SPATIAL AND TEMPORAL VARIATION OF MICROBIAL COMMUNITY STRUCTURE IN SURFICIAL SEDIMENTS OF COCHIN ESTUARY

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By

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I am dedicating this work to the Almighty God

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Certificate

*This is to certify that the research work presented in this thesis entitled "**SPATIAL AND TEMPORAL VARIATION OF MICROBIAL COMMUNITY STRUCTURE IN SURFICIAL SEDIMENTS OF COCHIN ESTUARY**" is based on the original work done by Mrs. Neetha Joseph (Reg. No. 3489), under my supervision at National Institute of Oceanography, Regional Centre, Kochi, 682018, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** in Faculty of Marine Sciences, Cochin University of Science and Technology, Kochi, 682018 and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition in any universities or institutes.*

Kochi -18
June, 2012

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DECLARATION

The research work presented in this thesis entitled "**Spatial and Temporal Variation of Microbial Community Structure In Surficial Sediments of Cochin Estuary**" submitted in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**, is a bonafide record of the research work done by me under the supervision of Dr. Shanta Achuthankutty, Chief Scientist, National Institute of Oceanography, Regional Centre, Kochi, 682018. No part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition in any universities or institutes

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CHAPTER 1

INTRODUCTION

Estuaries are ecologically and economically important ecosystem, which function as transition zone between marine and freshwater systems. Autochthonous and allochthonous organic carbon from a variety of sources (Peduzzi and Herndl 1992; Mann and Wetzel 1995; Conan et al. 1999) are processed in the estuary which are primarily dictated by tidal variations and fresh water influx. The organic carbon sequestered in a variety of biomolecules is released for biogeochemical cycles by the concerted action of various microorganisms (Fuhrman and Azam 1982; Strom et al. 1997). This process is mainly dictated by the hydrolytic potential of metabolically active microorganisms, which secretes various extracellular enzymes and degrades polymeric particulate organic matter into dissolved organic matter. However, recent studies indicated that various factors including anthropogenic inputs impinges the community structure of metabolically active microorganisms in the estuaries, which in turn influences the functioning of estuarine ecosystem. Hence an understanding of the community structure of metabolically active microbial population and its spatial and temporal variations is not only important for understanding the ecosystem but also for maintaining the microbial factories for better processing of anthropogenic inputs. Studies on benthic microbial communities are important in the estuary for a variety of reasons. Firstly, sediment being the repository of all dead materials of plants, animals and microbes, microorganism predominates over the mineralization of organic matter and the release of nutrients fueling benthic-pelagic coupling in the coastal system (Joergensen 1996; Joergensen 2000). For shallow-water systems, as much as 30 to 70% of the total primary productivity may be

deposited at the sediment-water interface. Thus, rapid microbial degradation of labile components takes place at the sediment surface which results in the re-mineralization and regeneration of inorganic carbon and nutrients (Rowe et al. 1975; Klump and Martens 1981; Azam 1998). Secondly, the microorganisms are the food resource for deposit-feeding invertebrates, which in turn, serve as food for many commercially important demersal fishes. The complex microflora community forms an essential element in the trophodynamics of detrital and benthic ecosystems (Fenchel and Jørgensen 1977). Microbes not only have the capability to transform or degrade natural compounds, but also recalcitrant xenobiotic compound as well (Ibekwe et al. 2004; Zhang et al. 2004), by enriching microbial populations endowed with the ability to degrade organic compounds available in their milieu. Further, sediments have proved as a reliable source of microbes and genes for the degradation or transformation of various organic compounds. The diversity of the organisms responsible for carrying out these various processes must be identified and characterized in order to address the processes limiting organic matter cycling in shallow marine ecosystems.

According to a current estimate 1 g of soil may harbor up to 10 billion bacteria of possibly 4000–7000 different species and a biomass density of 300–30,000 kg ha^{-1} (Rosselló-Mora and Amann 2001) and technically it is also one of the most challenging tasks as the large microbial diversity exhibit wide morphological, ecological and physiological characteristics. Depending on the ecosystem only 0.001-15% of the total number of visible cells can be retrieved by isolation (White and Findlay 1988; Torsvik and Øvreås 2002; Kassen and Rainey 2004), a fact which is now often designated “the great

plate count anomaly" (Balkwill et al. 1988). The traditional cultivation approaches for studying community in the shelf sediment has detected a mixture of both aerobic and anaerobic microbial groups, many of which fall within the Proteobacteria (Llobet-Brossa et al. 1998; Rusch et al. 2003; Hunter et al. 2006). Hence, traditional microbiological culture dependent techniques, apart from being time consuming also underestimate the species diversity.

