGTGCGAGATCCCGTTGGCCATCGCCGCCTGTCCGACGGCAGCGA
TAAAGCCGGCCAAGATTGAGGTCGGAGGCGAAAAGGTCAAAAGTGTGGCTGTCAATGAGCCCCGC
TGCATGTTCTGCGGTAACTGCTACAC

>DSR48 (329)
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TCTTTGGCCTGCTGCTTGAATATGTGCGGCGCGGTGCACTGCTCGGATATTGCTATCCTGGGCTACC
ATCGCAAGCCGCCCTTGATGGACCATGAATACCTGGACAAATTGTGCGAAATTCCGCTGGCCATCG
CGTCCTGTCCCACGGCAGCGCTCAAGCCGGGGAAAGTGGAACTGGAAGACGGCACCTCGGTTAA

>DSR49 (314)
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CCATCCTCGGCGTGCACCGCAAGCCGCCTTTCATCGAACACGAGCGTGTCAGCTCGGTCTGCGAGG
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CTGTGGCGGTGAGAAACGAGCGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR51 (338)
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TGGAAGACGGCACCAAGGTTAAAAGTATTATCGTAAACGAGCCCAGATGTATGTTCTGCGGTAACT
GCTACAC

>DSR52 (341)
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GTGCGGTCCACTGCTCGGACATCGCCCTCCTGGGGTATCACCGCAAGCCGCCCATCATCGACAACG
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AACTGCTACAC

>DSR53 (320)
AACATCGTCCACACCCAGGGCTGGATCCACTGCCACACCCCGGCTATCGACGCCTCCGGTATCGTC
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GCCCTGGCCTGCTGCCTCAACATGTGTGGTGCCGTGCACTGCTCCGATATAGCCATCCTCGGGTGCA
CCGCAAGCCGCCTTTCATCGAACACGAGCGTGTCAGCTCGGTCTGCGAGGTCCCGCTGGCCATCGC
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>DSR54 (323)
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ACATGAAAACCGTGGCCGTGAGGAACGAGCGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR57 (344)
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ATGATGACTTCTGCTCCATGCGGATGCCGGCTCAGGTGCGAATCGCTCTGGCCTGCTGTCTCAACAT
GTGCGGCGCTGTGCATTGCTCCGACATCGCCATCCTCGGCGTGCACCGCAAGCCGCCTTTCATCGA
ACACGAGCGTGTTAGCTCGGTCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACTGCGGCCAT
CAAGCCGGCCAAGGTGGATGACATGAAAACCGTGGCCGTGAGGAACGAGCGCTGCATGTTCTGCG
GTAACTGCTACAC

>DSR59 (257)
CGGATGCCGGCTCAGGTGCGAATCGCCCTGGCCTGTTGTCTCAACATGTGCGGCGCCGTGCATTGC
TCCGACATCGCCATCCTCGGGGTGCACCGCAAGCCGCCTTTCATCGAACACGAGCGTGTCAGCTCG
GTCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACTGCGGCCATCAAGCCGGCCAAGATAGAT
GACATGAAGACCGTGGCGGTGAGAAACGAACGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR63 (328)
AATTTGGCCCCTCCCGGGTATCCGTCAAGGCTGTTCTGGATGATCTCTACGATGACTCCTGCACAAT
GCGCATGCCGGCTCAGGTGCGAATTGCCCTGGCCTGCTGTCTCAACATGTGCGGCGCGGTCCACTG
CTCGGATATCGCCATGCTTGGCTATCACCGGAAACCGCCCATTATTGACCATGAGGTTCTCGACAA
CGTCTGTGAGATCCCATTGGTTATTACGGCCTGCCCCACTGCGGCCATTTCGCCTTCCAAGACCGAA
GACGGCAAAAAGACGGTCAAGGTCAAGAATGAACGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR64 (314)
TGGGGATATTCGGTCAAGGCGGTCCTGGACGATCTCTATGATGACTTCCCGCTCCATGCGGATGCC
GGCTCAGGTGCGAATCGCTCTGGCCAGCTGTCTCAACATGTGCGGCGCTGTGCATTGCTCCGACAT
CGCCATCCTCGGCGTGCACCGCAAGCCGCCTTTCATCGAACACGAGCGTGTCAGCTCGGTCTGCGA
GGTCCCGCTGGCCATCGCCGCTTGCCCCACTGCGGCCATCAGCCGGCCAAGGTGGATGACATGAAA
ACCGTGGCCGTGAGGAACGAGCGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR65 (326)
GCCCATTCGATGGCCTCCGGTATCGTCAAGGCTGTTCTGGATGATCTCTACGATGACTTCCGTGGTA
TGCGGATGCCGGCTCAGGTGCGAATCGCCCTGGCCTGCTGTCTCAACATGTGCGGCGCCGTGCATT
GCTCCGACATCGCCATCCTCGGGGTGCACCGCAAGCCGCCTTTCATCGAACACGAGCGTGTCAGCT
CGGTCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACTGCGGCCATCAAGCCGGCCAAGATAG
ATGACATGAAGACCGTGGCGGTGAGAAACGAACGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR68 (338)
GCACCGGCCCGGGCCACGGACGCCTCGGCTATGGTGAAAGCGGTGATGGACGAAGTTTTCGAACA
TTTTCTGGGGAAGACCTTCCCCGCCCAGGTCAGGATCTCGATGGCCTGCTGTCTAAACATGTGTGG
GGCTGTCCACTGCTCGGATATCGCCCTCTTGGGCTATCATCGAAAACCCCCCATCATTGACCATGAG
GTATTAGACAATGTGTGTGAGATTCCTCTGGTCATTGCTGCCTGCCCGACCGCGGCTATTTCGCCCT
CCAAGACTGAGGATGGGAAAAGGACCGTCAAGATCAGAGAGGAGCGCTGCATGTTCTGCGGTAAC
TGCTACAC

>DSR69 (347)
ACATCGTCCACACCCAGGGCTGTATCCATTGCCACACGCCGGCAACGGACGCCTCGGCTATGGTGA
AAGCGGTGATGGACGAAGTTTTCGAACATTTTCAGGGGATGACCTTCCCCGCCCAGGTCAGGATCT
CGATGGCCTGCTGTCTAAACATGTGTGGGGCTGTCCACTGCTCGGATATCGCCCTCTTGGGCTATCA
TCGAAAACCCCCCCATCATTGACCATGAGGTATTAGACAATGTGTGTGAGATTCCTCTGGTCATTGCT
GCCTGCCCGACCGCGGCTATTTCGCCCTCCAAGACTGAGGATGGGAAAAGGACCGTCAAGATCAG
AGAGGAGCGCTGCATG

>DSR70 (347)
ACATCGTCCACACCCAGGGGTACATCCATTGCCATACCCCGGCAACCGATGCCTCGGCTATGGTGA
AAGCGGTCATGGACGAGCTTTTCGATTATTTCGGACAGATGACCTTTCCCGCCCAGGTGAGGATAT
CCATGGCCTGCTGTCTGAATATGTGCGGTGCGGTCCACTGTTCCGACATCGCCCTCCTGGGTTATCA
CAGAAAACCGCCCATCGTTGACAATGATGTGCTGGCGAACGTGTGTGAAATTCCTCTGGTCGTTGC
GGCTTGCCCGACGGCGGCTATTTCTCCGACCAAGACAGAAGAAGGCAAAAAAAGCGTAAAGATTA
AACAGGACCGCTGCATG

>DSR71 (341)
GGGCCCCCCGACGCGTCTGGACCGGTAAAAGCCACCATGGATGCATTGTTTGATGACTTTACTGAC
ATGCGGCTGCCGGCACATCTTCGCGTGTCACTGGCATGTTGTTTAAATATGTGCGGTGCGGTGCATT
GCTCGGATATCGCCATTTTAGGCTATCACCGCAAGGTTCCCATGCTGGACCATGAATACCTGGATA
AATTTTGCGAAATTCCGCTGGCTATCTCCGCCTGTCCGACATATGCCATCAAACCGGCCAAAGTGA
CCTTGGAGGATGGCACCAAGGTCAAGAGTGTCGCGCTGAACGTGCCCCGATGTATGTTCTGCGGTA
ACTGCTACAC

>DSR72 (323)
GGGTGGTTCGTCAAGGCCGTGATGGACGAGCTTTACGACTATTTCCCCTCTATGACCCTGCCTGCCC
AGGTGAGAATTTCCCTGGCCTGCTGCCTGAACATGTGCGGCGCGGTGCACTGCTCGGACATCTCCA
TCCTCGGGATCCATCGTAAACCGCCTTTGATCGAGCACGATCGCGTGGATGCGGCGTGCGAGGTTC
CTCTGGCCATCTCTGCCTGCCCCACGGCCGCGATCAAGCCGAAGAAGATCAAGGTGGGCGACAAA
GAGGTGAAGAGCGTAGAGGTGAACGAGCCGCGGTGCATGTTCTGCGGTAACTGTTACAC

>DSR73 (314)
GGGGTTATTCCGTCAAGGCTGTTCTGGATGATCTCTACGATGACTTTTCGTGATATGCGGATGCCGG
CTCAGGTGCGAATCGCCTTGGCCTGCTGTCTCAACATGTGCGGCGCCGTGCACTGCTCCGACATCG
CCATCCTCGGGGTGCACCGCAAGCCGCCCCTCATCGAGCACGAGCGGGTGAGCAAAGTCTGCGAG
GTCCCGTTGGCCATCGCCGCTTGCCACACCGCGGCCATCAAGCCGGCCAAGGTGGATGACATGAAA
ACCGTGGCGGTGAGAAACGAGCGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR75 (340)
ATCGTCCACACCCAGGGATGGGTGCACTGCCACAGTGCATGTACAGATGCTTCAGGCATAGTGAAG
GCTGTCATGGATGAACTCTATGACTACTTCACAACAAAAGAACTGCCTAACAAAGTGAGACTTGCA
GTTGCGTGCTGTGTAAACATGTGCGGTGCTGTTCACTATTCTGACATCGCGGTGGTTGCCGTGCACA
GAAAAGTACCGACAGTAGACGAAGAAAATGTAGGGAAAATGTGTGAAATTCCGACAACTGTTGCT
TCACGACCGACTTCGGCTATCAGACCGAATCCAAAGACAAAATCAATTACTATTAATGATGAAAAG
TGCATGTACT

>DSR77 (345)
TTGGCCCCTCCGGGTATCGTCAAGGCGGTCCTGGACGATCTCTATGATGACTTCTGCTCCATGCGGA
TGCCGGCTCAGGTGCGAATCGCTCTGGCCTGCTGTCTCAACATGTGCGGCGCTGTGCATTGCTCCGA
CATCGCCATCCTCGGCGTGCACCGCAAGCCGCCTTTCATCGAACACGAGCGTGTCAGCTCGGTCTG
CGAAATTCCGCTGGCTATCTCCGCCTGTCCGACATATGCCATCAAACCGGCCAAAGTGACCTTGGA

GGATGGCACCAAGGTCAAGAGTGTCGCGCTGAACGTGCCCCGATGTATGTTCTGCGGTAACTGCTA
CACAAGGGCGAAT

>DSR79 (326)
GATGGGCCCCTCGGGGCTATGGTGAAAGCGGTCATGGACGAGCTTTTCGATTATTTCGGACAGATG
ACCTTTCCCGCCCAGGTGAGGATATCCATGGCCTGCTGTCTGAATATGTGCGGTGCGGTCCACTGTT
CCGACATCGCCCTCCTGGGTTATCACAGAAAACCGCCCATCGTTGACAATGATGTGCTGGCGAACG
TGTGTGAAATTCCTCTGGTCGTTGCGGCTTGCCCGACGGCGGCTATTTCTCCGACCAAGACAGAAG
AAGGCAAAAAAAGCGTAAAGATTAAACAGGACCGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR81 (326)
AGAAATTGCCCCTTCGGGGTATCGTCAAGGCGGTCCTGGACGATCTCTATGATGACTTCCCGCTTTA
TGCGGATGCCGGCTCAGGTGCGAATCGCTCTGGCCTGCTGTCTCAACATGTGCGGCGCTGTGCATT
GCTCCGACATCGCCATCCTCGGCGTGCACCGCAAGCCGCCTTTCATCGAACACGAGCGTGTCAGCT
CGGTCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACTGCGGCCATCAAGCCGGCCAAGGTGG
ATGACATGAAAACCGTGGCCGTGAGGAACGAGCGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR82 (323)
GAAATGGCCCCTTCGGGTATCGTCAAGGCGGTCCTGGACGATCTCTATGATGACTTCTGCTCCATGC
GGATGCCGGCTCAGGTGCGAATCGCTCTGGCCTGCTGTCTCAACATGTGCGGCGCTGTGCATTGCT
CCGACATCGCCATCCTCGGCGTGCACCGCAAGCCGCCTTTCATCGAACACGAGCGTGTCAGCTCGG
TCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACTGCGGCCATCAAGCCGGCCAAGGTGGATG
ACATGAAAACCGTGGCCGTGAGGAACGAGCGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR83 (319)
CGTCCACACCCAGGGATGGCTCCACTGCCACACCCCGGCCATCGATGCCTCCGGTGTCGTCAAGGC
CGTGATGGACGAGCTTTACGACTATTTCACCTCCATGACCCTGCCTGCCCAGGTGAGAATTTCCCTG
GCCTGCTGCCTGAACATGTGCGGCGCGGTGCACTGCTCGGACATCTCCATCCTCGGGATCCATCGT
AAACCGCCTTTGATCGAGCACGATCGCGTGGATGCGGCGTGCGAGGTTCCTCTGGCCATCTCTGCC
TGCCCCACGGCCGCGATCAAGCCGAAGAAGATCAAGGTGGGCGACAAAGAGGTG

>DSR84 (300)
AAATTGAGGCCCTGCCCCTCGTTTTGCAAGGCGGCGATGGCCAGCGGAATTTCGCACATCTTCGTC
CAGATATTCATGGTCCAGCAGCGGCGGTTTGCGGTGATAGCCCAAAATGGCGATATCCGAGCAGTG
AACCGCACCGCACATGTTCTGGCAGCAGGCCAGAGAGACCCGCAGATGGGCCCGCAGTCGATGAT
CTTTGAAATCATCAAAGAGTACGTCCATGGTGGCCTTGACCGGACCGGATGCATCCGTGGCCGGAG
TGTGGCAGTGAATCCAGCCCTGGGTGTGGACGATGTT

>DSR85 (322)
CAACATCGTCCAACCCAGGGCTGGATCCACTGCCACACCCCGGCTATCGATGCCTCGGGTATCGTC
AAGGCGGTCCTGGACGATCTCTATGATGACTTCTGCTCCATGCGGATGCCGGCTCAGGTGCGAATC
GCTCTGGCCTGCTGTCTCAACATGTGCGGCACTGTGCATTGCTCCGACATCGCCATCCTCGGCGTGC
ACCGCAAGCCGCCTTTCATCGAACGACGAGCGTGTCAGCTCAGTCTGCGAGGTCCCGCTGGCCATC
GCCGCTTGCCCCACCGCGGCCATCAAGCCGGCCAAGGTCGATGACATGAAGACCGTG

>DSR86 (212)
ATGTGCGGCGCCGTGCATTGCTCCGACATCGCCATCCTCGGGGTGCACCGCAAGCCGCCTTTCATC
GAACACGAGCGTGTCAGCTCGGTCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACTGCGGCC
ATCAAGCCGGCCAAGATAGATGACATGAAGACCGTGGCGGTGAGAAACGAACGCTGCATGTTCTG
CGGTAACTGCTACAC

>DSR89 (314)
GGGGGGTATCGGTCAAAGGCGGTCCTGGACGATCTCTATGATGACTTCTGCTCAATGCGGATGCCG
GCTCAGGTGCGAATCGCTCTGGCCTGCTGTCTCAACATGTGCGGCACTGTGCATTGCTCCGACATCG
CCATCCTCGGCGTGCACCGCAAGCCGCCTTTCATCGAACACGAGCGTGTCAGCTCAGTCTGCGAGG
TCCCGCTGGCCATCGCCGCTTGCCCCACCGCGGCCATCAAGCCGGCCAAGGTCGATGACATGAAGA
CCGTGGCGGTGAAAAACGAGCGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR90 (329)
GTCCATTTCGGATTGCCTCCCGGTATCGTCAAGGCTGTTCTGGATGATCTCTACGATGACTTCTGTT
CCATGCGGATGCCGGCTCAGGTGCGAATTGCCCTGGCCTGCTGTCTCGACATGTGCGGCGCCGTGC
ATTGCTCCGACATCGTCATCCTCGGGGTGCACCGCAAGCCGCCTTTCATCGAACACGAGCGTGTCA
GCTCGGTCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACTGCGGCCATCAAGCCGGCCAAGG
TGGATGACATGAAAACCGTGGCGGTGAGAAACGAACGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR91 (326)
GGGCCCCCGGGTTCAAAGGCCGTGATGGACGAGATGTTCGATCATTTTCCTATCCACAGCATGCCG
GCCAAGGTGCGGATCTCCCTGGCTTGCTGCCTGAACATGTGCGGAGCGGTTCACTGCTCGGATATC
GCCATTCTGGGATACCATCGCAAACCACCGATGCTCGATCACGAGTATCTGGATAAAATGTGCGAG
ATTCCGCTGGCCATCGCCGCCTGCCCGACGGCTGCCATCAAACCGTCGAAGGTCGAGTTCGAGGGT
GCGAAGGTCAATTCGGTGGCGGTGAAAAACGAGCGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR92 (340)
ACATCGTCCACACCCAGGGCTGGATCCACTGCCACACCCCGGCTATCGACGCCTCCGGTATCGTCA
AGGCTGTCCTGGACGACCTCTACGACGACTTCTGTGGTATGCGGATGCCGGCTCAGGTGCGAATTG
CCCTGGCCTGCTGCCTCAACATGTGTGGTGCCGTGCACTGCTCCGATATAGCCATCCTCGGGTGCAC
CGCAAGCCGCCTTTCATCGAACACGAGCGTGTCAGCTCGGTCTGCGAGGTCCCGCTGGCCATCGCC
GCTTGCCCCACTGCGGCCATCAAGCCGGCCAAGATAGATGACATGAAGACCGTGGCGGTGAGAAA
CGAACGCTGC

>DSR94 (308)
GGGGGAATGGATCCTTGTTTGACTATTTCCATTGGCAAGACACTACCCGCCCAGGTCAGGGTCTCC
ATGGCCTGCTGCCTGAACATGTGCGGTGCGGTCCACTGCTCTGATATCGCCATCCTGGGATATCACC
GCAAACCGCCCATGCTGGACCATGAATATCTGGACAAGATGTGCGAGATTCCTCTCGCCGTAGCGG
CCTGTCCTACGGCAGCGATCAAGCCGGCCAAGGTGGAGGTTGGCGGTGAAAAGCTCAATAGTGTG
GCGGTGAAAAATGAACGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR95 (327)
ACATCGTCCACACCCAGGGATGGATCCACTGCCACACACCGGCAACGGATGCATCCGGTCCGGTTA
AGGCCACCATGGATGTGCTTTTTGACGATTTTAAAGATCACCGACTGCCGGCCCATCTGCGCGTCTC
CTTGGCATGCTGTCWGGACATGTGCGGTGCGGTTCATTGCTCGGATATCGCCATTCTGGGATATCA
CCGCAAGCCGCCGCTGCTGGACCACGAATATCTGGACAAGATGTGCGAGGTTCCGCTGGCGATTGC
AGCCTGCCCGACGGCGGCCATCAAACCGGCTAAAGTTGAGTTTGAAGGACAGAAGGTCAACA

>DSR97 (342)
AGGTTCTTTTTTTTACCATATCAAACCGGCCACGATGCCTCCGGGCCCGTCAAGGCCACCATGGAC
GTGCTCTTTGATTACTTCAAGGAGCACAAACTGCCGGCCAAACTGCGTGTGTCTCTGGCCTGCTGCC
TGAATATGTGGGGCGCGGTGCATTGTTCCGACATCGCCATTTTGGGATACCACCGCAAACCGCCGT
TACTGGACCACGAATATCTGGATAAGATGTGCGAGATCCCGCTGGCCATCGCGGCCTGCCCGACGG
CCGCCATTAAACCGGCCAAGGTAGAGTTCGAAGGCGAAAAGGTCAACAGTGTGGCGGTCAATGAG
CCCCGCTGTATGTTCTGCGGTAACTGCTACAC

>DSR99 (349)
CATTGGGGCTTGTTCCACGCCGGCCCCGACGCGTCTGGACCGGTAAAAGCCACCATGGATGCATTG
TTTGATGACTTTACCGACATGCGGCTGCCGGCACATCTTCGCGTGTCACTGGCATGTTGTTTAAATA
TGTGCGGTGCGGTGCATTGCTCGGATATCGCCATTTTAGGCTATCACCGCAAGGTTCCCATGCTGGA
CCATGAATACCTGGATAAATTTTGCGAAATTCCGCTGGCTATCTCCGCCTGTCCGACATATGCCATC
AAACCGGCCAAAGTGACCTTGGAGGATGGCACCAAGGTCAAGAGTGTCGCGTTGAACGTGCCCCG
ATGTATGTTCTGCGGTAACTGCTACAC

>DSR100 (350)
GCCTTATTTGCTTCCGAAGTCCACCCCGGCATCGATGCCTCCGGTATCGTCAAGGCTGTTCTGGATG
ATCTCTACGATGACTTCTGTGGTATGCGGATGCCGGCTCAGGCGCGAATCGCCCTGGCCTGCTGTCT
CAACATGTGCGGCGCCGTGCGTTGCTCCGACATCGCCATCCTCGGGGTGCACCGCAAGCCGCCTTT
CATCGAACACGAGCGTGTCAGCTCGGTCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACTGC
GGCCATCAAGCCGGCCAAGATAGATGACATGAAGACCGTGGCGGTGAGAAACGAACGCTGCATGT
TCTGCGGTAACTGCTACAC

>DSR101 (338)
TCGTCCACACCCAGGGCTGGATCCACTGCCACACCCCGGCCATCGATGCCTCCGGTATCGTCAAGG
CTGATCTGGATGATCTCTATGATGACTTCTGCTCCATGCGGATGCCGGCTCAGGTGCGAATCGCTCT
GGCCTGCTGTCTCAACATGTGCGGCGCTGTGCATTGCTCCGACATCGCCATCCTCGGCGTGCACCGC
AAGCCGCCTTTCATCGAACACGAGCGTGTCAGCTCGGTCTGCGAGGTCCCGCTGGCCATCGCCGCT
TGCCCCACTGCGGCCATCAAGCCGGCCAAGGTGGATGACATGAAAACCGTGGCCGTGAGGAACGA
GCGCTGC

>DSR104 (341)
CATAATGTAAAACCCCGGGCCATCGATGCCTCCGGTATCGTCAAGGCTGTTCTGGATGATCTCTAC
GATGACTTCTGCTCCATGCGCATGCCGGCTCAGGTGCGAATTGCCCTGGCCTGCTGTCTCAACATGT
GCGGCGCCGTGCATTGCTCCGACATCGCCATCCTCGGGGTGCACCGCAAGCCGCCTTTCATCGAAC

ACGAGCGTGTCAGCTCGGTCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACTGCGGCCATCA
AGCCGGCCAAGATAGATGACATGAAGACCGTGGCGGTGAAAAACGAGCGCTGCATGTTCTGCGGT
AACTGCTACAC

>DSR105 (353)
GGGGCTTGGCCACCCCCGGCGACCGATGCCTCAGGGCCCGTGAAAGCAACCATGGATGTCCTCTTT
GATGACTTTACCAACATGAGGCTTCCGGCACAGGTCAGAGTGGCGCTCGCCTGCTGTCTGAACGTG
TGCGGTGCGGTCCACTGCTCGGATATTGCTATCCTCGGGTACCACCGCAAGCCGCCCTTGCTGGAC
CATGAATATCTGGACAAGATGTGCGAGATTCCTCTTGCCATAGCGGCCTGTCCTACGGCAGCCATC
AAGCCGGGAAAGGTGGAAGTCGGAGGCGAGAAGATAAAGAGTGTATCTGTGAACAATGATCGGTG
CATGTACTGCGGTAACTGCTACAC

>DSR106 (352)
CAGCTCTGGAAACGAGTCGTGCGGTCACCCGGCACGACGCTTCCTCATGGTCAAGCGCTCATGGAT
GATCTGTTTGACTATTTCCAGGGCATGACACTACCCGCCCAGGTCAGGGTCTCCATGGCCTGCTGCC
TGAATATGTGCGGTGCGGTCCACTGCTCTGATGTCGCCCTTCTGGGATATCACAGGAAACCACCGA
TCGTTGACAGTGAGGTCCTGGAGAACGTATGTGAGATCCCGCTGGTAATCGCGGCATGTCCCACAG
CGGCCATCTCCCCCACAAAGACGGAAGATGGAAAGAAGGCAGTAAAGATCAAGGATGAGCGCTGC
ATGTTCTGCGGTAACTGCTACA

>DSR109 (341)
TCGTCCACACCCAGGGTTTCATCCACTGTCATACCCCTGCAACCGATGCGTCCAGTATGGTAAAGG
CCACCATGGACGTGCTTTTTGATGATTTTAAAAAGATGCGTTTGCCGGCGCAGATGCGCGTCTCTTT
GGCCTGCTGCTTGAATATGTGCGGCGCGGTGCACTGCTCGGATATTGCTATCCTGGGCTACCATCGC
AAGCCGCCCTTGATGGACCATGAATACCTGGACAAATTGTGCGAAATTCCGCTGGCCATCGCGTCC
TGTCCCACGGCAGCGCTCAAGCCGGGGAAAGTGGAACTGGAAGACGGCACCTCGGTTAAAAGCAT
TATCGTCAAC

>DSR112 (335)
CCAAACCCCCGGCCAATCGATGCCTCCGGTATCGTCAAGGCTGTTCTGGATGATCTCTACGATGAC
TTCTGCTCCATGCGCATGCCGGCTCAGGTGCGAATTGCCCTGGTCTGCTGTCTCAACATGTGCGGCG
CCGTGCATTGCTCCGACATCGCCATCCTCGGGGTGCACCGCAAGCCGCCTTTCATCGAACACGAGC
GTGTCAGCTCGGTCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACTGCGGCCATCAAGCCGG
CCAAGATAGGTGACATGAAGACCGTGGCGGTGAAAAACGAGCGCTGCATGTTCTGCGGTAACTGC
TACAC

>DSR113 (341)
CACGGCACCCCCCCCGGGCCCATCGATGCCTCGGGTATCGTCAAGGCGGTCCTGGACGATCTCTAT
GATGACTTCTGCACCATGCGGATGCCGGCTCAGGTGCGAATTGCCCTGGCCTGCTGTCTCAACGTG
TGCGGCGCTGTGCATTGCTCCGACATCGCCATCCTCGGGGTGCACCGCAAGCCGCCTTTTATCGAA
CACGAGCGTGTCAGCTCGGTCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACTGCGGCCATC
AAGCCGGCCAAGGTGGATGACATGAAAACCGTGGCGGTGAGAAACGAGCGCTGCATGTTCTGCGG
TAACTGCTACAC

>DSR114 (318)
GCGTCCACACCCAGGGCTGGCTTCACTGTCACATATCGGCCGCCGATGCATCCGGTCCGGTGAAAG
CCACCATGGACGTGCTCTTCGATTATTTTAAAGAACACAAAATGCCGGCCAAACTGCGCGTATCTC
TGGCCTGTTGCCTGAACATGTGTGGCGCGGTGCATTGCTCTGACATCGCGATCCTGGGCTACCATCG
TAAACCGCCGATGCTGGACCGCGAATATCTGGACAAGATGTGCGAGATTCCGCTGCCATCTCCGCC
TGCCCGACGGCAGCTATTAAGCCGGCCAAGGTTGAGGTCGGAGGCGAAAAGGT

>DSR115 (341)
CGTGAGGTGCCCCCCCCGGCCTTCGATGCCTCCGGTATCGTCAAGGCTGTTCCGGATGATCTCTACG
ATGACTTCTGCTCCATGCGCATGCCGGCTCAGGTGCGAATTGCCCTGGCCTGCTGTCTCAACATGTG
CGGCGCCGTGCATTGCTCCGACATCGCCATCCTCGGGGTGCACCGCAAGCCGCCTTTCATCGAACA
CGAGCGTGTCAGCTCGGTCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACTGCGGCCATCAA
GCCGGCCAAGATAGATGACATGAAGACCGTGGCGGTGAAAAACGAGCGCTGCATGTTCTGCGGTA
ACTGCTACAC

>DSR116 (341)
ATTTTCAACACCCCCGGGCCTATCGATGCCTCGGGTATCGTCAAGGCGGTCCTGGACGATCTCTATG
ATGACTTCTGCTCCATGCGGATGCCGGCTCAGGTGCGAATCGCTCTGGCCTGCTGTCTCAACATGTG
CGGCGCTGTGCATTGCTCCGACATCGCCATCCTCGGCGTGCACCGCAAGCCGCCTTTCATCGAACA
CGAGCGTGTCAGCTCGGTCTGCGAGGTCCCGCTGGCCATCGCCGCTTGCCCCACCGCGGCCATCAA
ACCGGCCAAGGTGGATGGCATGAAAACTGTGGCGGTGAGAAACGAGCGCTGCATGTTCTGCGGTA
ACTGCTACAC

>DSR117 (345)
CGGATTGTTTTTCGGGCCGGGTTGCCACCCGGCACCGACGCTTCCGGCCCGGTCAAGGCCGTGATG
GACGAGATGTTCGATCATTTTCAGAGCCACAGCATGCCGGCCAAGGTGCGGATCTCCCTGGCTTGC
TGCCTGAACATGTGCGGAGCGGTTCACTGCTCGGATATCGCCATTCTGGGATACCATCGCAAACCA
CCGATGCTCGATCACGAGTATCTGGATAAAATGTGCGAGATTCCGCTGGCCATCGCCGCCTGCCCG
ACGGCTGCCATCAAACCGTCGAAGGTCGAGTTCGAGGGTGCGAAGGTCAATTCGGTGGCGGTGAA
AAACGAGCGCTGCATGTTCTGCGGTAACTGCTACAC

>DSR118 (344)
TTCCCTTTTTCACTCCGCCCGCGAACGGACGCATCCGGCGTTGTAAAGGCAGTAATGGACGACCTC
TGCGACTACTTCACCGGGATGGAAATACCTGGGAAACTGAGGATCGCCCTGGCGTGCTGCCTCAAC
ATGTGCGGCGCGGTCCACTGTTCAGATATCGCGATCCTCGGCATTCACCGCAGACCGCCGAAGATC
CTACACGACAACCTCAACAAGATCTGCGAGATCCCCAACGTCGTGGCTTCCTGCCCGACGGCGGCG
ATCCGGCCAGCGACGGTGGAAGGAATGACTTCCGTCGAGCTGGATGAGGAACTGTGCATGTTCTGC
GGTAACTGCTACAC

>DSR119 (341)
GATATAGTCCCAACCCCGGCCATCGACGCCTCGGGTATCGTCAAGGCGGTCCTGGACGATCTCTAT
GATGACTTCTGCTCCATGCGGATGCCGGCTCAGGTGCGAATTGCCCTGGCCTGCTGTCTCAACATGT
GCGGCGCTGTGCATTGCTCCGACATCACCATCCTCGGCGTGCACCGCAAGCCGCCTTTCATCGAAC
ATGAGCGTGTCAGCTCGGTCTGCGAGGTCCCGCTGGCCATCGCCGCTTACCCCACTGCGGCCATCA
AGCCGGCCAAGGTGGATGACATGAAAACCGTGGCCGTGAGGAACGAGCGCTGCATGTTCTGCGGT
AACTGCTACAC

>DSR121 (348)
ATTTCCCGGGCCCGGGCCACCGACGCGTCTAGACCGGTAAAAGCCACCATGGATGCATTGTTTGAT
GACTTTCCCGACATGCGGCTGCCGGCACATCTTCGCGTGTCACTGGCATGTTGTTTAAATATGTGCG
GCGCGGTGCATTGCTCGGATATCGCCATTTTAGGCTATCACCGCAAGGTTCCCATGCTGGACCATG
AATACCTGGATAAATTTTGCGAAATTCCGCTGGCTATCTCCGCCTGTCCGACATATGCCATCAAACC
GGCCAAAGTGTCCTTGGAGGATGGCACCAAGGTCAAGAGTGTCGCGCTGAACGTGCCCCGATGTAT
GTTCTGCGGTAACTGCTACAC

>DSR124 (332)
ATCGTCCACACCCAGGGCTGGATCCACTGCCACACCCCGGCTATCGACGCCTCCGGTATCGTCAAG
GCTGTCCTGGACGACCTCTATGACGACTTCTGTGGTATGCGGATGCCGGCTCAGGTGCGAATTGCC
CTGGCCTGCTGCCTCAACATGTGTGGTGCCGTGCACTGCTCCGATATAGCCATCCTCGGGTGCACCG
CAAGCCGCCTTTCATCGAACACGAGCGTGTCAGCTCGGTCTGCGAGGTCCCGCTGGCCATCGCCGC
TTGCCCCACTGCGGCCATCAAGCCGGCCAAGACAGATGACACGAAGACCGTGGCGGCGAGAAACG
AA

3. 16S rRNA sequences of DGGE analysis (Sequence length bp in brackets)

GU968631
>EB7 (134)
CAGGGAATCTTGTCCAATGGGCGAAAGCCTGAGACAGCGACGCCGCGTGAGGGATGAAGGCCTTCGGG
TTGTAAACCTCTTTCAGGAGGGACGAAATTGACGGTACCTCCAGAAGAAGCCCCGGCCAACTACGT

GU968619
>EB17 (135)
CAAGGAATATTGTGCAATGGGCGAAAGCCTGACACAGCGACGCCGCTTGAGGGAAGAAGGCTTTCGGG
TTGTAAACCTCTTTTCTCAGGGAAGATAATGACGGTACCTGAGGAATAAGTCTCGGCTAACTACGTG

GU968620
>EB3 (155)
TGAGGAATATTGGGCAATGGACGCAAGTCTGACCCAGCCATGCCACGTGCAGGATGACGGCGCTATGC
GTTGTAAACTGCTTTTATACAGGAAGAAACCCCCGAACGTGTTCGGGGTTGACGGTACTGTAAGAATA
AGCATCGGCTAACTCCGTG

GU968621
>EB14 (160)
TGGGGAATTTTGCGCAGTGGGCGAAAGCCTGACGCAGCGACGCCGCGTGTGCGATGAAGGCCTTTGGG
TCGTAAAGCACTGTCGAGAGGGACGAAAACCCATCGGGGATAATACGGTGGGCTTGACGGTACCTCTA
AAGGAAGCACCGGCTAACTCTGTG

GU968622
>EB5 (135)
TGGGGAATCTTGGACAATGGGGGCAACCCTGATCCAGCCATGCCGCGTGAACGATGAAGGCCTTAGGG
TTGTAAAGTTCTTTCAGCAGGGAGGATAATGACGGTACCTGCAGAAGAAGCCCCGGCTAACTCCGTG

GU968623
>EB21
CAGGGAGTATTGCACAATGGGCGCAGGCCTGATGCAGCGACGCCGCGTGAGGGATGAAGGCTTTCGGG
TTGTAAACCTCTTTCAGTGGGGAAGAAGCGCAAGTGACGGTACCCACACAAGAAGGCCCGGCCAACTA
CGTG

GU991352
>EB6 (140)
TGAGGAATTTTGCGCAATGGGGGAAACCCTGACGCAGCAACGCCGCGTGGGTGAAGAAGGCTTTCGGG
TCGTAAAGCCCTGTCAGGTGGGAAGAACCGTTTCGATACTAATAATGTCGAAGCCTGACGGTACCACC
AGAGGAAGCACCGGCCAACTCCGTG

GU968624
>EB13 (135)
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GU968625
>EB18 (160)
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GAAGAAGCACCGGCTAACTCTGTG

GU968626
>EB 8 (160)
TGAGGAATATTGGGCAATGGGCGAAAGCCTGACCCAGCCACGCCGCGTGTGTGATGAAGGCCTTCGGG
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TAGCAAGCACCGGCTAACTCTGTG

GU968627
>EB10 (161)
TGAGGAATTTTGCGCAATGGGGGAAACCCTGACGCAGCGACGCCGCGTGGAGGACGAAGGCCTTCAGG
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GAAGGAAGCCCCGGCTAACTCCGTG

GU968628
>EB19 (160)
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GAAGAAGCACCGGCTAACTCCGTG

GU968637
>EB11 (132)

AATATTGCACAATCGACGCAAGTCGGATGCAGCAACGCCGCGTGGACGATGAAGGCCTTCGGGTTGTA
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GU968638
>EB2 (135)
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GU968629
>EB16 (135)
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GAAGAAGCACCGGCTAACTCCGTG

GU968630
>EB12 (160)
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TTGTAAAGCACTTTTATCGGGAAGGAAATCTCAGGGTTAATACCCCCTGGGCCTTGACGGTACCCGAA
GAATAAGCACCGGCTAACTCAGTG

GU968618
>EB15 (135)
CAGGGAATCTTGTCCAATGGGCGAAAGCCTGAGACAGCGACGCCGCGTGAGGGATGAAGGCCTTCGGG
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GU968632
>EB1 (161)
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AAAGGAAGCACCGGCTAACTCCGTG

GU968633
>EB4 (161)
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GU968634
>EB 20 (136)
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GU968635
>EB22 (132)
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GU968636
>EB9 (161)
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AGAGGAAGCACCGGCCAACTCCGTG

4. 16S rRNA sequences of clone library (Sequence length bp in brackets)

FJ268553
>PL186 (1008)
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CCTTGCGAGGGAAAGCTTTAGCGCCCTGGGATGAGCCCGCGTCGGATTAGCTTGTTGGTGAGGTAACGGC
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CCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGCAAACGTTGTTCGGAATTACTGGGCGTA
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GTGGCGCAGCTAACGCATTAAGCACACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTG
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CTGTCGTCAGCTCGTGTCGTGAGATGTT

FJ268510
>PL 123 (951)
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GGACTGCTTGTGTAGTAACGCTACAGGCTCGATCGAGCTGTCGAGGAGATCGCACATGACTGACACGCAG
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ATTGTCAAGTCAGAGGTGAAAGCCCTGGGCTCAACCTGGGAATGCCTTTGAAACTAGCAGTCCAGAGTCC
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TAATTCGAAGCAACGCGCAGAACCTTACCAGCCCTTGACATGCCCGTCGCGGATCTCGGAGACAGGATCC
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FJ268571
>PL 208 (988)
TGGCGGCAGGCCTAACACATGCAAGTCGAACGCTGAAGCAATGAAGAGTGGCAGACGGGTGAGTAACGCG
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TGTCGTGA

FJ268570
>PL 207 (993)
CAACTGGCGGCGGCCTCCACATGCTAGTCGAGCGCGCAGCCCCTTCGGGTGGTGGGCGAGCGGCGGACGG
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TGCCGTTCAGTGTCGCAGCTAACGCGATAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
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TGCATGCTGTGTC

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>PL 179 (1036)