Over the past 10 years, several approaches have been attempted to resolve this problem. One was to use geochemical markers such as n-alkanes, alcohols, sterols, alkenones, and fatty acids which are used to identify the carbon inputs from various organisms in marine environments (Volkman et al. 1992; Tolosa et al. 2003). There are great numbers of different kinds of fatty acids in the lipids of micro-organisms, and different micro-organisms have different combinations of these fatty acids. These can be easily identified by gas chromatography (Moss et al. 1980; Moss 1981; Vestal and White 1989; Cavigelli et al. 1995). These signature molecules are valuable phenotypic markers for characterizing pure cultures (Guckert et al. 1991; Vainshtein et al. 1992). FA is now used extensively in bacterial phylogenetic and taxonomic classification of pure isolated culture (Lechevalier 1977; Taylor and Parkes 1983; Kaneda 1991; Vainshtein et al. 1992; Koga et al. 1993). A variety of other compounds, such ester and ether lipids, are used in microbial ecology and related fields like organic geochemistry to detect different groups of organisms or their remains in natural or artificial ecosystems (Alexander 1977; Findlay 1996). Further, it can also provide information on the overall structure of active microbial

communities and the biomass of certain groups of microorganism (Parkes 1987). Phospholipid fatty acid (PLFA) analysis is a biochemical method that does not rely on culturing of microorganisms (Dubey et al. 2006; Ramsey et al. 2006). Phospholipids, essential membrane components of living cells, have a relatively high turnover rate and are not known to accumulate outside living cells (White and Findlay 1988). PLFA are 'signatures' of different microbial groups (Tunlid and White 1992). They are found in reasonably constant amount in bacterial cells (White et al. 1979). Since PLFA are structurally diverse and exhibit a relatively high degree of biological specificity, they are employed as biomarkers for the identification of well studied groups of microorganisms (Parkes 1987; Tunlid and White 1992). Using biomarkers in PLFA composition, the microbial communities can be classified into four groups viz. (1) microeukaryotes biomarker: (polyunsaturated fatty acids), (2) aerobic prokaryotes and eukaryotes (monounsaturated fatty acids and 18:2 branched fatty acids), (3) gram-positive prokaryotes and other anaerobic bacteria (branched fatty acids with C15 and 16) and (4) sulphate reducing bacteria (SRB) and other anaerobic prokaryotes (biomarkers: branched fatty acids with C17 and methyl branched fatty acids) (Findlay et al. 1990).

PLFA represents a quantitative and sensitive method for determining the interactions between members of consortia in terms of biomass, community structure, nutritional status, and metabolic activities (Ringelberg et al. 1997). This technique delivers relatively sensitive and reliable information through a reasonably straightforward methodology (Findlay et al. 1989; Rajendran et al. 1992a; Bååth et al. 1998). It has been advocated as a

rapid and inexpensive alternative for describing complex microbial communities and it has been found to be more discriminatory than other methods (Bossio and Scow 1998; Steer and Harris 2000; Ramsey et al. 2006). An advantage of PLFA analyses is that the same technique and even the same sample can be used to determine both fungi and bacteria. Despite the usefulness of this method, there are some major limitations (Haack et al. 1994) such as appropriate signature molecules are not known for all organisms in a sample, cannot be used to characterize microorganisms to a fine taxonomic species level, since the method relies heavily on signature fatty acids to determine gross community structure, any variation in these signatures would give rise to false community estimates created by artifacts in the method and the types of fatty acids vary with growth conditions and environmental stresses. However, continuing analysis of the species composition should help to resolve some of these patterns.