GCTGGCGGCATGCCTTACACATGCAAGTCGAACGGCAGCACGGGTGCTTGCACCTGGTGGCGAGTGGCGA
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GGCTCTAATACAGTCGGGGGATGACGGTACCGGAAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGC
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CCGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTGGTGACTGGCAGGCTAGAGTATGGCAGAGGG
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CTGGGCCAATACTGACGCTCATGCACGAAAGCGTGGGGAGCAACAGGATTAGATACCCTGGTAGTCCACG
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GCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGAT
GTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGACATGGTCGGAATCCCGCTGAGAGGTG
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EU071379

>PL 11 (918)

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AGGTAGTTGGTGGGATAAAAGCTTACCAAGCCGACGATCTGTAGCTGGTCTGAGAGGACGATCAGCCACA
CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGC
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CACGCCCTAAACGATGTCAACTGGTTGTTGGGTCTTAACTGACTCAGTAACGAAGCTAACGCGTGAAGTT
GACCGCCTGGGGAGTACGGCCGCAAGGTTGAGACTCAAAGGAATTGACGGGGACCCGCACAAGACGGTGG
ATGATGTGGTTTAATTCGATGCAACGCGAAAAACCTTACCCACCTTTGACATGTACTGGAATCCTTTAGA
GATAGATG

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>PL 63 (991)

CCAGAAGCTTGCTTCCGGCTGACGAGTGGCGGACGGGTGAGTAATACATAGGAATCTGCCCATGTAGTGG
GGGACAACCCGGGGAAACTCAGGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCTTCGGACC
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ACGCCAGCACACTGGACTAGACACGCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGACAATGGGC
GAAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTTGGGAA
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ATAAGTTCACCGCCTGGGGAGTACGGCCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
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CAGAAATGATTGGTGCCTTCGGGAACGCAGTGACAGGTGCTGCAAAGCTGTCGTCAGCTCGTGTCGTGAG
ATGTTGGGTTA

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>PL 148 (972)

GGGCGCTGACGAGTGGCGGACGGGTGAGTAATGCGTAGGAATCTGCCCAGTAGCGGGGGACAACTTGGGG
AAACTCAAGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCTTCGGACCTTGCACTATTGGAT
GAGCCTACGTCGGATTAGCTTGTTGGTGGGGTAACGGCCCACCAAGGCGACGATCCGTAGCTGGTCTGAG
AGACGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGG
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AGCAGGGACGAAAAGCTGAGGCTAATACCCTCAAGTCTTGACGTAACCTAAAGAAGAAGCACCGGCTAAC
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ACCCTGGTAGTCCACGCCGTAAACGATGTCAACTACCGTTGGGTTCATAAGAACTTAGTGGCGCAGCTAA
CGCGATAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCA
CAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGCCCTTGACATCCAGGGAA
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>PL 149 (968)

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AGATGAGCCTGCGTCGGATTAGCTTGTTGGTAGGGTAATGGCCTACCAAGGCGACGATCCGTAGCTGTCT
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TGGAGTGTGGTAGAGGGAAGCGGAATTCCGGGTGTAGCGGTGAAATGCGTAGATATCCGGAGGAACACCA
GTGGCGAAGGCGGCTCCTGACCAACACTGACGCTCAGCACGAAAGCGTGGGGAGCAAACAGGATTAGATA
CCCTGGTAGTCCCGCCCTAAACGATGAGAACTAGCCGTTGGGAGTATTGAAACTCTTAGTGGCGCAGCTA
ACGCGATAAGTTCTCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGCCCTTGACATCTAGGAA
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>PL 67 (978)

TGCAGTCGAACGGTAACGCGAGGAGCTTGCTCCTTGGCGACGAGTGGCGGACGGGTGAGTAATACTTAGG
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CCTACCAAGGCGACGATCCGTAGCTGGTCTGAGAGGACGATCAGCCACACTGGGACTGACCGGCCAGACT
CTACGGAGCAGCAGTGGGATATTGGACAATGGGGGCAACCCTGATCCAGCAATGCCGCGTGTGTGAAGAA
GGCCTGCGGGTTGTAAAGCACTTTCAGTAGAGAAGAAAAGCTTACGGTTAATACCCGTGAGTCTTGACGT
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AATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAGAACCTTACCTGC
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>PL 139 (949)

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AAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA
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FJ268563
>PL 200 (1063)
CTGGCGGCATGCCTAACACATGCAAGTCGAACGGTAACAGGACGCTGTGATCCCTTCGGGGTGAACTTGG
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>PL 198 (1062)
GCTGGCGGCATGCTTAACACATGCAAGTCGAACGGTACAGGTCGCTGTGATCCCTTCGGGGTGAACTTGA
CGCTGACGAGTGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCCAGAAGTGGGGGACAACCCGAGGAAA
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CGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGCCCTTGACATCCTC
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GTGTCGTGAGAT

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>PL 87 (980)
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AGCTCTGTCAGGTGGGAAGAACCCTCCGTCGATTAATAATTGACGGAGCTGACGGTACCACCAGAGGAAG
CACCGGCTAACTCCGTGCCAGCAGCCGCGGTAACACGGAGGTGCAAGCGTTGTTCGGAATCGCTGGGCG
TAAAGGACGCGCAGGCGGCCCGGTAAGTCGGAAGTGAAATCCGTCGGCTCAACCGACGAACGGCTTCCGA
TACTGCCGGACTTGAGTACGGGAGAGGAAAGCGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATGGG
AAGACACGGTGCGAAGGCGCTTTCTGGCCGATACTGACCCCACGCGTGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCTGTAAACGGTGAGTACTAGGTGTAGGGGGTACCGACCCCCCCTGTGC
CGCAGCTAACGCACTAAGTACTCCGCCTGGGGACTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGG
GGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGATTTGACA
TCCCCGGACCACCCTGGAAACAGAGTTTTCCCACTTCGGTGGACCGGGTGACAGGTGCTGCATGGCTGTC

FJ268501
>PL 101 (1040)
TCGTAACACATGCAAGTCGAACGAGAAACTCCGAGCTTGCTCGGAGGAGTAAAGTGGCGCACGGGTGAGT
AACGCGTGGGTAACCTGCCCTTGAATTCGGGATAACTTCGGGAAAGCGTTGCTAATACCGGATACCGTCC
CGAGAACTGTGGTTCTTGAGATGAAAGGTGACCTCTCCACGGAAGTTGCCGTTCGGGGAGGGGCCCGCGT
CCCATTAGCTTGTTGGTAGGGTAACGGCCTACCAAGGCGACGATGGGTAGCTGGTCTGAGAGGATGATCA
GCCACACTGGCACTGAACACGGCAGACTCTACGGGAGGCAGCAGTGAGGAATTTGGCGCAATGGGGAAAC
CCTCACGCAGCAACGCCGCGTGAGTGAAGAAGGCCTTCGGGTCGTAAAGCTCTGTCAAGAGGGGAAAAAT
CCTTCTGATTAACAGTCTAAAGGACTGATGGTACCTCTAGAGGAAGCACGGGCTAACTCCGTGCCAGCAG

CCGCGGTAATACGGAGGGTGCAAGCGTTGTTCGGAATTACTGGGCGTAAAGAGCGTGTAGGCGGTTTGGC
AAGTCAGATGTGAAATCCTTCCGCTCAACGGGAGAAGTGCATTTGAAACTGCCATTCTAGAGTATGGGAG
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CTCTCTGGACCAATACTGACGCTGAGACGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGANGAGAACNAGGTGTAGCGGGTATTGACCCCTGCTGTGCCGTAGCTAACGCATTAT
GTTTCCGCCTGGGGAGTACGGTCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTCAATTCGACGCAACGCGAAGAACCTTACCTGGGCTTGACATCCTCTGACAGCCCCGGA
AACGGGGTCTTCCCTGCTTGCAGGGACAGAGTGACAGGTGCTGCATGGTCTGTCGTCACG

FJ268503

>PL 103 (976)

CTTGCGGAAAAGAGTAAAGCGGCGCACGGGTGAGTAATGCATGAATAACCTGCCCTCAGGTCTGGGATAA
CCCCGTGAAAATGGAGTTAATACCGGATGATATCCATTTAACCATGGTTGAATAGGTTAAAGGTGGCCTC
TGCTTGCAAGCTATCGCCTGGAGAGGGGTTCGTGTCCCATCAGTTAGTTGGTAGGGTAATGGCCTACCAA
GGCTAAGACGGGTAGCTGGTCTGAGAGGACGACCAGCCACATTGGCACTGAAACACGGGCCAGACTCCTA
CGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGCGAAAGCCTAACGCAGCGACGCCGCGTGAGGGAAGA
AGGCCTTGGGTCGTAAACCTTTGTCAGGTGGGAAGAACCCCTCCCTATCAAATAGGTTTGGAGGGTTGAC
GGTACCACCCAAGGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTG
TTCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGCTTTGTAAGTCAGGTGTGAAATACTCTTGCTCAAC
GAGAGAATTGCACTTGAAACTGCATTGCTTGAGTACGGGAGAGGGAAGTGGAATTCCAGGTGTAGCGGTG
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CGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGGCACTAGGTGT
AGGGGGTTTATCCCTCTGTGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGTCGCAAGGCT
AAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAA
GAACCTTACCTGGGCTTGACATCTCCCGAATTCCGATGAAAATCGGTAGTGCCCCTTCGGGGGAAC

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>PL 190 (1057)

CACATGCAAGTCGAACGAGAAACTCCGAGCTTGCCGGAGGAGTAAAGTGGCGCACGGGTGAGTAACGCGT
GGGTAACCTGCCCCTGAAACTGGGATAACTTCGCGAAAGCGTTGCTAATACCGGATATCGTCCTTTAACT
ATGGTTTTTGGGATGAAAGGTGACCTCTTCATGAAAGTTGCCGTTCAGGGAGGGGCTCGCGTACCATTAG
CTTGTTGGTAGGGTAATGGCCTACCAAGGCTACGATGGTTAGCTGGTCTGAGAGGATGATCAGCCACACT
GGAACTGAAACACGGTCCAGACTCCTACGGGAGGCAGCAGTGAGGAATTTTGCGCAATGGGGGAAACCCT
GACGCAGCAACGCCGCGTGAGTGAAGAAGGCCTTCGGGTCGTAAAGCTCTGTCAAGTGGGAAAAAATCCT
TGCGATTAATAATCGGAAGGACTGATGGTACCACTAGAGGAAGCACCGGCTAACTCCGTGCCAGCAGCCG
CGGTAATACGGAGGGTGCAAGCGTTGTTCGGAATTACTGGGCGTAAAGAGCGTGTAGGCGGTTTGGCAAG
TCAGATGTGAAAGCCCTGGGCTCAACCCAGGAAGTGCATTTGAAACTGCCATTCTTGAGTATGGGAGAGG
AGAGTGGAATTCCCGGTGTAGAGGTGAAATTCGTAGATATTGGGAGGAACACCCGTGGCGAAGGCGACTC
TCTGGACCAATACTGACGCTGAGACGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
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CTCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA
GCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTGGATTTGACATCCTCTGACAGCCCCGGAAA
CGGGGTCTTCCCTGCTTGCAGGGACAGAGTGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGG

FJ268534

>PL 158 (1047)

CGCTGGCGGCGTGCCTAACACATGCAAGTCGAACGAGAAAATTCTGCTTGGCGGGATGAGTAAAGTGGCG
CACGGGTGAGTAACGCGTGGGTAACCTGCCCTTGAATCGGGGATAACTTCGCGAAAGCGTAGCTAATACC
GGATACTGTCCCTAAGGCTATGGCCCTGGGGATGAAAGGTGACCTCTTCATGAAAGTTGCCGTTTGGGGA
GGGGCTCGCGTACCATTAGCTTGTTGGCGGGGTAATAGCCCACCAAGGCTACGATGGTTAGCTGGTCTGA
GAGGATGATCAGCCACACTGGAACTGAAACACGGTCCAGACTCCTACGGGAGGCAGCAGTGAGGATTTTG
CGCAATGGGGAAACCCTGACGCAGCAACGCCGCGTGAGTGAAGAAGGCTTTCGGGTCGTAAAGCTCTGTC
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CTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTGTTCGGAATTATTGGGCGTAAAGAGCGT
GTAGGCGGTTTGGTAAGTCAGATGTGAAAGCCCTGGGCTCAACCCAGGAAGTGCATTTGATACTGCCATC
CTTGAGTATGGGAGAGGAGAGTGGAATTCCAGTGTAGAGGTGAAATTCGTAGTATTGGGAGACACCGTGC
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AAGTTCTCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTGGATTTGACATCCTCTGACAGTCCC
GGAAACGGGATCTTTCCGACTTGTCGGAACAGAGTGACAGGTGCTGCATGGCTGTCGTCAGCTCGTG

FJ268495

>PL 94 (986)

TCGTACGAGAAATCCCGAGCTCGCTTGGGAGATAAAGTCGGCGCACGGGTGAGTAACGCGTGGGTAACCC
ACCTTCGAATCTGGGATAACTCCGCGAAAGCGGTGCTAATACCGGATAAGACCACAGCCGTTTCGGCGGC
AGAGGTAAAAGCTGACCTCTCCATGGAAGTTAGCGTTCGAGGACGGGCCCGCGTCCTATCAGCTTGTTGG
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AACACGGTCCAGACTCCTACGGGAGGGCAGCAGTGAGGAATTTTGCGCAATGGGGGAAACCCTGACGCAG
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TAATAATGTCGAAGCCTGACGGTACCACCAGAGGAAGCACCGGCCAACTCCGTGCCAGCAGCCGCGGTAA
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TTATGACGCCAAGGGAGAACTTACCTGGGCTTGACATCCCGACACCTGAACATGTCTCCCTTCTTACCAG
GTAACG

FJ268498

>PL 98 (965)

CTTCGGGTGGCGAGTACAGTGGCGCACGGGTGAGTAACACGTGGACAATCTACCCTTGAGACTGGAATAA
CTCGCTGAAAAGCGGGATAATGCCGGATATGACTACGGGAGGCATCTTCTGTAGTTAAAGGCGGGGACCC
TTCGGGGCCTGTCACTCTTGGATGAGTCCGCGTCCCGTCAGCTTGTTGGTAGGGTAATGGCCTACCAAGG
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GGAGGCAGCAGTGGGGAATATTGCGCAATGGGCGAAAGCCTGACGCAGCGACGTTGCGTGAGGGAAGAAG
GCTCTCGGGTTGTAAACCTCGTCGGGGGGGAAGAAATTCCTAACGGTAATAACCGTTAGGATTGACGGTA
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GAATTGCCTATGATACTGCAGGGCTTGAGTACTGGAGAGGGAAGCGGAATTCCTGGTGTAGCGGTGAAAT
GCGTAGATATCAGGAGGAACACCGGTGGCGAAGGCGGCTTCCTGGACAGATACTGACACTGAGGAGCGAA
AGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGGGTACTAGGTGTGAGG
ACGATTTAAAGTCTTCGTGACGCAGCTAACGCAATAAGTACCCCACCTGGGAAGTACGGCCGCAAGGTTG
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGAAG
AACCTTACCTGGGCTTGACATCCGGAGAACCCCTTGGAAACTTGGGGGTGCCCGG

FJ268517

>PL 133 (1010)

GCCTAACACTGCAAGTCGTACGAGAAATCCCGAGCTTGCTTGGGAGAGTAAAGTGGCGCACGGGTGAGTA
ACGCGTGGGTAACCCACCTTCGAATCTGGGATAACTCCGCGAAAGCGGTGCTAATACCGGATAAGACCAC
AGCCGTTTCGGCGGCAGAGGTAAAAGCTGACCTCTCCATGGAAGTTAGCGTTCGAGGACGGGCCCGCGTC
CTATCAGCTTGTTGGTGGGGTAAAGGCCTACCAAGGCAGCGACGGGTAGTGGTCTGAGAGGATGATCAGC
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CTGGGACTACGGTCGCAACACTAAAACTCAAAGGAATTGACGGGGCCCGCACAAGCGGTGGAGCATGTGG
TTTATTGCGCAAAGCGAAGAACATACCCGGCTACATCCCGACAACCCAACGAAACAACGCCTACCACAAC
GGTGGACCGGGTGACAGGTGCTGCATGGCT

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>PL 202 (1068)

GCTGGCGGCGTGCCTAACACATGCAAGTCGTACGAGAAATCCCGAGCTTGCTTGGCACACTAAAGTGGCG
CACGGGTGAGTAACGCGTGGGTAACCCACCTTCGAATCTGGGATAACTCCGCGAAAGCGGTGCTAATACC
GGATAAGACCACAGCCGTTTCGGCGGCAGAGGTAAAAGCTGACCTCTCCATGGAAGTTAGCGTTCGAGGA
CGGGCCCGCGTCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCAGCGACGGGTAGCTGGTCTGA
GAGGATGATCAGCCACACTGGAACTGGAACACGGTCCAGACTCCTACGGGAGGCAGCAGTGAGGAATTTT
GCGCAATGGGGGAACCCTGACGCAGCAACGCCGCGTGGGTGAAGAAGGCTTTCGGGTCGTAAAGCCCTGT
CAGGTGGGAAGAACCGTTTCGATACTAATAATGTCGAAGCCTGACGGTACCACCAGAGGAAGCACCGGCC
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GACTTGAGTACGGGAGAGGAGGGGGGAATTCCCGGTGTAGAGGTGAAATTCGTAGATATCGGGAGGAATA
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GTAGCTAACGCATTAAGTGCTCCGCCTGGGGACTACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGG

GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTGGGCTTGACAT
CCCCGGACAACCCTGGAAACATGGTCTTCCACTTCGGTGGACCGGTGACAGGTGCTGCATGGCTGTCGTC
AGCTCGTGTCGCGAGATG

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>PL 171 (1070)

CGCTGGCGGCGTGCCTAACACATGCAAGTCGTACGAGAAATCCCGAGCTTGCTTGGGAGAGTAAAGTGGC
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CGGATAAGACCACAGCCGTTTCGGCGGCAGAGGTAAAAGCTGACCTCTCCATGGAAGTTAGCGTTCGAGG
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AGAGGATGATCAGCCACACTGGAACTGGAACACGGTCCAGACTCCTACGGGAGGCAGCAGTGAGGAATTT
TGCGCAATGGGGGAAACCCTGACGCAGCAACGCCGCGTGGGTGAAGAAGGCTTTCGGGTCGTAAAGCCCT
GTCAGGTGGGAAGAACCGTTTCGATACTAATAATGTCGAAGCCTGACGTACCACCAGAGGAAGCACCGGC
CAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGAG
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TAGATACCCTGGAAGTCCACGCCGTAAACGATGAGCACTAGGTGTAGCGGGTATTGACCCCTGCTGTGCC
GTAGCTAACGCATTAAGTGCTCCGCCTGGGGACTACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGG
GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTGGGCTTGACAT
CCCCGGACAACCCTGGAAACATGGTCTTCCACTTCGGTGGACCGGTGACAGGTGCTGCATGGCTGTCGTC
AGCTCGTGTCGCGAGATGTT

FJ268511

>PL 124 (985)

GCTCGCTTGGGAGAGTAAAGTGGCGCACGGGTGAGTAACGCGTGGGTAACCTACCCTTGAATCGGGGATA
ACTCCGCGAAAGCGGAGCTAATACCGGATAAGACCACGGCCGCTCCGGGGCGAGGTAAAAGCTGACCTCT
CCATGGAAGTTAGCGTTTGGGGACGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAAGGCCACCAAGGC
AACGACGGGTAGCTGGTCTGAGAGGAGATCAGCCACACTGGAACTGGACACGGTCAGACTCTACGGAGGC
AGCAGGAGGAATGCGCATGGGAACCTGCGAGCACCGCGGGTGAGAGGCTTTCGGGTCGTAAAGCCTGTCA
GGTGGGAGAACCGATCCGGCACTAATATTGCCGAACCTGACGGTACCACCAGAGGAAGCACCGGCCAACT
CCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGAGCGTGT
AGGCGGTTAGGAAAGTCAGATGTGAAAGCCCTGGGCTCAACCCAGGAAGTGCATTTGAAACTTCCTAACT
TGAGTACGGGAGAGGAGGGGGGAATTCCCGGTGTAGAGGTGAAATTCGTAGATATCGGGAGGAATACCGG
TGGCGAAGGCGCCCCTCTGGACTGATACTGACGCTGAGACGCGAAAGCGTGGGGAGCAAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGAGCACTAGGTGTAGCGGGTATTGACCCCTGCTGTGCCGTAG
CTAACGCATTAAGTGCTCCGCCTGGGGATTACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTGGGCTTGACATCCCC
GGACAGCCCTGGAAACAGGGTCTTCCACTACGGGGGACCGGGTGACAGGTGCTGCATGGCTGTCGTCAGC
TCGTG

FJ268562

>PL 199 (1072)

CTGGCGGCGTGCCTAACACATGCAAGTCGTACGAGAAATCCCGAGCTTGCTTGGGAGAGTAAAGTGGCGC
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GATAAGACCACAGCCGCTCCGGGGCGAGGTAAAAGCTGACCTCTCCATGGAAGTTAGCGTTTGGGGACGG
GCCCGCGTCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCTACGACGGGTAGCTGGTCTGAGAG
GATGATCAGCCACACTGGAACTGGAACACGGTCCAGACTCCTACGGGAGGCAGCAGTGAGGAATTTTGCG
CAATGGGCGAAAGCCTGACGCAGCAACGCCGCGTGGGTGAAGAAGGCTTTCGGGTCGTAAAGCCCTGTCA
GGTGGGAAGAACCGTCCGGCACTAATATTGCCGAACCTGACGGTACCACCAGAGGAAGCACCGGCCAACT
CCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGAGCGTGT
AGGCGGTTAGGAAAGTCAGATGTGAAAGCCCTGGGCTCAACCCAGGAAGTGCATTTGAAACTTCCTAACT
TGAGTACGGGAGAGGAGGGGGGAATTCCCGGTGTAGAGGTGAAATTCGTAGATATCGGGAGGAATACCGG
TGGCGAAGGCGCCCCTCTGGACTGATACTGACGCTGAGACGCGAAAGCGTGGGGAGCAAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGAGCACTAGGTGTAGCGGGTATTGACCCCTGCTGTGCCGTAG
CTAACGCATTAAGTGCTCCGCCTGGGGACTACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTGGGCTTGACATCCCC
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GTGTCGCGAGATGTTGGGTTAA

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>PL 169 (972)

TTCAGCTTGCTGAAGCTAGTAAAGTGGCGCACGGGTGAGTAACGCGTGGGTAATCTACCTCCTAATTAGG
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GCCTCTCCATGGAAGCTACTGTTAGGAGATGAGCCCGCGTACCATTAGCTCGTTGGTAGGGTAATGGCCT
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TCCTACGGGAGGCAGCAGTGAGGAATTTTGCGCAATGGGGGAAACCCTGACGCAGCAACGCCGCGTGAGT
GATGAAGGCCTTCGGGTCGTAAAGCTCTGTCAAGTGAGAAGAACCTGCCCTGTGCTAATATCACCGGGCA
CTGACGGTACCACTGAAGGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAACACGGGGGGTGCAAG
CGTTATTCGGAATCACTGGGCGTAAAGAGCGCGTAGGCGGTCTCTTAAGTCAGATGTGAAAGCCCGGGGC
TCAACCCCGGAAGTGCATTTGAAACGAAGGGACTTGAGTATGGGAGAGGGAAGTGGAATTCCTGGTGTAG
CGGTGAAATGCGTAGATATCAGGAGGAACACCGGTGGCGATGGCGACTTCCTGGACCAATACTGGACGCT
GAGGCGCGAAGGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCAGTAAACGTTGAACACT
AGGTGTAGCGGGTATTGACCCCTGCTGTGCCGCAGCTAACGCATTAAGTGTTCCGCCTGGGGAGTACGGC
CGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACG
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>PL 183 (1063)

GCGGCGTGCTTAACACATGCAAGTCGCACGAGAACGCTTCAGCTTGCTGAAGCTAGTAAAGTGGCGCACG
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GATATCTCAAAAACTTCGGTTTTTGAGATCAAAGGTGGCCGCTCCATGGAAGCTACTGTTAGGAGATGAG
CCCGCGTACCATTAGCTTGTTGGTAGGGTAATGGCCTACCAAGGCAACGATGGTTAGCTGGTCTGAGAGG
ATGACCAGCCACACTGGAACTGACACACGGTCCAGACTCCTACGGGAGGCAGCAGTGAGGAATTTTGCGC
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GTGGGAAGAACCTGCCCTGTGCTAATATCACCGGGCACTGACGGTACCACTGAAGGAAGCACCGGCTAAC
TCCGTGCCAGCAGCCGCGGTAACACGGGGGGTGCAAGCGTTATTCGGAATCACTGGGCGTAAAGAGCGCG
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TTGAGTATGGGAGAGGGAAGTGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAGGAACACCG
GTGGCGATGGCGACTTCCTGGACCAATACTGACGCTGAGGCGCGAAGGCGTGGGGAGCAAACAGGATTAG
ATACCTGGTAGTCCACGCAGTAAACGTTGAACACTAGGTGTAGCGGGTATTGACCCCTGCTGTGCCGCAG
CTAACGCATTAAGTGTTCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGAAGAACCTTACCTGGGTTTGACATCCCG
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TCGTGTCGTGAGA

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>PL 132 (988)

GTGCTTAACACATGCAAGTCGAACGAGAAAGCACCTTCGGGTGCGAGTAGAGTGGCGCACGGGTGAGTAA
CGCGTGGATAATCTGCCCTTGAGTTTGGGATAACCAGCCGAAAGGCGGGCTAATACCGGATAAAATAGGC
AGCATAAGTCTGATAATAAAAGGTTTACCGCTTGAGGATGAGTCCGCGTCCCATTAGCTGGTTGGTAGGG
TAAAGGCCTACCAAGGCACATGGTAGCTGTCGAGAGGATGATCAGTCACACTGGAACTGGAACACGGTCC
AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGACGCAGCGACGCCGCGT
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CACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGACGGTGCAAGCGTTGTTCGGATTTATTGGGCG
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AGGAACACCAGTGGCAAGGCGGCTACCTGGACCGATCGACGCTGAGATGCGAAAGCGTGGGGAGCAAACA
GGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGAGTACTAGGTGTTGCGGGCTTAACCTGCAGTGT
CGCAGCTAACGCATTAAGTACTCCGCCTGGGAAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGACCTTACCTGGGCTTGACAT
CCTGAGAACTCTTGGAAACTAGAAGTGCCCTTCGGGGAACGAAGAGACAGGTGCTGCATGGCTGTCGTCA
GCTCGTGT

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>PL 147 (966)

TGTAGACGAGTGGCACACGGGTGAGTAACGCGTGGGTAATCTGCCCTCGGGTTCGGGACAACTCCGGGAA
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GCTACCGCCGGAGGATGAGCCCGCGTCCCATTAGCTTGTTGGTGAGGTAAAGGCTCACCAAGGCGGCGAT
GGGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG
CAGTCGGAATTTTGGGCAATGGGGGCAACCCTGACCCAGCAACGCCGCGTGAGGGATGAAGGCCCTCGGG
TCGTAAACCTCTGTCGGGAGGGAAGAACGTTCTCCGGGTGAATAATCCGGTGAGCTGACGGTACCTCCAA
AGGAAGCGCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGCGCGAGCGTTGTTCGGAATCAT
TGGGCGTAAAGCGCGCGTAGGCGGCTTGGCAAGTCGGGTGTGAAAGCCCGGGGCTCAACCCCGGAATTGC
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GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGTGCTAGGTGTCGCGGGTATTG
ACCCCTGCGGTGCCGTCGTTAACGCATTTAGCACCCCGCCTGGGGAGTACGGTCGCAAGGCTAAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTA
CCTGGCCTTGACTTCTGGGGAACCTCCTCGAAAGTGGAGGGTGCCTTCGGAGCCAA

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>PL 151 (1070)
GCGTGCCGTAACACATGCAAGTCGTACGAGAAAGTCGCCTTCGGGTGGCGAGTACAGTGGCGCACGGGTG
AGTAACACGTGGACATCTACCCTTGAGACTGGAATAACTCGCTGAAAAGCGGGATAATGCCGGATATGAC
TACGGGAGGCATCTTCTGTAGTTAAAGGCGGGGACCCTTCGGGGCCTGTCACTCTTGGATGAGTCCGCGT
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>PL 159 (1055)
TCAGGACACCTGGCGGCTGCCTAACACATTGACAAGTCGAGCGCGAAATTGGGGGGCACTCCCCGAGTAG
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GTGAC

FJ268507
>PL 118 (1009)
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GTCGTGAGATGTTGGGTTAAGTCCCGCAA

FJ268513
>PL 127 (993)
CCTTCCGGGATGAGTAAAGTGGCGCACGGGTGAGTAACACGTGGATAATCTACCTCTAAGTGAGGGATAA
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TAAGTGCCCCGCCTGGGGAGTACGGTCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
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>PL 100 (981)

CCTCGGGAGAGTAAAGTGGCGAACGGGTGAGTAAAGCGAAGGTAATCTACCCTTAAATTAGGGATAACTT
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C

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>PL 74 (990)

CCCTTGCCAGGATGAGTAAAGTGGCGCACGGGTGAGTAACACGTGGGTAATCTGCCTCTAAGCTAGGGAT
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>PL 121 (1014)

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>PL 187 (1061)

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CCGTGAGGTGT

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>PL 193 (1079)

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>PL 93 (993)

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GGTTAAGTCCCGC

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>PL 138 (960)

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>PL 70 (925)
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GGCTGCCGTCAGCTC

EU445348
>PL 73 (1148)
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>PL 106 (1007)
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GAGGTGCTGCATGGCTGTCGTCAGCTCA

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>PL 161 (989)
TTGGCGGCGTGGATAGGCTGCAGTCTGCGAGTATCTCCTTCGGGGGCGAAAAGCGCGCAAGGGAGAGTAA
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>PL 212 (1030)

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>PL 145 (929)

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AACCTGGAGTAATACTCGATGTGGTCCTCCGGGATCAAAGGCTTCGGTCGCCTTGGGAGCGGGGAACGCC
CTATCGGTTTTGTAGTAACGCTACCAAGCCGAAACGGTAGCGGGTGGAAGCACGACCCGCCGCATCGGAC
TGAACACTGCCCGACTCCTACGGGAGGCTGCAGCAACGAATCTTCCGCAATGCGCGAAAGCGTGACGGAG
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ACCCAAAGGAAGGGCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGCCCGAGCGTTGCGCGG
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AATTGCTTGGTGAACCGCTCGTCTTGAGGCAGTAGGGGCTGTCGGAACGATAGGTGGAGCGGTGAAATGC
GTTGATATCTATCGGAACGCCAAGGTGAAGACAGGCAGCTGGGCTGTCCTGACGCTGAGGTACGAAAGCG
TGGGGAGCGAACGGGATTAGATACCCCGGTAGTCCACGCCGTAAACGATGTGCACTAGATCGAGGAGGTT
CTGACGCATTCTCGGTGAAGCAAAGTGTTAGTGCACCGCCTGGGAGTCGGTCCAAGGCTAAAACTCAAAG
GAATTGACGGGGGCTCACACAAGCGGTGGAGCATGTGGCTTAATTCGAAGCAACGCGAAGAACCTTATCC
TGGGTTGACATGCACGGATTAGCTTCCTGAAAGGGAAGTGACGCCTTCGGGTGGAACGTCCACAGGTGCT
GCATGGCTGTCGTCAGCTC

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>PL 146 (1035)

GTGGCTAAGGCATGCAAGTCGAACGATGGTGGAAGGTAACGGAAGCACATTTAGTGGCGAACGGGTGAGG
AATAGATAGGTAACGTACCCCGGACTCAGGAATAGCGGGGAACTTCGGTCCCTTTGCGAAAGTGCCGGTA
ATACCTGATGATATCACCGCATCGCATGGGGTGGTGATCAAGGGTCCGCCGGTCTGGGATCGGCCTATCT
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AGCAACGCGAAGAACCTTACCTGGGTTTGACATGCTAGGATGCACCCGTGAAAGCGGGCTATGCTGCCTT
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>PL 97 (930)

GATTAAAGCCCCTTCGGGGGTGTATAAAGTGGCGAAAGGGCGAGGAATACTTTTCCACGTACCCCGAGGT
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TGGGAGCGGGGAACGCCCTATCAGGTTGTTGGTGAGGTAACGGCTCACCAAGCCGATGACGGGTAGCGTG
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>PL 211 (972)

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TGCTGTCAGGGTTCAGCAACACAATGAGCAGACCCAAAGGAAGGGCCGGCTAACTTCGTGCCAGCAGCCG
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AGCATGTGGCTTAATTCGAAGCAACGCGAAGAACCTTATCCTGGGTTGACATGCACGGATTAGCTTCCTG
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>PL 204 (1013)

GGCGGCATGGCTAAAACATGCAAGTCGAACGATGAAAGCACCTTCGGGTGTGCATGAAGTGGCGAAAGGG
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>PL 205 (1031)

CGGCGTGGCTAAGACATGCAAGTCGAACGGACCATTAATTTATTGTAGTTAGTGGCGAAGGGTTAGTAAC
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>PL 156 (1060)
GGCGTGGCTAAGGCATGCAAGTCGAACGGACGTACCTGGAAGGAAACGGAAGGGTATGTTAGTGGCGAAC
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>PL 157 (1014)
TGGCTAAGGCATGCAAGTCGAACGGACGTACCTGGAAGGAAACGGAAGGGTAGTTAGTGGCGAACGGGTG
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CAAAGGAATTGACGGGGGCTCACACAAGCGGTGGAGCATGTGGATTAATTCGAAGCAACGCGAAGAACCT
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AGGTGCTGCATGGCTGTCGTCAGCTCGTGCTGTG

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>PL 185 (1038)
AACGCTGGCGGCATGGCTAAGACATGCAAGTCGAACGGACTATTTTGAAGAGTAATCGGATTAATAGTTA
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AATGCCGGATGATGTCATCTGTTTAATGCAGATGACCAAAGATTTATCGGCCCCTGAGCGGCCTATTTCC
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CGAAGCAACGCGCAGAACCTTACCTGGGTTTGACATGTATGGATGCCTATCTGGAAACAGAGTCGGCTGC
CCTCGGGTGAAACTTACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATG

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>PL 65 (862)
CCTGTGCTTCAGGATAGCGTCAGAAAACTTGTAACCATCCGAAAGGGGCGGTAATACTATAATACGGATA
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>PL 195 (1051)

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G

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>PL 62 (993)

ACATGCAAGTCGAACGAGAACCCATTCTTCCGATGGGGGACAGTGGCGAACGGGTGAGTAACACGTTGGT
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GTCGTCAGCTCGT

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>PL 75 (1110)

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>PL 153 (969)

CAAGTCGAACGAGGCCCTTGTATTCGTAGAAGGTGTCCTAGTGGCGAACGGTGAGTAACACGTTGGTGAC
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TGCCGCCTGGGGACTACGACCGCAAGGTAAAACTCAAAGGAATTGACGGGACCCGCACAAGCAGCGGAGC
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>PL 96 (934)

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ATTGACGGGGGCCCGCACAAGCAGCGGAGCGTGTGGTTTAATTCGAGGATACGCGAAGAACCTTACCTGG
GCTTGACATGTTCGTAGTAGGAAGCCGAAAGGTGACCGACCCTTCGGGGAGCGATCACAGGTGCTGCATG
GCTGTCGTCAGCTCGTGCCGTGAG

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>PL 104 (977)

GGAGGAGTGTCCTAATGGCGACGGGTGAGTAACACGTTGGTGACCTACCCCGAGAGGGGGACAACCCGGG
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GTTTAATTCGAGGCTACGCGAAGAACCTTACCTGGGCTAGACATGTTCGTAGTAGGAAACCGAAAGGTGA
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>PL 201 (1035)

AACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAGGCCCTCTTCCTTCGGGAGGAGGTGTCCTAGT
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GGCCTATCAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGGGGACCTGAGAGGGTGAC
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GGCAAGCGTTATCCGGATTTACTGGGCGTAAAGCGCGTGCAGGCGGTTTGGTAAGTCGGACGTGAAAGCT
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GGTGTAGCGGTGAAATGCGTAGATATCTGGAGGAACACCAGTGGCGAAGGCGGCCTCCTGGACTGTCCCT
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TCGAGGCTACGCGAAGAACCTTACCTGGGCTTGACATGTTCGTAGTAGGAAGCCGAAAGGTGACCGACCC
TTCGGGGAGCGATCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTG

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>PL 82 (1123)

CCCAATGTGGAATTCGCCCTTAGAGTTTGTCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATACATG
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GACCTACCCCGAAGAGGGGGACAACCCTGGGAAACTAGGGCTAATACCCCATGTGATCCTAAGGATTAGA
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TCGAAACTACCAGGACTAGAGGGCAGCAGAGGGAGGTGGAATTCCAGGTGTAGCGGTGAAATGCGTAGAT
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GTG

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>PL 178 (1027)

GGCGGCGTGCCTAATACATGCAAGTCGAACGGGGAGTATTAATTTCGGTTTTTACTCCTAGTGGCGAACG
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GCTAGTTGGTAAGGTAACGGCCTACCAAGGCGATGACGGGTAGGGGACCTGAGAGGGTGGTCCCCCACAC
TGGCACTGAAACACGGGCCAGACACCTACGGGTGGCAGCAGTAGGGAATATTGCACAATGGGCGAAAGCC
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>PL 102 (1006)

CCCTAATACTGCAGTCGAACGAGGAGCCATTGTATTCGTACGGTGGTTTCCTAGTGGCGAACGGGTGAGT
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CTCAAAGGAATTGACGGGGACCCGCACAAGCAGCGGAGCGTGTGGTTTAATTCGATGCTACGCGAAGAAC
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PL 119 (938)

GGGTCGGCGAGCGGCGAACGGCTGAGTAACACGTTGGAACCTGCCCCAAAGTGAGGGATAACTACGAGAA
ATCGTAGCTAATACCGCATATGCCAGAAATGGTAAAGCTTCGGCGCTTTGGGAGGGGCCTGCGACGTATC
AGGTAGTTGGTGAGGTAATGGCTCACCAAGCCTCTGACGCGTAGCTGGTGTGAGAGCACGACCAGCCAGA
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>PL 180 (1036)

CGCTAGCGGCAGGCCTAATACATGCAAGTCGAGGGGCAGCATCATCGCTTGCGATGGATGGCGACCGGCG
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CCATGGTACCATAAAGTAGCATTGCTTAATGGTTAAAGATTTATTGGTAAAGGATGGGCATGCGTCTGAT
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TGGCACTGAGATACGGGCCAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGGTCAATGGGCGAGAGCC
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GGAGTACGCTCGCAAGAGTGAAACTCAAAGGAATTGACGGGGGTCCGCACAAGCGGTGGAGCATGTGGTT
TAATTCGATGATACGCGAGGAACCTTACCTGGGCTAGAATGCGAGGGAATGATTCAGAGATGGATCAGTC
TTCGGACCCGAAGCAAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTT

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>PL 163 (988)