Benthic ecologists have analyzed biomarker compounds of different ecosystems to determine microbial community structure (Bobbie and White 1980; White and Findlay 1988), to assess changes in community composition (Volkman et al. 1980; Taylor and Parkes 1983; Guckert et al. 1985; Guckert et al. 1991; Vainshtein et al. 1992; Cavigelli et al. 1995; Boon et al. 1996; Guezennec and Fiala-Medioni 1996; Elferink et al. 1998; Elvert et al. 2003; Zhang and Bennet 2005) nutritional status and to determine metabolic status(Bobbie and White 1980; Federle 1986; Guckert et al. 1986; Kaneda 1991; Tunlid and White 1992; Findlay and Dobbs 1993; Kieft et al. 1994; Frostegård et al. 1996) and have revealed diverse bacterial and archaeal communities over a range of salinity, pH and temperature

(Rajendran et al. 1992a). Being degraded rapidly upon cell death, it is also frequently used as a measure of living microbial communities (White et al. 1979; Findlay et al. 1990; Ringelberg et al. 1997).

Cochin Estuary (CE) located at Lat. 9º5' - 10ºN and Long. 76º15' - 76º25'E is a micro-tidal (amplitude 1 m), bar-built estuary constituting a network of shallow canals situated on the southwest coast of India. It is one of the productive ecosystems and is also rich in biodiversity. It opens into the Arabian Sea and these entry regions are relatively deeper with depth ranging 5 –15 m which are flushed during flood and ebb tides whose maximum range is about 1m. The upper reach of the estuary is shallow (2 – 5 m deep) with little or no tidal influence and the salinity is markedly low. Six rivers and several tributaries discharge ~20,000 Mm^3 of freshwater annually into the estuary, making it the largest wetland along the west coast of India (Srinivas et al. 2003). The estuary receives heavy freshwater influx during the summer monsoon (June to September) when 60% of annual rainfall occurs (Madhupratap 1987; Srinivas et al. 2003). The large amount of organic matter transported through this estuary (Balachandran et al. 2003) might have a significant effect on the system's metabolism. Salinity remains near zero over a large part of the CE during the summer monsoon period, but soon after, as river discharge gradually diminishes, tidal influence (salinity) gains momentum to play an important role in the ecology of the system (Madhupratap 1987; Menon et al. 2000). Seasonal changes in the surface temperature are not well marked in the estuary. These hardly exceed 3 - 4º C and generally fall within the range of 28 - 31ºC. Recently, estuaries have become vulnerable ecosystems (GESAMP 1990), CE is also

undergoing dramatic alteration in aquatic habitats and environmental deterioration due to increased anthropogenic activities (Menon et al. 2000; Qasim 2003). CPCB, (1996) have reported release of 0.104×10^{6} m^3d^{-1}untreated effluents from industries and 0.26×10^{3} $m^3d^{-}1$ from domestic sectors in CE and there is a definite possibility of yearly increase with increase in urbanization. The increased nitrogen loads stimulate eutrophication (Galloway et al. 1995).

Most of the studies in CE have been to understand the spatial and temporal variations in the physical, chemical and biological characteristics of Cochin estuary and the influence of anthropogenic inputs (Gopalan 1983; Gopinathan 1984; Balachandran 2006; Jyothibabu 2006; Madhu 2007). However sedimentary studies are restricted to trace metals (Balachandran et al. 2005) and benthic studies (Jayaraj 2006). Very limited microbial studies have been carried out in CE. These studies are either on specific bacteria using traditional or molecular approaches (Thomas et al. 2006; Parvathi et al. 2009; Jose et al. 2011; Parvathi et al. 2011) or on bacterioplanktonic dynamics (Thottathil et al. 2008a; Thottathil et al. 2008b). Despite, surficial sediments being sites of nutrient regeneration (Kemp and Boynton 1992) overall studies are very scanty compared to water column. Few studies from other regions have delved on the seasonal variability in microbial biomass and community structure (Findlay and Watling 1998; Smoot and Findlay 2001; Findlay et al. 2008; Steger. et al. 2011) from temperate zones. From the tropical estuary, there is only one report from Visakhapatnam harbour, east coast of India on the sediments' microbial community structure (Harji et al. 2010). It is obvious that there is a dearth in general on surficial study from