TTGAAAGCGGCGGACGGGTGAGTAACGCGTGGGTAACCTGCCCATACAAGGGATAGCCACTGGAAACGGT
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CCTGATGCAGCAACGCCGCGTGAGCGAAGAAGGCTTTCGGATCGTAAAGCTCTGTCCTAAGGGAAGAAAA
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TTAGTTGA

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>PL 140 (953)

GGTCCTGGAAAGCGGCGAACGGGTGAGTAACGCGTGGGAAACCTGCCCCCGAGATGGGGATAACCCTCCT
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GCTAACGCATTAAGTGCTCCGCCTGGGGAGTACGCCCGCAAGGGTGAAACTCAAAGGAATTGACGGGGGC
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>PL 165 (1032)

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AAAGCCTGACACAGCGACGCCGCGTGGGGGATGAAGGCCTTCGGGTCGTAAACCCCTTTTCTCAGGGAAG
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CTAGCGTTATCCGGATTTACTGGGCGTAAAGAGGGCGTAGGCGGTTTTTCAAGTCGGATGTAAAATCTCC
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CCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCGTGTGGTTTAATTCGAT
GCAACGCGAAGAACCTTACCAAGGCTTGACATGTCGGAAGTAGCCACCCGAAAGGGAAGCCACCTGTAAG
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>PL 141 (970)

GGCGAACGGGTGAGTAACGCGTAAGTAACCTGCCCCCAAGTAGGGATAACTCTAAGAAATTGGAGCTAAT
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CAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCGTGTGGTTTAATTCGATGCA
ACGCGAAGAACCTTACCAAGGCTTGACATGTCGGAAGTAGCGACCCGAAAGGGGAGCCACCTGTTAAGTC
AGGAGCCGACACAGGTGCTGCATGGCTGTCGTCGCTCGTGCCGTGAGGTGTTGGGTTAAG

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>PL 184 (1028)

AAACGCTGGCGGCGTGCTTTATGCATGCAAGTCGAACGGTTTCGATTTATCGGAACAGTGGCAGACGGGT
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ATTTATTGGGCGTAAAGAGGGCGTAGGCGGCTTTTCAAGTCGGATGTAAAATCTCCCGGCTCAACTGGGA
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GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACACCGTAAACGATGGACACTAGGTATAGGGA
GTATCGACCTCTCTGTGCCGAAGCTAACGCTTTAAGTGTCCCGCCTGGGGATTACGGCCGCAAGGCTAAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCGTGTGGTTTAATTCGATGCAACGCGAAGAA
CCTTACCAAGGCTTGACATGTCGGAAGTAGCGACCCGAAAGGGAAGCGACCTGTTAAATCAGGAGCCGTC
ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTGGGTT

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>PL 134 (1012)

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CCCAGACTCCTACGGGAGGCAGCAGCAAGGAATATTGTGCAATGGGCGAAAGCCTGACACAGCGACGCCG
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CCTCTCTGTGCCGAAGCTAACGCGTTAAGTGTCCCGTCTGGGAACTACGGCCGCAAGGCTAAAACTCAAA
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>PL 64 (1113)

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>PL 155 (1030)

TAAACCTGGCGCTGCTTATGCTGCAGTCGAACGTTCTCTTTGGTAAGGATAGTGGCGAAGGGTGAGTAAC
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GATACTGTTGGGCTAGAGGGCAGCAGGAGGAGGTGGAATTCCCGGTGTAGTGGTGAAATGCGTAGATATC
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CGAACAGGATTAGATACCCTGGTAGTCCACACTGTCAACGATGGACACTAGGTATAGGGAGTATCGACCC
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AATTGACGGGGGCCCGCACAAGCAGCGGAGCGTGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAG
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>PL 71 (1093)

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>PL 194 (1007)

CGGCGCGCCTTACGCATGCAAGTCGCACGGACCTTCGGGCCAGTGGCGGACGGCTGAGTAACGCGTAGGT
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AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAACACGTAGGTGGCAAGCGTTGTCCGGATTTACTGGG
CGTAAAGGGCGTGTAGGCGGTTCGTTAAGTCCGGTGTGAAATCTCCCGGCTCAACTGGGAGCGGCCATCG

GATACTGGCGAACTAGAGGGGGGCAGAGGAAGGCGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATC
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CAAACCGGATTAGATACCCGGGTAGTCCACGCCCTAAACTGTGGATGCTAGGTGTGGGGGGTGTCGACCC
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>PL 61 (965)

CGGACGGGTGCGTAACACGTGAGCAACCTGCCTGGAAGAGGGGGATAACCCGGGGAAACCCGGGCTAATA
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ATGGGCTCGCGGCCTATCAGCTTGTTGGTGAGGTAACGGCTCACCAAGGCGATGACGGGTAGCTGGTCTT
AGCGGATGATCAGCCACACGGGGACTGAGACACGGCCCCGACTCCTACGGGAGGCAGCAGTGGGGAATTT
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GTCAGGAGGGAAGAACTGATTCGGGTTAATACCCTGGATCTCTGACGGTACCTCCAGAGAAGCGCCGGCT
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GCGCAGGCGGCTTGTTAAGTCCTGTGTGAAAACCCGCGGCTCAACCGCGGAATGCCACAGGATACTGGCG
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>PL 81 (1174)

CCCAATGTGGAATTCGCCCTTAGAGTTTGTCCTGGCTCAGAACGAACGCTGGCGGCGTGGATTAGGCATG
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CAAATGGTGGAGCATGTGGTTTAATACGACGATACGCGTGGAACCTTACCAGGACTTGACATTTAGTGGA
AAGCCTATGGAAACATAGGCCCTCCCTTCCGAGACTGCTAAACAGGTGTTGCATGGCTGTCGTCAGCTCG
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>PL 188 (1069)

ACGCTGGCGGCGTGGATTAGGCATGCAAGTCGAACGATTCTTTAAGAGGGGGACAGTGGCAAACGGGTGA
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>PL 76 (1209)

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>PL 131 (1004)

GGCGTGGTTTAGGCATGCAAGTCGTACGATCGTAGGATAGTGGCAAAAGGGTGAGTAACACGTGGGTAAT
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>PL 99 (962)

CTTGCACTCTCTGAGAGTGGCGGACGGGTGAGTAACACGTAGGTGATCTACCTCTGGATAGGGGATAGCC
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CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGATACGCGAGAAACCTTACCAGGGCTTGACATGTA
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>PL 84 (1005)

TGCTGGCACTCTGAGAGTGGCGGACGGGTGAGTAACACGTAGGTGATCTACCTCTGGATAGGGGATAGCC
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>PL 66 (954)

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>PL 181 (1099)

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>PL 91 (988)

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>PL 128 (1019)

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TGGGGAGTACGGTCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG
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>PL 160 (927)

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>PL 206 (1004)

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>PL 90 (1149)

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>PL 77 (1153)

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TTTAATTCGACGCAACGCGAAGAACCTTACCTGGGTTTGACATGCACGGGACAGGAGCAGAGACGTTCCC
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>PL 172 (1068)

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>PL 79 (1160)

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>PL 167 (974)

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>PL 107 (983)

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>PL 152 (978)

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>PL 106 (1007)

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>PL 80 (1044)

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>PL 197 (1072)

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>PL 85 (1008)

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>PL 126 (935)

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EU071385
>PL-19(1064)
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TGTCGTCAGCTCGT

5. 16S rRNA sequences of Anaerobic bacteria
>gi|166210327|gb|EU407233.1| Staphylococcus gallinarum strain AN42 16S ribosomal RNA gene, partial sequenceAATCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTTCCCCTTCGGGGGACAAAGTGAC
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CCCTTAAACTTAGTTGCCATCATTAAGTTGGGCACTCTACGTCGACTGCCGGTGACAAACCGGAGG
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AATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGT
AGTCTGCAACTCGACTACATGA

>gi|166210326|gb|EU407232.1| Pseudomonas geniculata strain AN41 16S ribosomal RNA gene, partial sequence
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GGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAG
GAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTACTACAATGGT
AGGGACAGAGGGCTGCAAGCCGGCGACGGTAAGCCAATCCCAGAAACCCTATCTCAGTCCGGATT
GGAGTCTGCAACTCGACTCCATGAAG

>gi|166210325|gb|EU407231.1| Bacillus sp. AN38 16S ribosomal RNA gene, partial sequence
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CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTC
AGTTGGGCACTCTAAGGTGACTGCCGGTGACCAAACCGGAGGAAGGTGGGGATGACGTCAAATCA
TCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGAACAAAGGGTCGCGAAGCCGC
GAGGTGAAGCCAATCCCATAAATCCATTCTCAGTTCGGATTGCAGGCTGCA

>gi|166210324|gb|EU407230.1| Staphylococcus arlettae strain AN37 16S ribosomal RNA gene, partial sequence
TCTTGACATCCTTTGACCACTCTAGAGATAGAGCTTTCCCCTTCGGGGGACAAAGTGACAGGTGGT
GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAA
ACTTAGTTGCCAGCATTTAGTTGGGCACTCTAGGTTGACTGCCGGTGACAAACCGGAGGAAGGTGG
GGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAATACAA
AGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGC
AACTCGACTACATGAA

>gi|166210323|gb|EU407229.1| Bacillus sp. AN22 16S ribosomal RNA gene, partial sequence
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AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGC
ATTTAGTTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAA
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>gi|166210321|gb|EU407227.1| Bacillus sp. AN13 16S ribosomal RNA gene, partial sequence
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TGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC
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GCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGT

>gi|166210319|gb|EU407225.1| Vibrio sp. AN6 16S ribosomal RNA gene, partial sequence
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CGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCC
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GCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTC
GACTCCATGAA

>gi|166210318|gb|EU407224.1| Bacillus drentensis strain AN4 16S ribosomal RNA gene, partial sequence
GTCTTGACATCCTCTGACACTCCTAGAGATAGGACGTTCCCCTTCGGGGGACAGAGTGACAGGTGG
TGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTG
ATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTG
GGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACA
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CAACTCGCCTGC

>gi|166210317|gb|EU407223.1| Vibrio sp. AN3 16S ribosomal RNA gene, partial sequence
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GGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATAC
AGAGGGCAGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGTGCGTCGTAGTCCGGATTGGAGTC
TGCAACTCGACTCCA

>gi|166210316|gb|EU407222.1| Vibrio sp. AN2 16S ribosomal RNA gene, partial sequence
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CGAGTAATGTCGGGAACTCCAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTC
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AGCTAGCGATAGTGAGCGAATCCCAAAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACT
CCATG

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ORIGINAL PAPER

16SrRNA and enzymatic diversity of culturable bacteria from the sediments of oxygen minimum zone in the Arabian Sea

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Abstract Sediment underlying the oxygen minimum zone of the eastern Arabian Sea is rich in organic matter. Bacteria in this sediment–water interface are of great ecological importance as they are responsible for decomposing, mineralizing and subsequent recycling of organic matter. This study has for the first time addressed the phylogenetic and functional description of culturable bacteria of this region. Genotypic characterization of the isolates using amplified rDNA restriction analysis (ARDRA) followed by 16SrRNA sequencing grouped them into various phylogenetic groups such as Firmicutes, Gammaproteobacteria, Low G+C Gram positive bacteria, Actinobacteria and unaffiliated bacteria. Among the enzyme activities, phosphatase was predominant (52%) and was associated with all the phylotypes followed by amylase (37%) and gelatinase (33%). These hydrolytic enzymes were expressed at a wide range of temperature and pH. Firmicutes expressed most of the hydrolytic activities, consistent with a role in degradation of organic matter. Multiple enzyme expression (≥3) was exhibited by Actinobacteria (100%), followed by unaffiliated group (62.5%) and Firmicutes (61.5%). Besides hydrolytic enzymes, the phylotypes also elaborated functional enzymes such as nitrate reductase and catalase (58 and 81% of the isolates, respectively). In the oxygen minimum zone, the diversity was high with 28 phylotypes. Culturable bacterial assemblages encountered were *Bacillus* sp., *Halobacillus* sp., *Virgibacillus* sp., *Paenibacillus* sp., *Marinilactibacillus* sp., *Kytococcus* sp., *Micrococcus* sp., *Halomonas* sp. and *Alteromonas* sp. The high diversity and high percentage of extracellular hydrolytic enzyme activities of the culturable bacteria reflect their important ecological role in biogeochemical cycling of organic matter in the oxygen minimum zone.

Keywords Bacteria · 16SrRNA · Extracellular hydrolytic enzymes · Oxygen minimum zone · Sediment · Arabian Sea

Abbreviations
OMZ Oxygen minimum zone
AS Arabian Sea

Introduction

Oxygen minimum zones (OMZ) are unique ecosystems in the most productive coastal areas and open oceans. The high oxygen demand along with flux of

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labile organic matter and limited intermediate water ventilation lead to an intense, basin wide mid depth layer of oxygen depleted waters (<0.5 ml l^{-1} dissolved oxygen concentration), which impinges on the continental slope, extending from water depths of 150 to 1500 m (Wyrtki 1962; Kamykowski and Zentera 1990; Helly and Levin 2004; Cowie 2005). Naqvi et al. (2006) have shown an increase in OMZ as oceans are losing dissolved oxygen at high rates in intermediate water depths. Arabian Sea (AS) which covers 59% of oxygen minimum area of the world oceans (Helly and Levin 2004) is known for high primary productivity and vertical decrease in the dissolved oxygen concentrations in the water column from 0.5 ml l^{-1} to non-detectable levels. Sediment underlying the OMZ is rich in organic matter and a primary site of sedimentary organic carbon burial with high sedimentation rates (Hedges and Keil 1995). The organic matter in the sediments below the OMZs is contributed by the detritus exported from the overlying water column along with considerable fluvial and aeolian inputs (Cowie 2005). By virtue, organic particles get modified by bacterioplankton during sinking to deeper depths so these settled organic matters do not contain any of its original components. The aerobic heterotrophic bacteria are capable of producing certain hydrolytic enzymes depending on the supply and nature of organic matter (Kristensen 1995). Being a zone of high organic matter, we hypothesize that the continental margin sediments underlying the OMZ harbour numerous bacteria with hydrolytic enzymes responsible for transformation of old, refractory and structurally complex organic matter that settles on the ocean floor. Very little effort has been made to understand this microbial community and its function. Though 16SrRNA based techniques are widely used and provide taxonomic classification in a culture-independent approach (Woese et al. 1985; Pace et al. 1985; Amann et al. 1995) they do not elaborate their metabolic activities in biogeochemical cycles. In this study we have made an attempt to understand the diversity using 16SrRNA and extracellular hydrolytic enzyme activities of the aerobic culturable heterotrophic bacteria inhabiting the OMZ sediment of the eastern Arabian Sea (200, 500 and 1000 m contours) along the western continental margin of India.

Materials and methods

Collection of sediment samples

Sampling was carried out during an inter-disciplinary cruise of FORV Sagar Sampada (#254). The sampling stations were in the western continental margin of India (Fig. 1). Samples were collected along a transect off Goa at 200, 500 and 1000 m depth contours using a box corer. The collected sediment samples were sub-sampled using sterile syringes and stored at 4–5°C until analysis.

Isolation of bacteria

Bacteria were isolated after serial dilution of sediment samples and plating on standard nutrient medium (Hi Media, India) prepared in aged sea water. The composition of the medium was peptic digest and sodium chloride −5 g each; beef and yeast extract −1.5 g each and 15 g of agar per litre of seawater. The plates were monitored at 24-h interval for a period of 1 week in order to isolate even the slow growing bacteria. Morphologically different isolates (150) from the three depth zones were isolated and purified for genotypic and enzymatic studies.

Genotypic characterization of isolates

Genomic DNA was extracted and purified from bacterial cultures grown in nutrient broth (Hi Media, India) using standard protocol (Ausubel et al. 2003).

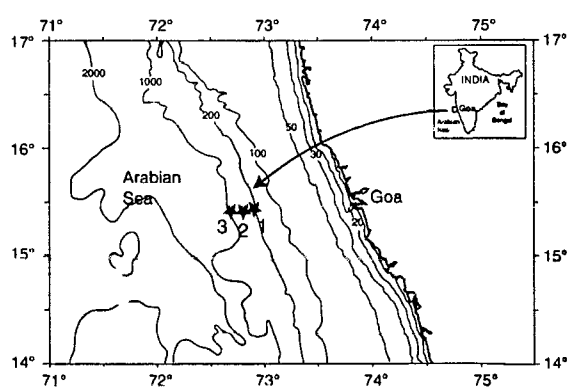

Fig. 1 Location of sampling stations

16SrRNA genes were amplified using the primers 8F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) (Lane 1991), using thermal cycler (Eppendorf, Germany). Amplified Ribosomal DNA Restriction Analysis (ARDRA) was performed to group the isolates into different phylotypes. 16SrRNA gene was digested using the four base-cutting restriction enzyme *Hae*III (1 U) at 37°C for 1 h. The restricted products were electrophoresed in 1.2% agarose gel and viewed on Gel Imaging System (Kodak Gel Logic). The patterns in the gels were compared using Bionumerics Software Version 4.6 (Applied Maths, Belgium). Representative phylotypes were sequenced and compared with sequences in NCBI database using BLAST software (www.ncbi.nih.nlm.gov). The sequences were submitted in the GenBank (GenBank accession numbers: GQ 900674–GQ 900701).

Screening of enzymes

Enzymatic activities were determined by agar diffusion method as per the protocols given in manual of general bacteriology (Gerhardt et al. 1981). Enzyme activities were tested in nutrient agar medium of pH 7.5 in sea water amended with respective substrates (amylase—0.2% soluble starch; gelatinase—0.4% gelatin; nitrate reductase—0.1% potassium nitrate; citrate synthase—0.2% sodium citrate). The cultures that showed growth or clearing zones when tested with respective reagents were considered as positive cultures. DNase activity was determined by the growth of blue colonies on DNase test medium while phosphatase activity was determined by the development of pink colouration on phenolphthalein agar when subjected to ammonia fumes. Urease activity was detected by pink colouration of urease agar medium. Deposition of the calcium chloride-lipid complex in degradative zones detected in medium amended with 1% Tween 80 and Tween 20 showed esterase and lipase activity, respectively. Cytochrome oxidase and catalase activities were determined with *N,N,N,N*-tetramethyl-*p*-phenyl-diaminedihydrochloride and hydrogen peroxide solutions, respectively. Utilization of amino acids by the isolates was assessed by their ability to grow in 1% of ornithine, arginine, lysine and tryptophan. The isolates were also tested for enzyme activity at pH 6 and 7 and temperature 4, 20 and 28°C. Data from the three stations were pooled for a homogenous representation of the OMZ region.

Results and discussion

Phylotypes distribution

The sediment–water interface is a zone of high microbial abundance and processes/fluxes occurring are of great biogeochemical importance especially in the continental margin in the AS (Walsh 1991) creating unique sedimentary environment. Though earlier study in Pacific Ocean has reported low species richness (Levin 2003), cultivation-independent studies of the sediments in OMZs of Pacific and Atlantic oceans have shown enormous bacterial diversity (Schippers and Neretin 2006; Liu et al. 2003a, b). Further, this technique has facilitated the detection of uncultured bacteria which at times constitute 99% of the total bacteria (Olsen et al. 1986; Schloss and Handelsman 2004). Notwithstanding the prospective of cultivation-independent techniques to provide taxonomic identification; in this study we have adopted pure culture technique to link bacterial species with their functions. In the present study, ARDRA of the culturable bacterial isolates categorized them into various phylotypes which were then sequenced for identification. ARDRA analysis showed 28 phylotypes, affiliated to Firmicutes, Proteobacteria, Low G+C Gram positive bacteria and Actinobacteria (Table 1). Being the first report from OMZ, the results are being discussed with those obtained using different methods of direct analysis of 16SrDNA of culturable bacteria of sediments from other ecosystems such as Shimokita peninsula, Southern Okinawa trough and mangroves (Kobayashi et al. 2008; Dang et al. 2009; Dias et al. 2009). Unlike the deep sea sediments of Pacific Ocean where the dominant groups were Gammaproteobacteria and Alphaproteobacteria (Kato et al. 1996; DeLong et al. 1997; Wang et al. 2004), the OMZ sediment in the AS was dominated by Firmicutes. Interestingly, most of the sequences had high similarity (>98%) with earlier reported culturable bacterial strains of the deep sea sediment (Dang et al. 2009). However, few sequences showed lesser similarity and these were categorized as unaffiliated bacteria and the possibility of new species endemic to this region cannot be ruled

out. The genera obtained from the OMZ sediments were *Bacillus* sp., *Halobacillus* sp., *Virgibacillus* sp., *Paenibacillus* sp., *Marinilactibacillus* sp., *Kytococcus* sp., *Micrococcus* sp., *Halomonas* sp. and *Alteromonas* sp. (Table 1). *Bacillus* was the dominant genus in the OMZ sediment unlike in Okinawa Trough and southwestern Okhotsk Sea where *Halomonas* was the major group (Dang et al. 2009; Inagaki et al. 2003). In addition, Low G+C Gram positives and unidentified groups were also obtained. High bacterial diversity was reported for OMZ waters of the AS by culture independent method (Fuchs et al. 2005). However in the sediment, most of cultivable bacteria were restricted to Firmicutes and a few representatives of other phyla, in spite of the fact that the sediment was rich in organic matter. This may be due to the competition for the variable and limited labile substrates that reach the bottom of the sea from the overlying euphotic zone especially in the slope of the AS. The sediment carbohydrate content gives the best idea of concentration of readily assimilable organic matter which ranged from 1.33 to 17.99 mg g^{-1} of dried sediment with higher concentration in the middle region of the shelf and the least at the base of the slope of the AS (Bhosle et al. 1978). Occurrences of a few dominant species have been reported form different areas, in spite of different methodologies used for typing the isolates (Giuliano et al. 1999; Uphoff et al. 2001). The occurrence of a few dominant phylotypes may be habitat adaptations enabling them to survive in the OMZ environment.

Extracellular enzymatic activity and its ecological implications

Studies have revealed that the oxic and anoxic bacteria are able to degrade organic matter at similar rates (Kristensen 1995; Hall et al. 1998). However, aerobic heterotrophic bacteria are capable of producing certain hydrolytic end products of their metabolism that are effective in initiating degradative pathways of old, refractory and structurally complex organic matter (Mayer 1994; Hedges and Keil 1995; Kristensen 1995; Hall et al. 1998) compared to anaerobic bacteria and thus play an important role in nutrient cycling. The present study covers the continental slope of the eastern AS which is an excellent site of deposition and preservation of organic carbon (>4%) (Paropkari et al. 1992). The unsuitability of the sedimentary organic matter for direct assimilation by the bacteria leads to the production of several extracellular enzymes that makes it more assimilatory (Hoppe 1991; Meyer-Reil 1991).

Among the studied enzymes, 52% of the OMZ isolates expressed phosphatase (Fig. 2a). Phosphatase is known to be prevalent in marine bacteria (Hoope 2003) and these bacteria have been reported in sediments of east coast of India (Ayyakkannu and Chandramohan 1971). An increased level of phosphate concentration in the pore waters of suboxic continental margins is due various factors such as microbial degradation of organic matter (Froelich et al. 1988; Rottenberg and Berner 1993), desorption from iron oxides (Sundby et al. 1992; Slombe et al. 1998) and dissolution of fish debris (Suess 1981). It has been reported that benthic phosphorous regeneration and consequent dissolution of phosphate in the sediment–water interface are observed under oxygen-depleted conditions (Ingall and Jahnke 1994). The occurrence of DNase among the OMZ isolates provides a source of phosphorous by degrading the extracellular DNA in the sediment. Dell'Anno and Corinaldesi (2004) have demonstrated that the extracellular DNA concentration as well as the degradation of DNA in sediments was higher than that in water. Also they have suggested that preservation of DNA in deeper sediment layers may be favoured in the benthic systems with higher sedimentation rates. It is not only a source of phosphorous but also a suitable carbon and nitrogen source for prokaryotic metabolism (Jorgensen et al. 1993; Dell'Anno and Danovaro 2003). Therefore phosphatase and Dnase are the two enzymes that play an important role not only in phosphorous cycling but also in the carbon and nitrogen cycling of OMZ sediments.

Carbohydrates and proteins generally form about <90% of the biopolymeric carbon in marine sediments (Poremba 1995; Azam 1998). In the Chilean margin, it has been reported that most of the photosynthetically produced particulate protein in the photic zone gets degraded in the upper water column up to 300 m and only 2% reaches the sediment–water interface (Pantoja et al. 2004). Studies by Bhosle et al. (1989) in shallower sediments of Arabian Sea in the western continental margin, have demonstrated that the total carbohydrate in the sediments was high depending on the season. Furthermore, Alagarsamy (2003) has reported that

Table 1 Nearest neighbours of the phylotype representatives in the GenBank

Phylotype representative	Phylogenetic group	Accession numbers	Nearest neighbour	Similarity (%)
G12-15A	Firmicutes	GQ 900675	*Halobacillus* (FJ237396)	97
G12-10A	Firmicutes	GQ 900678	*Bacillus licheniformis* (FN298317)	99
G12-11B	Firmicutes	GQ 900681	*Bacillus* sp. (DQ078995)	96
G12-8A	Firmicutes	GQ 900685	*Halobacillus mangrovi* strain (DQ888316)	99
G9-1A	Firmicutes	GQ 900686	*Halobacillus* sp. (AB305183)	99
G9-2	Firmicutes	GQ 900687	*Bacillus* sp. (FJ977613)	89
G9-6	Firmicutes	GQ 900688	*Bacillus* sp. (DQ868675)	99
G9-15A	Firmicutes	GQ 900689	*Virgibacillus* sp. (FJ477404)	97
G9-18B	Firmicutes	GQ 900690	*Halobacillus* sp. (AB305183)	99
G9-12	Firmicutes	GQ 900693	*Paenibacillus* sp. (DQ854976)	99
G6-4	Firmicutes	GQ 900694	Uncultured Firmicutes (EU753617)	98
G6-13A	Firmicutes	GQ 900696	*Halobacillus* sp. (AB198765)	96
G6-10	Firmicutes	GQ 900698	*Marinilactibacillus* sp. (DQ344859)	96
G12-23A	Actinobacteria	GQ 900676	*Kytococcus sedentarius* (AY881239)	98
G12-22B	Actinobacteria	GQ 900677	*Micrococcus* sp. (EF491952)	97
G12-14A	Low G+C Gram positives	GQ 900683	Low G+C Gram positive organism (AB002642)	92
G12-12A	Low G+C Gram positives	GQ 900684	Low G+C Gram positive organism (AB002643)	94
G6-15	Low G+C Gram positives	GQ 900695	Low G+C Gram-positive bacterium (AF348730)	99
G6-16	Proteobacteria	GQ 900700	*Halomonas* sp. (EU781513)	98
G6-9	Proteobacteria	GQ 900701	*Alteromonas hispanica* (EU529840)	99
G12-11A	Unidentified bacteria	GQ 900674	Glacial ice bacterium (AF479338)	99
G12-21B	Unidentified bacteria	GQ 900679	Uncultured bacterium (FJ479457)	99
G12-21A	Unidentified bacteria	GQ 900680	Uncultured compost bacterium clone (DQ346631)	99
G12-1A	Unidentified bacteria	GQ 900682	Marine sediment bacterium (AY936960)	95
G9-15B	Unidentified bacteria	GQ 900691	Uncultured compost bacterium (DQ346631)	97
G9-5	Unidentified bacteria	GQ 900692	Marine sediment bacterium (AY911022)	94
G6-8	Unidentified bacteria	GQ 900697	Uncultured bacterium (AM183073)	93
G6-14	Unidentified bacteria	GQ 900699	Uncultured compost bacterium (DQ346631)	97

the contribution of hydrolysable carbohydrates and amino acids to the total organic carbon in Oman margin sediments is only 10% and the remaining being recalcitrant in nature. Proteolytic and starch hydrolyzing microflora are well documented in non-OMZ sediments of India (Nair et al. 2000; Raghukumar et al. 2006). In the OMZ sediments amylase and gelatinase were expressed by 37 and 33% of the isolates, respectively reflecting the presence of these biopolymers. These biopolymeric compounds are degraded by the benthic bacteria extracellularly and their oligomeric products become available as substrates for the microbial uptake. Studies have shown that marine heterotrophic bacteria are capable of synthesizing extracellular proteases which breakdown proteins into smaller units that can act as immediate precursors for protein synthesis and other microbial cell metabolism (Pantoja and Lee 1994; Mudryk and Podgorska 2006). Studies have also shown that the lipolytic bacteria are of great significance as they aid in the processes of modification and transformation of lipid compounds (Wakeham

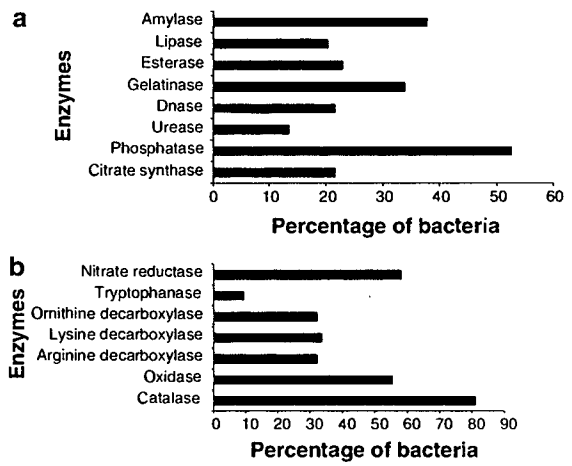

Fig. 2 Percentage of bacteria producing **a** hydrolytic enzymes in OMZ, **b** other enzymes in OMZ

1995). In situ lipase activity experiments conducted by Boetius and Lochte (2000) in the North East Atlantic demonstrated that the lipids which are the nutritional compounds of the deep sea microorganisms, get rapidly consumed during sedimentation in the water column. However, in the AS, bacterial and terrestrial fatty acids are enriched in the sediments within the oxygen minimum zone (Schulte et al. 2000). Thus the production of lipase enzyme by the bacterial isolates reflects the high lipase activity in the sediments (Middelboe et al. 1995). The aerobic isolates also expressed hydrolytic enzymes at wide range of pH and temperature (Table 2). Maximum amylase activity was at 28°C both at neutral (44%) and acidic (30%) conditions. Interestingly, about 21 and 48% of the isolates were able to produce amylase enzyme at wide range of temperature and pH, respectively. However, the highest Dnase activity was observed with 52% positive isolates at 20°C in neutral conditions, while high acidic Dnase activity was recorded at 4 and 28°C (12% each), respectively. Other enzymes such as arginine decarboxylase, lysine decarboxylase, ornithine decarboxylase, tryptophanase (lyases) and the oxidoreductases such as catalase, oxidase and nitrate reductase were also produced by most of the OMZ isolates (Fig. 2b). About 32, 33 and 32% of OMZ isolates expressed arginine, lysine and ornithine decarboxylases, respectively and 58% of the isolates were able to reduce nitrate. Nitrate is an abundant electron acceptor in the Arabian Sea (Devol et al. 2006). It has also been demonstrated that denitrification is the major mode of respiration in the sub-oxic zones of the Arabian Sea (Codispoti et al. 2001; Naqvi 1994). Inagaki et al. (2003) and Biddle et al. (2005) found that aerobic bacteria from the deep sea sediments of Peru margin and Okhotsk Sea formed a major part of heterotrophs and can be facultative in nature. Oxidase enzymes are generally present in bacteria which possess an aerobic type of metabolism for the regeneration of NAD in the respiratory system (Nishiyama et al. 1997). Oxidase was present in more than 50% of the OMZ isolates. Later studies on these enzymes revealed the presence of NADH oxidases belonging to peroxiredoxin oxidoreductases that are not only involved in the regeneration of NAD, but also in scavenging of peroxides (Poole et al. 2000). Thus, the anaerobic or facultative anaerobic bacteria without respiratory systems or peroxide scavenging systems are able to survive in the aerobic conditions with the help of these enzymes (Niimura et al. 1990). About 81% of the OMZ isolates were able to express catalase enzyme. Rocha and Smith (1995) have demonstrated that this enzyme helps in the detoxification of oxygen byproducts even in anaerobic conditions. Such diverse and abundant hydrolytic enzyme producers have been reported from organic and methane rich deep sub seafloor of off Shimokita Peninsula (Kobayashi et al. 2008). These enzyme activities signify the role of culturable bacteria in the degradation and mineralization of organic matter and the production and activity depend primarily on the availability, distribution, type and concentration of the organic substrates (Boetius 1995). These complex organic matters may be relatively fresh or partially degraded organic carbon (Paropkari et al. 1992).

Among the various groups, Firmicutes was the major enzyme producing group expressing all the enzymes tested and was found to be metabolically versatile (Fig. 3). Though Actinomycetales are

Table 2 Percentage of bacteria producing enzyme at different temperature and pH

Enzymes	Temperature (4, 10, 28°C)	pH (6, 7, 8)
Amylase	21	48
Lipase	3	24
Gelatinase	3	15
DNase	9	21

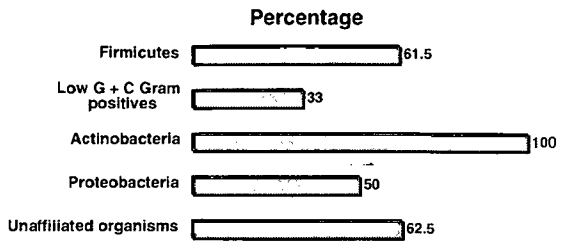

Fig. 3 Multiple hydrolytic enzymes (≥3) by phylogenetic groups

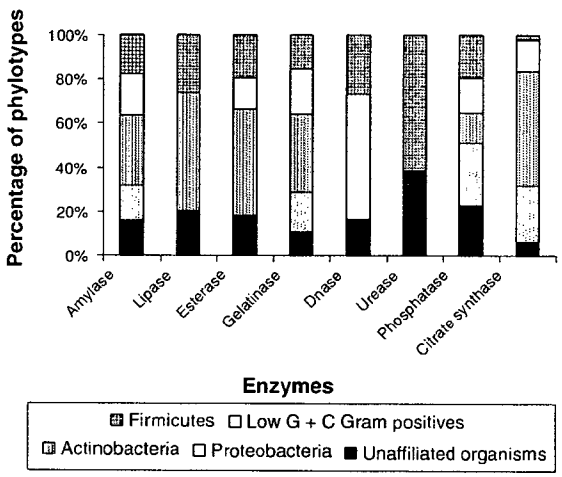

Fig. 4 Extracellular hydrolytic enzyme production by different phylogenetic groups

known to be a producer of numerous important enzymes (Nakai et al. 1987), in the present study they were restricted to the production of only six out of the eight hydrolytic enzymes tested. Interestingly, 61.5% of Firmicutes exhibited multiple enzyme expression (≥3) (Fig. 3). The unidentified group also formed a major producer of all the enzymes tested and exhibited multiple expressions (62.5%) with only marginal difference with the Firmicutes. Though Actinomycetales produced only six out of the eight hydrolytic enzymes tested, highest multiple expressions of enzymes were shown by this group with all the representatives expressing three or more enzymes. Proteobacteria showed the highest phosphatase activity (100%) followed by unaffiliated bacteria (81%). Phosphatase enzyme production has been reported in heterotrophic bacteria falling in Proteobacteria and Firmicutes (Chróst and Overbeck 1987). The phylogenetic group with the highest Dnase activity was shown by Low G+C Gram

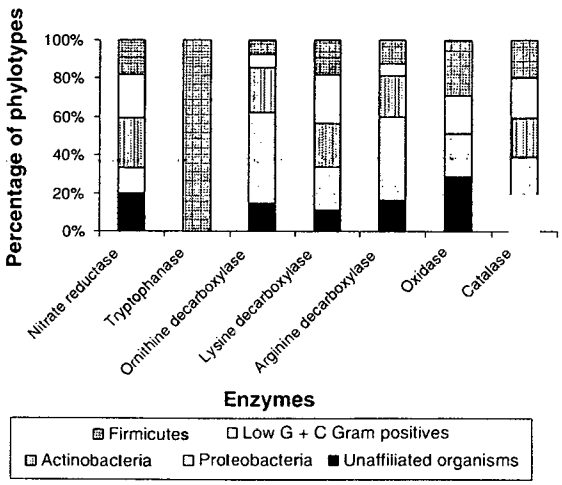

Fig. 5 Percentage of the phylogenetic groups exhibiting other enzymes

positive group (42.8%). Amylase activity was observed in all phylogenetic groups with the highest in the Actinobacteria (100%) followed by Low G+C Gram positives (57%). A similar trend was observed in gelatinase activity. Though esterase activity was observed in four phylogenetic groups lipase activity were restricted to only three phylogenetic groups. Interestingly, Actinobacteria formed the dominant group showing esterase and lipase activity (Fig. 4). The percentage of phylogenetic groups exhibiting other enzymes is depicted in Fig. 5.

The significance of metabolically active aerobic culturable bacteria in cycling of organic matter has been reiterated in our present study. The OMZ sediments of the AS harbour diverse bacteria which have enormous catabolic efficiencies. The supply of organic material to the OMZ sediment is a key factor in stimulating the production of extracellular hydrolytic enzymes. These hydrolytic enzymes are responsible for organic matter mineralization and thereby play important role in in situ biogeochemical processes.

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ORIGINAL PAPER

16S rRNA-based bacterial diversity in the organic-rich sediments underlying oxygen-deficient waters of the eastern Arabian Sea

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Abstract The eastern Arabian Sea has a unique and permanent oxygen minimum zone (OMZ) that extends along the western continental margin of India. The sediment below this region is rich in organic matter. This study describes the bacterial community structure and diversity in OMZ sediments of the eastern Arabian Sea (AS) through 16S rRNA clone library analysis. Phylogenetic analysis of the sequences demonstrated that phylum Proteobacteria (52%), followed by Planctomycetes (12.7%), Chloroflexi and an unidentified bacterial group (8.8% each) were represented in the library. Deltaproteobacteria was the dominant class (62.5%) in phylum Proteobacteria with clones falling in orders Syntrophobacterales and Desulfovibrionales. Few minor phylogenetic groups, corresponding to Spirochetes, Firmicutes, Acidobacteria and Verrucomicrobia were found. Unidentified candidate groups falling in OP11, OP8 and OP3 were represented by 0.9, 2.9 and 3.9%, respectively and two clusters of the cloned sequences in this study showed very low identity to known sequences. This is the first report that discusses the phylogenetic groups in the OMZ sediments of eastern Arabian Sea individually and compares it with available data from marine hypoxic locales and water mass. LIBSHUFF statistics revealed high richness of the bacterial community of the Arabian Sea OMZ (AS-OMZ) compared to the other regions.

Keywords Bacterial diversity · Cultivation-independent · Oxygen minimum zone · Sediments · Eastern Arabian Sea

Introduction

Oxygen minimum zones (OMZ) are unique habitats with pronounced oxygen deficiency and are permanent features in the most productive regions of all oceans where dissolved oxygen concentrations at intermediate depths are as low as 0.5 ml l^{-1} (Kamykowski and Zentera 1990; Stramma et al. 2008). The seabed below the OMZ is a region of "large organic food falls" due to the deposition of particulate organic matter from the productive waters above, harbouring enormous and unique microbial biomass. Studying the diversity and distribution of natural microbial communities of marine sediments has been a long-standing challenge in microbial ecology. Over the last decade, the understanding of microbial diversity and dynamics in marine sediments has significantly increased due to the development of culture-independent methods which circumvented the associated culture biases (Pace et al. 1986; Giovannoni et al. 1990; Amann et al. 1995; Fuhrman and Davis 1997). Despite the two decades of advent of molecular techniques, studies have focussed mainly on functional gene diversity of anoxic and OMZ sediments (Schaefer et al. 2007; Schippers and Neretin 2006; Liu et al. 2003a, b). Thus very little has been studied on the bacterial diversity of OMZ.