estuary; hence a study was undertaken in the CE to understand the microbial community structure (ester link-PLFA analysis), bacterial diversity (FAME), abundance, its seasonality and interrelationship to various physio-chemical parameters of the water column and geochemical characteristics of the sediment. This study covered four stations representing the estuary as it is hypothesized that bacterial communities in the estuary differ at 1-3 km scale caused by a combination of local nutrient dynamics, salinity and allocthonous and autocthonous carbon pools. As the estuary undergoes marked seasonal changes and bacteria respond to these changes sampling was carried out during the pre-monsoon, monsoon and post monsoon seasons. This thesis is the first report on the spatial and temporal variation in bacterial communities of Cochin estuary and the role of environmental factors in structuring it.

CHAPTER 2

REVIEW OF LITERATURE

This chapter is an attempt to review the available literature on benthic microbial community of shallow water systems and its significance at national and international levels. The content is presented under the following sub-heads.

- General Introduction
    - Estuary
    - PLFA –EL Biomarkers
    - Varied Applications
- Benthic Microbial community
    - International Scenario
    - National Scenario
- Scope and objective

2.1. Introduction to Estuary

Estuaries are one of the critical land–ocean interfaces controlling the anthropogenic and terrestrial fluxes and fates of materials. It is regarded as a complex ecosystem with diverse habitats viz. sea grass beds, mangrove swamps, creeks, land bays. This extremely dynamic system is influenced by a host of physical, biogeochemical, and meteorological factors. Estuarine areas are characterized by naturally derived organic matter that originates from autochthonous production, the open sea, surrounding salt marshes, river drainage (Andrade et al. 2003; DeLong and Karl 2005) and anthropogenic contribution (Baldwin et al. 2005; Giovannoni et al. 2005). The organic carbon demand in these ecosystems is met by combination of autochthonous and allochthonous sources (Peduzzi and Herndl 1992; Mann and Wetzel 1995; Conan et al. 1999). Over the last 30 years, microbial

ecologists have been examining the microbes and processes and are the most important zones in the biogeochemical process of carbon. They are the major consumers of organic matter (DOM) thereby transferring their energy to the next trophic level (Sanders et al. 1992), and responsible for transformation and mineralization of organic matter i.e. in the recovery of organic matter from detritus to living biomass or in its remineralisation back to inorganic compounds, forming the link between the dissolved and particulate organic carbon (Ducklow and Carlson 1992; Shiah and Ducklow 1994). Study on microbial community in coastal and estuarine ecosystems is a central paradigm of any ecological study (Pomeroy and Wiebe 1991; Ducklow and Carlson 1992). Microbial ecology addresses the identity and functioning of microorganisms in their natural environment. Biologically dominated ecosystems generally show high biomass of heterotrophic bacteria because these organisms are responsible for the bulk of organic carbon utilization and respiration in the sea. They are the most abundant component. These bacteria often dominate the biomass of microbial food webs making their role in nutrient and energy fluxes crucial for the organization of marine ecosystems (Rappé and Giovannoni 2003). The ability to measure microbial community is a pre-requisite for the systematic study of microbial biogeography and community assembly. Microbial communities include viruses, eubacteria, archaea bacteria, fungi, protozoa, and algae (Kemp et al. 1990). Prokaryotic microorganisms are universally distributed in marine systems (Wikner et al. 1999). Of all the prokaryotes, eubacteria being ubiquitous and diverse make up significant components of the ecosystem

2.2. PLFA –EL Biomarkers

The determination of the microbial community in complex sediments has presented problems as the classical techniques of identifying bacteria based on culture method most often recovers a small fraction of the large proportion of microorganisms. Classical numerical taxonomic approach for determining community structure is cumbersome, time consuming, expensive, and only reveals the presence of microbes, that can be cultured on the medium chosen. Numerous methods using biochemical analyses to measure the biomass, presence, and activity of microbes under natural conditions have been discovered, and their uses and shortcomings have been reviewed (Parkes 1987; Rasheed and Balchand 1995; Forman 1998; Cunha et al. 2000). Analysis of biomarker compounds, as a measure of viable microbial biomass, has been proposed as an alternative method to determine microbial community structure. PLFAs are key components of microbial cell membranes, and because different groups of microorganisms produce unique types and suites of fatty acids, the structure of the microbial community can be examined by looking at the set of PLFAs extractable from a sediment sample (White et al. 1996; Conley 1998; Häggblom et al. 2000). The biomarkers used for community studies are given in table below.