The Indian Ocean covers ca 59% of the total global OMZ area, inclusive of the Arabian Sea and Bay of Bengal (Helly and Levin 2004). The AS-OMZ extends from 150 to

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1,500 m water depth and impinges on the continental slope (Wyrtki 1971; von Stackelberg 1972; Codispoti et al. 2005). Most of the bacteriological studies in the eastern Arabian Sea have been limited to pelagic region (OMZ and non-OMZ) dealing with abundance (Ramaiah et al. 1996; Ducklow et al. 2001), taxonomic diversity (Riemann et al. 1999; Fuchs et al. 2005) and functional gene diversity (Woebken et al. 2008; Ward et al. 2009). However, very little is known about the diversity and phylogenetic affiliation of bacteria in the underlying sediments of the AS-OMZ despite its contribution to the biogeochemical cycles (Boetius et al. 2000; Hedges et al. 2001). For better understanding of the ecosystem and the processes, it is imperative to carry out an exhaustive accounting of the contributing bacterial populations and their diversity. It is difficult to isolate and cultivate bacteria representative of natural communities and this limitation is even more pronounced in extreme and unique environments (Boivin-Jahns et al. 1996). Although diversity relies on the use of culture-independent methods, these techniques have biases (Farrelly et al. 1995; Acinas et al. 2004; Morasch et al. 2001; Chandler et al. 1997) which may influence the results. In spite of these caveats, among the various culture-independent tools 16S rDNA is, nevertheless, a widely employed technique to describe the composition of complex microbial community and to gain a descriptive overview of possible difference among communities (Yakimov et al. 2005). The present study provides a detailed 16S rDNA clone library analysis of the AS-OMZ sediment. This being the first study, has contributed to our fragmented knowledge on the biogeographical studies of sediment bacterial populations.

Methods

Collection of sediments

Sediment samples were collected from off Goa region of AS-OMZ, onboard FORV Sagar Sampada (Cruise # 254) from three different locations. Samples were handled aseptically and preserved in liquid nitrogen until DNA extraction.

DNA extraction from sediments

DNA was extracted using a modification of the method of Zhou et al. (1996). The crude DNA extract from different OMZ locations were purified using the UltraClean Soil DNA kit (MoBio Laboratories, USA), according to manufacturer's instructions except that the bead-beating step was avoided (Sorensen et al. 2004). The purified DNA was estimated using Nanodrop spectrophotometer (NanoDrop Technologies, Inc., USA) and pooled.

Clone library construction

The purified DNA was subjected to PCR for the amplification of 16S rRNA genes using the primers 27F: AGAGTTTGTCCTGGCTCA and 1090R: GCTCGTTGCGG GACTTAACC that amplify 1,099 bp fragment of 16S rDNA (Amann et al. 1995). Amplification was carried out in a 50 µl reaction mixture containing 50 ng of purified DNA (i.e., 5 µl from a 1:5 diluted genomic DNA), 3 mM concentration of $MgCl_2$, 2.5 mM of each dNTP, 10 pM of each primer and 3 U/µl of high fidelity Taq polymerase in 1X of Taq buffer (Bangalore Genei, India). The PCR conditions followed were: initial denaturation at 94°C for 5 min, 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 2 min and final extension at 72°C for 10 min. The PCR amplification was performed in a Thermal cycler (Eppendorf, Germany). The 16S rRNA gene amplicons were separated on 1.5% (w/v) agarose gel electrophoresed in Tris acetic acid-EDTA (TAE) buffer stained with 0.5 µg/ml final concentration of ethidium bromide (BioRad, USA). The gels were further visualized using the gel documentation system (Eastman Kodak Company, USA). The amplified PCR products were cloned using a TOPO TA cloning kit (Invitrogen, USA) designed for high cloning efficiency of long PCR products following manufacturer's instructions (Zhou and Gomez-Sanchez 2000). A total of 180 clones was obtained out of which 132 were positive. Recombinant plasmids were extracted using a QIA-prep Spin Miniprep kit (Qiagen, USA). The plasmids were screened for 16S rDNA inserts by PCR using the primers M13F: GTAAAACGACGGCCAG and M13R: CAGGAAACAGCTATGAC which are specific for the vector. Sequencing was carried out by a commercial company (BioServe Biotechnologies Pvt. Ltd, India).

Phylogenetic analysis

A total of 115 sequences obtained from the clone library were assembled using DNA Baser sequence assembly software Version 2 (DNA Baser, USA) and analysed for putative chimeric sequences using Chimera_Check software of Ribosomal Database Project (Cole et al. 2003) and Pintail Software (Ashelford et al. 2005). Similarities among sequences were calculated from the phylogenetic tree and sequences showing ≥97% sequence similarity were grouped into the same OTU (Stackebrandt and Goebel 1994). As the percentage of identical sequences was negligible (i.e. no two sequences showed 100% similarity) all the sequences were taken for the phylogenetic analysis. A total of 103 different

sequences were aligned using multiple sequence alignment tools Clustal X version 1.83 (Thompson et al. 1997). A PHYLIP tree was generated by neighbour-joining method and edited using MEGA (Kumar et al. 2004). Bootstrap analysis was carried out using 1,000 iterations.

Diversity analysis

The clone library of AS-OMZ was compared with those of other anoxic sediments from Gulf of Mexico, North Sea and South China Sea (28, 60 and 87 sequences, respectively) to understand the similarity between the libraries. Selected 16S rRNA gene sequences of the above libraries were retrieved from the Genbank database (NCBI) (Accession numbers: EU886379–EU886464 (Liao et al. 2009); FM179819–FM179914 (Wegener et al. 2008); AM745141–AM746064 (Orcutt et al. 2010). DOTUR software was used to assign sequences to operational taxonomic units (OTU), perform rarefaction analysis and calculation of diversity indices (Schloss and Handelsman 2005). Sequences with distance values of 0.03 ($\geq$97% sequence similarity) corresponding to the species level were grouped into a single operational taxonomic unit (Stackebrandt and Goebel 1994). Rarefaction curves with 95% confidence intervals were constructed by plotting the number of OTUs observed against the number of sequences sampled in each of the clone libraries. The diversity indices included the Shannon–Wiener diversity index, Simpson diversity index, non-parametric richness estimators such as Chao1 richness estimate and the abundance-based richness estimator (ACE).

LIBSHUFF analysis was performed to compare the four clone libraries for homologous C_X (D) and heterologous C_{XY} (D) coverage using the software weblibshuff (http://libshuff.mib.uga.edu) (Singleton et al. 2001). For DOTUR and LIBSHUFF analysis, all the sequences were aligned (multiple sequence alignment) using Clustal X in PHYLIP format, followed by the generation of distance matrices in the DNADIST program of PHYLIP (version 3.68), which were further used as the input files in the respective programs. The partial 16S rRNA gene environmental clone sequences were deposited in the Genbank database under accession numbers EU071379-EU071386, EU445347-EU445355 and FJ268480-FJ268573.

Results

Phylogenetic analysis

The abundance of each taxonomic group is given in Fig. 1. The clones were distributed among 14 phyla. The majority of the clones (52%) belonged to Proteobacteria followed by

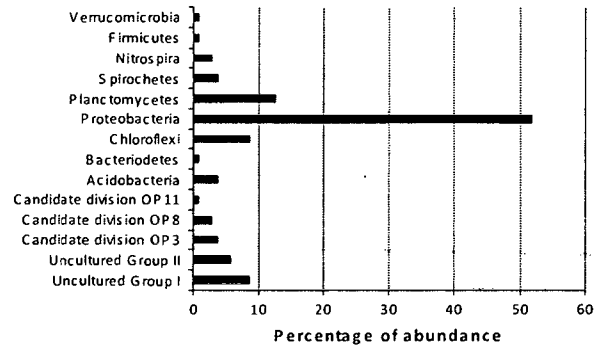


Fig. 1 Abundance of phylogenetic groups in the AS-OMZ sediments

Planctomycetes (12.7%) and Chloroflexi (8.8%). Firmicutes, Bacteriodetes and Verrucomicrobia formed 0.9% each of the total sequences. Acidobacteria and Spirochetes were 3.9% each. *Nitrospira* was represented by 2.9%. Interestingly, there were three uncultured candidate divisions OP3, OP8 and OP11 accounting for 3.9, 2.9 and 0.9% of the sequences, respectively.

The phylogenetic affiliations of all the clones are represented in Fig. 2 and are compared to the sequences from the other non-OMZ marine sediments such as mangroves, hydrothermal vents, shelf sediments, etc.

The dominant phylum, Proteobacteria was represented by Deltaproteobacteria (62.5%), Gammaproteobacteria (25%), Alphaproteobacteria (8%) and Betaproteobacteria (4%). The flagged groups in Fig. 2 such as Deltaproteobacteria, Planctomycetes and Chloroflexi were the dominant ones and their phylogenetic affiliation are shown separately. However, the other classes of Proteobacteria are elaborately represented in supplementary material of the paper (Figs. S1, S2, S3—see Electronic Supplementary Material for this paper). In OMZ sediments class Alphaproteobacteria were represented by a few sequences and they showed affiliation to both cultured and uncultured clades (Fig. S1). One of the sequences showed similarity to the order Rhizobiales. The Betaproteobacteria clustered with culturable taxa and showed high similarity to *Burkholderia cepacia* (Fig. S2). In the case of Gammaproteobacteria, majority showed similarity to *Thiorhodospira sibirica*, while the rest were affiliated to Vibrionales (Fig. S3). However, fifteen clones could not be assigned to any known phyla and are placed under two clusters.

Among the Deltaproteobacteria, 73% showed similarity to the sulphate-reducing bacteria falling in the orders Syntrophobacterales and Desulfovibrionales while 26% did not show any similarity to any cultivated representatives of the class (Fig. 3).

None of the sequences were similar to the SAR clusters that are found in the OMZ waters. Interestingly, representatives of orders Desulfurellales, Bdellovibrionales,

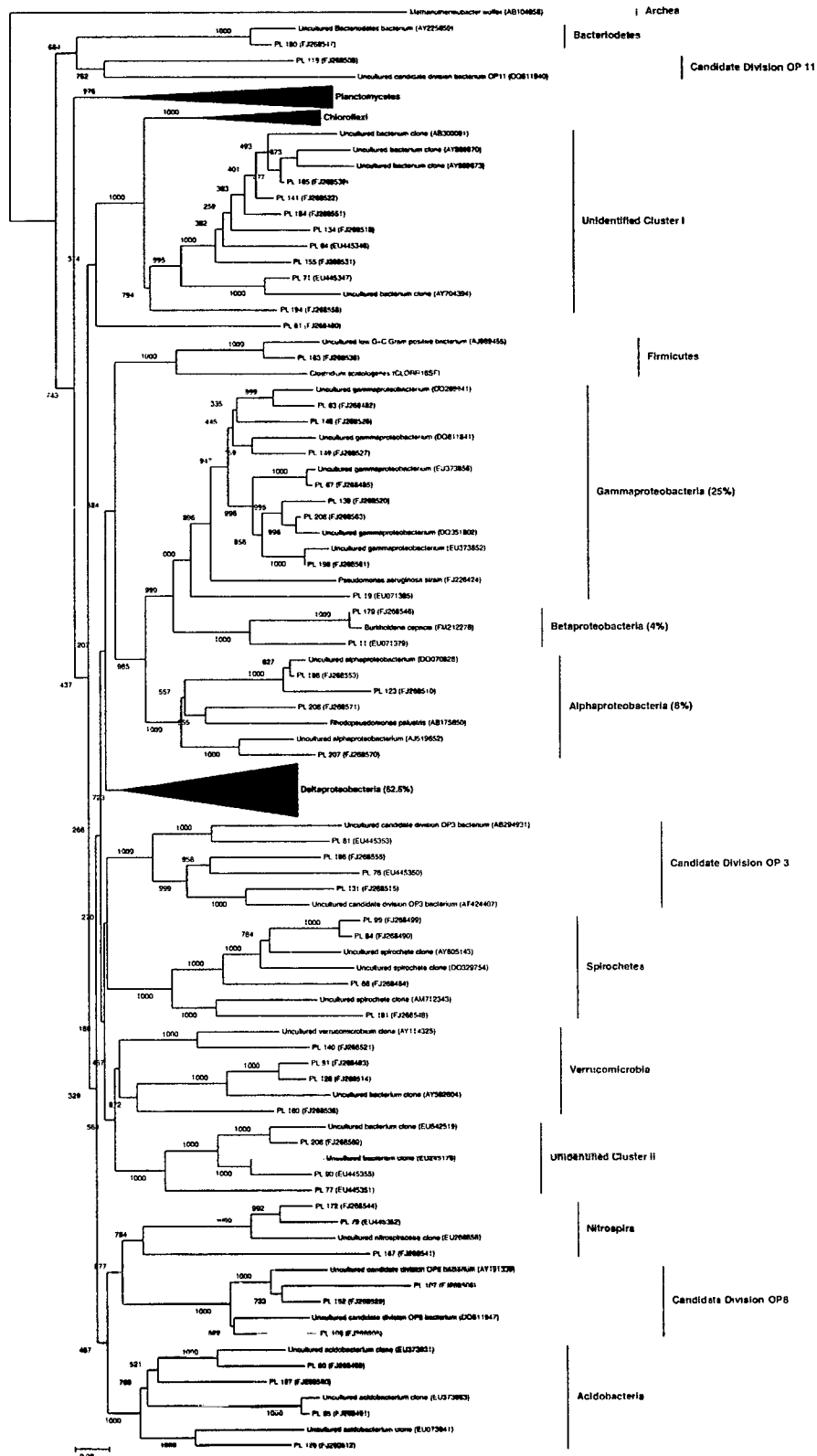


Fig. 2 Phylogenetic tree of 16S rRNA gene sequences of AS-OMZ sediments and their closest marine sediment sample entries in the GenBank. Sequences with prefix 'PL' are results of the present library. Groups flagged represent the three most dominant groups. Tree topology was inferred by neighbour-joining method, using 1,000 bootstrap iterations. *Methanothermobacter wolfeii* is the outgroup. *Scale bar* shows 2% sequence divergence. Numbers in parenthesis represent the percentage of abundance of classes Alpha-, Beta-, Gamma- and Delta-proteobacteria in the phylum Proteobacteria

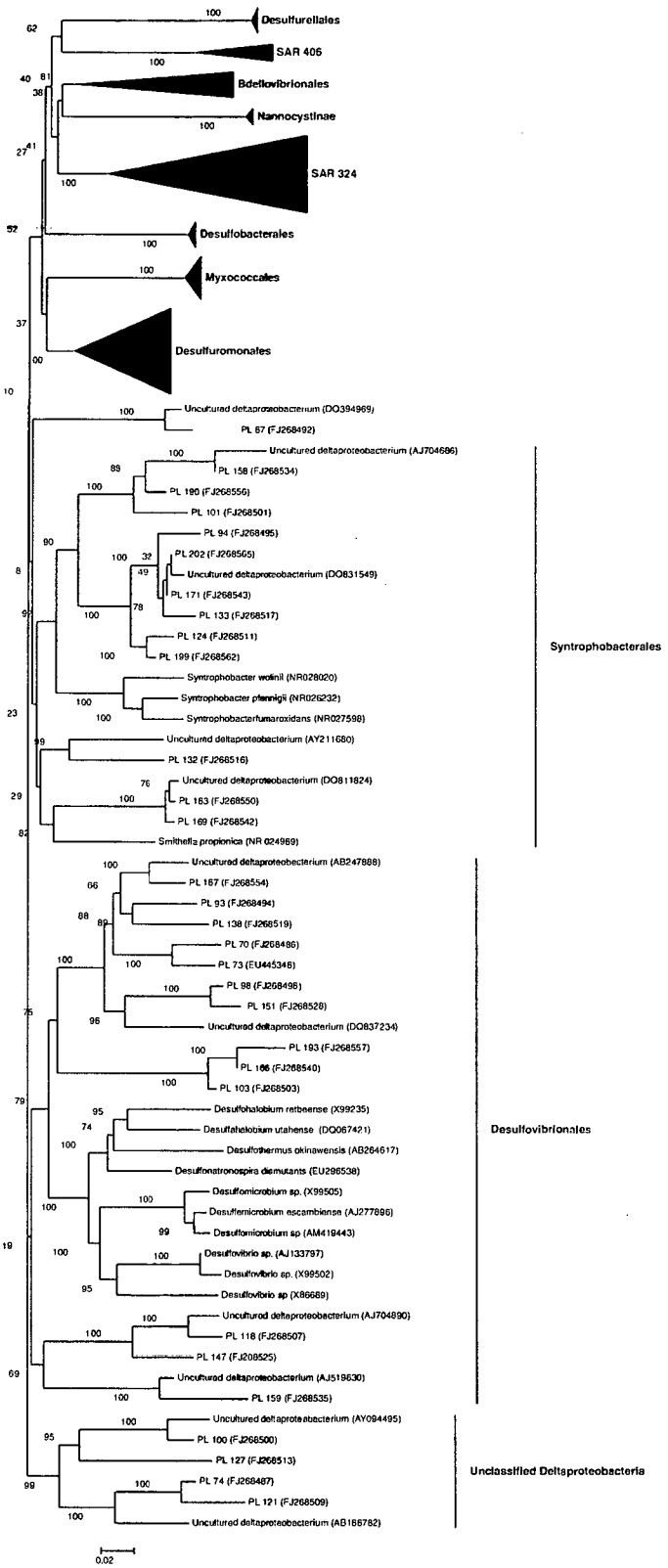


Fig. 3 Phylogenetic tree of 16S rRNA gene sequences of AS-OMZ sediments showing affiliation within the Deltaproteobacteria. The flagged group represent orders of the class Deltaproteobacteria

Nannocystinae, Desulfobacterales, Myxococcales and Desulfromonales were not obtained.

Phylum Planctomycetes formed the dominant group (12.7%) after Proteobacteria, suggesting its importance in the OMZ (Fig. 4). In this phylum 92% of the clones did not belong to the cultured representative and showed similarity to the uncultured representatives from other areas like sediments of mangrove, Black Sea and shelf sediments, except a clone which showed a close resemblance to reported uncultured Planctomycetes from the OMZ waters of Eastern Pacific Ocean. The sequences of the present study did not show any resemblance to the Candidatus *Scalindua* cluster which was present in the AS-OMZ waters.

The phylum Chloroflexi comprised the third dominant group and most species were affiliated to *Anaerolinea thermophila* (Fig. 5). The sequences showed close similarity to the uncultured representatives of the organic-rich sediments of mangrove and continental slopes.

Bacterial diversity and richness

To understand the bacterial diversity and richness of the AS-OMZ sediment clone library, comparisons were made with suboxic sediment clone libraries from the South China Sea, North Sea and Gulf of Mexico. The DOTUR analysis showed that the AS-OMZ sediment clone library had the highest number of OTUs (90) at an evolutionary distance of $\geq$0.03 (species level), followed by the South China Sea sediment library with 74 unique OTUs. The lowest number was recorded in the Gulf of Mexico sediment with only 23 OTUs (Table 1).

Rarefaction curve showed that the AS-OMZ sediment library showed close to plateau (data not shown). The Shannon–Wiener diversity index and Chao 1 richness estimator were the highest for the AS-OMZ sediment library with values 4.4 and 484.6, respectively (Table 1). The distribution of Delta C as a function of evolutionary distance (D) showed that delta C was maximum at 0.2 genetic distances in all the combinations using LIBSHUFF analysis. The bacterial community composition differed significantly among the four clone libraries ($P = 0.001$) (Fig. 6).