Table 2.1 Common biomarkers used in microbial ecology

Biomarker class	Organisms	Examples
PLFA	Bacteria and eukaryotes	Bacteria (i14:0, i15:0, a15:0,18: 1ω7c, cy19:0) Algae (20:5ω3, 18: 3ω3) Fungi (18: 2ω6) Actinomycetes (10Me17:0, 10Me18: 0) Sulphate reducers (i17:1, 10Me16:0) Methanotrophs (16:1ω8c, 18:1ω8c)
Sterols	Eukaryotes	Higher fungi (ergosterol)
Hopanoic acids	Bacteria	Cyanobacteria, methanotrophs
Ether lipids	Archaea	Methanogens (hydroxy-archeols) Crenarchaea (cyclic tetra-ether lipids)
D-Amino acids	Bacteria	D-Alanine

10*Me*-C16:0, *i*C17:0 and *ai*C17:0 for SRB, Short C13 to C21 iso- and anteiso-branched fatty acids for bacteria (Parker et al. 1967; Lechevalier 1977; Murty et al. 1985; Nair et al. 1993). Cyclopropane fatty acids represents certain classes of gram-negative bacteria, as well as lactobacilli and clostridia (Lechevalier 1977; Uzaki and Ishiwatari 1986; Levin et al. 2001; Schefuß et al. 2004). Mid-chain branched saturated fatty acid for SRB in the marine environments (Ishiwatari et al. 1977; Meyers et al. 1984; Vainshtein et al. 1992) iC15:0 or aC15:0 or both for strains of Desulfovibrio (Fogel et al. 1992; Vainshtein et al. 1992; Rabouille et al. 1993; Uchida et al. 2005) cy17:0 and cy19:0 for Desulfobacter and Desulfobacterium (Newell and Field 1983; Meyer et al. 2005).

Based on these markers the microbial communities can be classified into four groups viz. (1) microeukaryotes biomarker: polyunsaturated fatty acids, (2) aerobic prokaryotes and eukaryotes (monounsaturated fatty acids and 18:2 branched fatty acids), (3) gram-positive prokaryotes and other anaerobic bacteria (branched fatty acids with C15 and 16), and (4) sulphate reducing bacteria (SRB) and other anaerobic prokaryotes (biomarkers: branched fatty acids with C17 and methyl branched fatty acids) Perry et al.

(1984) has identified a group of fatty acids as valid markers for bacteria. These include iso- and anteiso-branched chain acids; 10-methylpalmitic acid; cyclopropyl 17:0 and 19:0 acids of which 19:0 cyclo (11,12) is unique to bacteria; cis-vaccenic acid; and the 15:1, 17:1 w6 and w8 isomers especially when these occur in pairs; iso cyclo 7-15:l and iso cyclo 9-17:l are branched unsaturated fatty acids apparently unique to bacteria. The nutritional status of bacteria in aquatic sediment could be inferred from the trans/cis ratio of monoenoic acids (Guckert et al. 1986). The reported ratio for bacterial cultures and sediment is normally less than 0.1 (Meyer-Reil 1983; Bianchi and Levinton 1984; Guckert et al. 1985; Nichols et al. 1986) increasing to more than unity during starvation. In some bacterial species, the cyclopropane fatty acids accumulate in the stationary phase (Pusceddu 1997; Danovaro et al. 1999; Levin et al. 2001) or with adverse growth conditions (Montagna 1984), resulting in a decrease in C_{18} mono-unsaturated fatty acids and an increase in C_{19} cyclopropane fatty acids.

2.3. Varied Applications

Fatty acids are considered as “signature” molecules (Moss et al. 1980; Moss 1981; Vestal and White 1989; Tunlid and White 1992; Cavigelli et al. 1995). Since then it has been recognized to be of great value in the understanding of phylogenic and taxonomic classifications (Lechevalier 1977; Uzaki and Ishiwatari 1986; Alongi 1988). PLFA can serve as fingerprints of special microbial groups and can be used as a taxonomy signature for bacterial classification (Taylor and Parkes 1983; Kaneda 1991; Vainshtein et al. 1992; Koga et al. 1993). Pioneering work on fatty acid composition of several bacterial strains was carried out by Dowling et al.