Discussion

Phylogenetic analysis

Sedimentary microorganisms are poorly understood as the majority are uncultivated and most are phylogenetically distinct from those in terrestrial environments (D'Hondt et al. 2002). Bacteriological studies of OMZ are quite recent, and most of them pertain to the water column. In the water, unusually diverse bacterial groups, which are mostly exclusive to the region, have been reported (Fuchs et al. 2005; Stevens and Ulloa 2008). Schippers and Neretin (2006) have attempted to quantify the microbial community in near-surface and deeply buried marine sediments from the Peru continental margin. Despite their obvious ecological importance, microbial communities thriving in the sediments below the OMZ have scarcely been studied, with the exceptions of certain functional gene-based studies (Liu et al. 2003a, b; Schaefer et al. 2007). Since being the first in eastern Arabian Sea, the phylogenetic groups of our study are discussed individually and are also compared with available published data from other non-OMZ sediments and OMZ water.

The phylogenetic analysis of the clones derived from OMZ sediments of AS showed a wide representation of various taxonomic groups as the eastern Arabian Sea sediments underlying the OMZ consist of laminated mud, rich in organic matter (Paropkari et al. 1993), which forms an excellent habitat for the microorganisms to flourish. Gupta (2000) in his review on the phylogeny of prokaryotes states that phylum Proteobacteria is of biological significance as it is the most dominant and diverse group of the microbial assemblage. Occurrence of classes of Proteobacteria in sediments has been reported from water or/and sediment with the dominance of Proteobacteria in certain regions (Yakimov et al. 2002; Knittel et al. 2003). Alphaproteobacteria are encountered in marine habitats, especially in the pelagic zone (Hugenholtz et al. 1998). The occurrence of Gammaproteobacteria has been reported among culturable and non-culturable bacteria of pelagic waters (Lopez-Garcia et al. 2001; Fuhrman and Davis 1997) and sediments (Hugenholtz et al. 1998; Cho and Giovannoni 2004). In the OMZ sediment, Proteobacteria was the most prevalent phylum (52%) represented by both cultivable and non-culturable classes viz. Alpha-, Beta-, Gamma- and Delta-proteobacteria. The Deltaproteobacteria, which include the anaerobic sulphate-reducing bacteria, were the most abundant class (62.5%), suggesting the prevalence of sulphate reduction in this region. Syntrophobacterales and Desulfovibrionales were the predominant groups. However, 26% of the sequences did not show any resemblance to cultured representatives, but were closely related to clones detected at cold seeps, mud volcano and harbour sediments (Fang et al. 2006; Losekann et al. 2007; Zhang et al. 2008). Sulphate concentrations vary from 39.8 to 12.7 mM in the pore waters of the eastern Arabian Sea, indicating sulphate reduction (Karisiddaiah et al. 2006). The presence in this zone of sulphate-reducing bacterial communities indicates the probable biological agents responsible for this process. Similar functional diversity of sulfate-reducing bacteria of the eastern Pacific continental

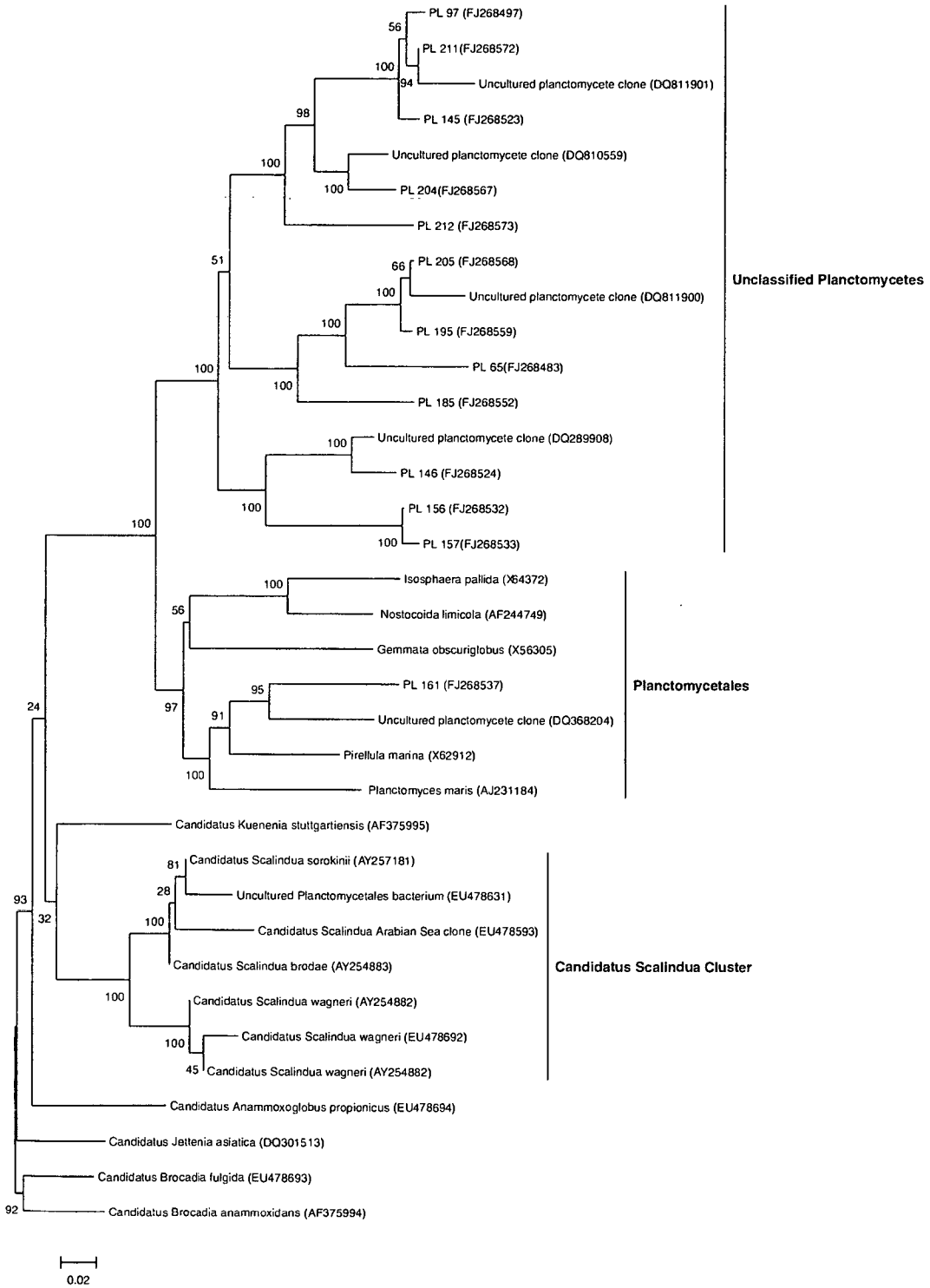


Fig. 4 Phylogenetic tree of 16S rRNA gene sequences of AS-OMZ sediments showing affiliation within the Phylum Planctomycetes

margin (Liu et al. 2003b) and microbial diversity of sulfate-reducing and methanogenic subsurface sediments of the Benguela Upwelling System (Schaefer et al. 2007) have been reported. The occurrence of high numbers of sulphate reducers has been reported in other marine sediments (Gray and Herwig 1996; Ravenschlag et al. 1999; Bowman and

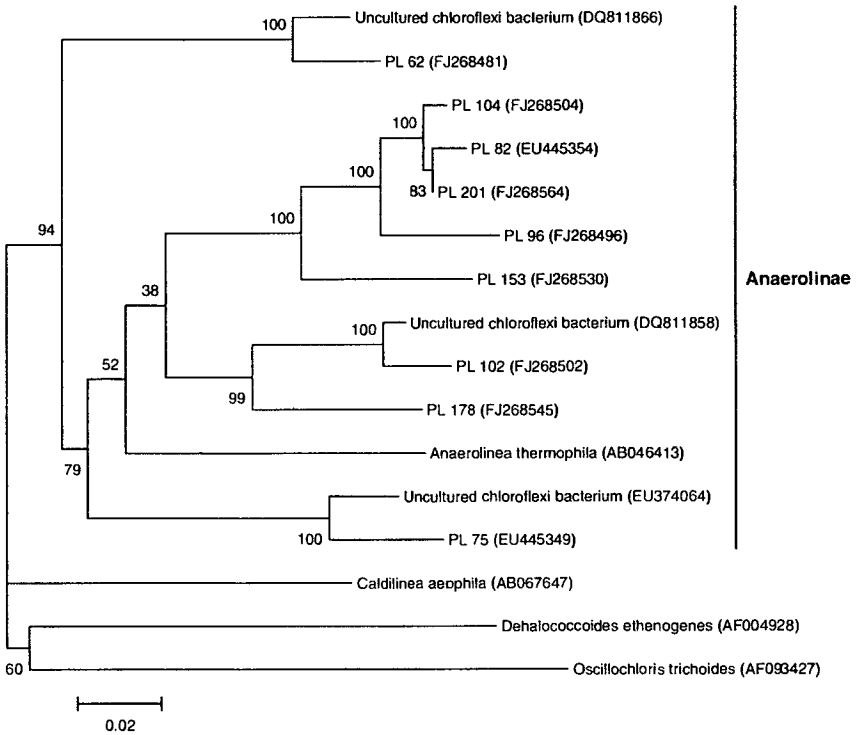
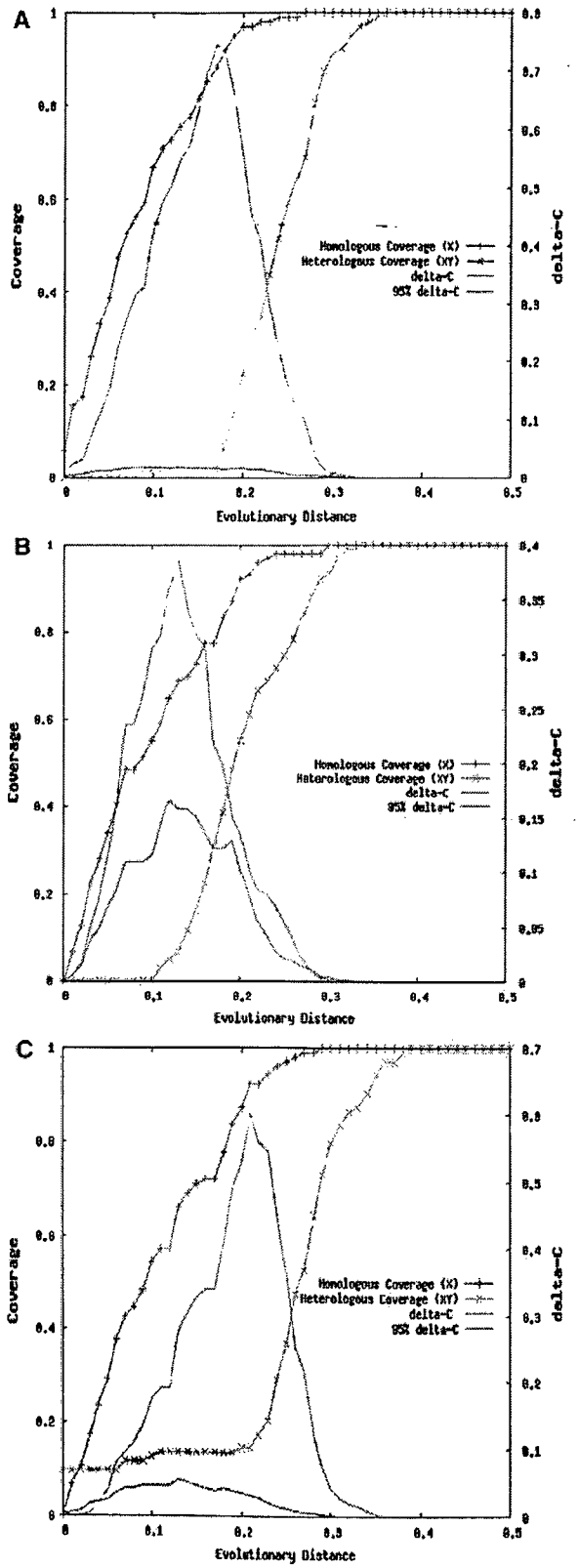


Fig. 5 Phylogenetic tree of 16S rRNA gene sequences of AS-OMZ sediments showing affiliation within the Phylum Chloroflexi

Table 1 Diversity indices of the four 16S rDNA clone libraries

Sediment	No. of unique OTUs	Abundance-based coverage estimator (ACE)	Chao I richness estimate	Shannon–Wiener index (*H*)	Simpson index (1/*D*)
Arabian Sea OMZ	90	484.6	485	4.4	0.003
North Sea	47	139.7	121	3.7	0.009
South China Sea	74	757	442.5	4.1	0.007
Gulf of Mexico	23	114.8	118	3.04	0.018

McCuaig 2003; Bissett et al. 2007). Dominance of this group was not only restricted to the Mid-Atlantic Ridge (Lopez-Garcia et al. 2003) but was also reported in Tokyo and Sagami Bays (Urakawa et al. 1999, 2000). Planctomycetes, though sparsely studied, contains species that are significant because of their abundance in marine environment (Glockner et al. 2003) and their role in anaerobic ammonium oxidation (anammox) (Alldredge 2000; Strous et al. 1999; Kuypers et al. 2005; Thamdrup et al. 2006). A few clones seem to group with the anammox lineages, suggesting that they are especially abundant in surface sediment. This monophyletic and deeply branching bacterial phylum has also been reported in permanently anoxic basins such as the Black Sea, Golfo Dulce (Kuypers et al. 2003; Dalsgaard et al. 2003), OMZ waters (Hamersley et al. 2007; Woebken et al. 2008) and other marine sediments (Musat et al. 2006). Members of the phylum Chloroflexi, exhibits unusual metabolic diversity like anoxygenic photosynthesis, organotrophy and reduction of chlorinated hydrocarbons (Jackson et al. 1973; Maymo-Gatell et al. 1995). This phylum was represented by only family Anaerolinae in the AS-OMZ. Various other groups such as Firmicutes, Bacteroidetes, Acidobacteria, *Nitrospira*, Spirochetes and Verrucomicrobia reported from marine realms were represented as a minority in the AS-OMZ. These are reported from marine sediments and water and their tight association with particles is thought to be helping them for the degradation of recalcitrant compounds (Ravenschlag et al. 1999; Polymenakou et al. 2005; Martin-Cuadrado et al. 2007; Quaiser et al. 2008). Among them the Bacteroidetes group is known to take part in organic matter degradation and has been isolated from wide range of habitats (Chang et al. 2003). The Verrucomicrobia are known to include both aerobic and anaerobic heterotrophs (Hedlund et al. 1997; Chin et al. 2001). Hence occurrence of the anaerobic members of this group in the sub-oxic



◀ **Fig. 6** Comparisons of AS-OMZ sediment clone library with **a** South China Sea, **b** Gulf of Mexico, **c** North Sea. Homologous (*red*, ——) and heterologous (*green*, ——) coverage *curves* are depicted. *Blue line* (——) indicates the value of $(C_X - C_{XY})^2$ for the original samples at each value of evolutionary distance (**d**). *Pink line* (——) indicates 95% delta-C. (Color figure online)

sediments in the present study is not unusual. Candidate divisions which involve groups that have no cultivated representatives in environment are assigned as OP series (Hugenholtz et al. 1998). In the OMZ sediments, Candidate divisions were spread in OP3, OP8 and OP11. Uncultured groups I and II which were not assigned to any taxonomic group formed a major part of the library. The OP11 candidate divisions are widespread and phylogenetically diverse (Harris et al. 2004) and have been detected primarily in the anaerobic ecosystems (Barns et al. 1994; Ludwig et al. 1997; Dojka et al. 2000).

Though sediments may seem unlikely to support ubiquitous dispersal of microorganisms compared to water, some bacterial taxa have shown widespread distribution in marine sediments. This may be due to environmental selection. Though the phylum Acidobacteria with a few culturable representatives is well represented in soils, they are only rarely encountered in marine habitats (DeLong et al. 2006; Quaiser et al. 2008). Five phylogenetic groups (Proteobacteria, Planctomycetes, Chloroflexi, Verrucomicrobia and Candidate division OP 11) present in the OMZ water column of the AS (Fuchs et al. 2005) were also found in the sediments. Though all the four classes of Proteobacteria (Alpha, Beta, Gamma and Delta) were present in AS-OMZ water and sediments, Alphaproteobacteria were found to be under represented in OMZ sediments compared to that in AS waters (Fuchs et al. 2005). Similar assemblages may be due to transportation of pelagic population to the seabed via constant detritus rain, followed by the genetic exchange between pelagic and benthic populations. Similarly, certain groups which were present in the AS pelagic waters such as Cytophaga, Nitrospira, Actinobacteria and Cyanobacteria were absent in the sediment (Fuchs et al. 2005). Interestingly, though SAR clades are common in the pelagic zone (Fuchs et al. 2005), it was not detected in the sediment. Betaproteobacteria include ammonia oxidizers such as *Nitrosospira*-like clusters, which have been well documented in the OMZ waters off north Chile and marine sediments (Molina et al. 2007; Freitag and Prosser 2004), were not recorded in the AS-OMZ sediment. Thus few groups were cosmopolitan and rest were exclusive. Coolen et al. (2002) opined that the relative abundance of organic matter in the AS sediment may alter the microbial community. The presence of Chloroflexi related SAR 202 cluster, which forms a monophyletic subgroup in the phylum, has been reported in the oxic (Morris et al. 2004)

and the oxygen minimum zone waters (Stevens and Ulloa 2008), but did not occur in our study. Also phylum Bacteroidetes was represented by less than 2% in the OMZ sediments whereas in the waters their relative abundance was 2–10% (Kirchman 2002; Abell and Bowman 2005; DeLong et al. 2006). The characteristic of this group is that only one predominant family occurs in an environment. In the sediment, we also observed the presence of only Flavobacteriaceae family (order Flavobacteriales) which have also been reported in the hydrothermal sediments (Lopez-Garcia et al. 2003). Thus phylogenetic analysis of AS-OMZ suggests that bacterial diversity was unexpectedly high compared to other hypoxic locales and quite distinct from the water mass.

Statistical analysis

Comparative analysis of diversity and paired reciprocal comparisons using LIBSHUFF statistics between the AS-OMZ sediment library and libraries of Gulf of Mexico (Orcutt et al. 2010), North Sea (Wegener et al. 2008) and South China Sea (Liao et al. 2009), showed high number of OTUs which differed significantly. This is further supported by the higher values of the diversity indices and richness. However, ACE was 484.6 at a distance of ≥ 0.03 less than the reported value of 757 for the South China Sea sediment clone library (Liao et al. 2009). The difference between these hypoxic regions may be due the selective pressure that shape diversity within communities (Dunbar et al. 1999). The OMZ sediments represented a greater diversity of bacteria, which may be due to the accumulation of less recalcitrant organic matter. The diversity analysis of the OMZ clone library of the AS showed that the diversity indices and estimates were high, though the coverage was 22%. Since the homologous coverage of our library with respect to evolutionary distance was high at $D < 0.2$, it suggests that the library contained the representatives of the original community. In the present study, the coverage was comparatively higher than the three areas and hence it is reasonable to suggest that the data were representative of the in situ bacterial community in spite of the drawbacks of the clone library method (Sogin et al. 2006). Though the biogeochemical implications of the phylogenetic diversity observed in marine microbial communities are not yet well understood, the bacterial species composition is significant in controlling the rates and patterns of organic matter hydrolysis (Riemann et al. 1999). This study is the first information on the bacterial diversity in the OMZ sediments, and provides the baseline for further studies on the microbial groups along with functional probes for a better understanding of the OMZ ecosystem.

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