(1986), further knowledge from these studies were used in interpretation of natural prokaryotic communities (Karl and Novitsky 1988; Rajendran et al. 1992a-b; Elferink et al. 1998; Kuwae et al. 1998; Elvert et al. 2003) as analysis of the samples carry information of the community structure (Volkman et al. 1980; Meyers et al. 1984; Guezennec and Fiala-Medioni 1996). The prokaryotic and microeukaryotic components in the microbial community can be defined by fatty acid analysis (Bobbie and White 1980). It can be used to estimate the viable microbial biomass in marine sediments (White et al. 1979). Furthermore phospholipid fatty acid (PLFA) composition also characterizes the structure of the microbial consortia, and this approach has been extensively used to describe microorganism community structure as well as biomass abundance (White et al. 1979; Douglas et al. 1987; Balkwill et al. 1988; Ibekwe and Kennedy 1998; Rütters et al. 2002) in sediments. This method has been extensively applied by microbiologists, marine chemists and geologists to address various environmental issues. This method has been utilized to compare different marine depositional environments (Van Duyl and Kop 1990), as a criterion for pollution (White et al. 1998) and for tracing the origin of detritus and marine organic surface deposits (Volkman and Johns 1977; Boon et al. 1978; Scala and Kerkhof 2000). Specific branched monoenoic and hydroxy fatty acids have been used to implicate Desulfovibrio in marine oozes (Boon et al. 1977) and the ratio of palmitoleic to palmitic acids was linked to seasonal succession in phytoplankton communities (Jeffries 1970). Lipid biomarkers, the preserved structural skeletons of biological molecules, can be used to identify potential source organisms (Summons et al. 1999), to establish possible links

between modern microbial communities and their ancient counterparts (Brocks et al. 1999; Brocks et al. 2003; Jahnke et al. 2004), and to illuminate the evolutionary histories of organisms and their environments (Brocks et al. 2003). It has been used extensively as proxies for determining microbial biomass and community composition in modern microbial ecosystems (White et al. 1997; Fang et al. 2006), characterize and compare sessile and planktonic microbial populations (Werker and Hall 1998) and has provided insight into important changes in microbial communities in wastewater treatment plants for improved planning of the operation of wastewater treatment plants, to estimate the microbial community structure and metabolic activity in polluted soils (Zelles 1999; Kozdrój and van Elsas 2001b; Pinkart et al. 2002); to identify contamination of surface waters with polluted soil adjacent to agricultural production fields and to wooded riparian zones (Banowetz et al. 2006); to quantify and to evaluate the adaptation of microorganisms to a particular condition, such as acid environments (Quivey Jr et al. 2000), to establish the microbial community distribution in terms of structure (Sundh et al. 1997; Kozdrój and van Elsas 2001a) and to determine the relative changes in abundance of microorganisms, such as bacteria and fungi (Zeller et al. 2001) in determining the interactions between members of consortia in terms of biomass, community structure, nutritional status, and metabolic activities (Ringelberg et al. 1997) and assessing changes in community composition (Ishiwatari et al. 1977; Guckert et al. 1985; Guckert et al. 1991; Vainshtein et al. 1992; Guezennec et al. 1996; Guezennec and Fiala-Medioni 1996; Elvert et al. 2003; Zhang and Bennet 2005), nutritional status (Guckert et al. 1986; Kieft et al. 1994), metabolic

activity (Kaneda 1991) and environmental stress (DeLong and Yayanos 1985; Guckert et al. 1986; Kamimura et al. 1993; Yano et al. 1998; Fang et al. 2004). Specific patterns of FAME can also indicate physiological stress in certain bacterial species (Pinkart et al. 2002). PLFA has also been used in extreme environments to determine microbial community structure and biomass in the deep subsurface of several levels and shafts in mines Pfiffner et al. (2006). The figure below gives the number of publications related to PLFA. There were 708 hits, most of them in recent 5 years.

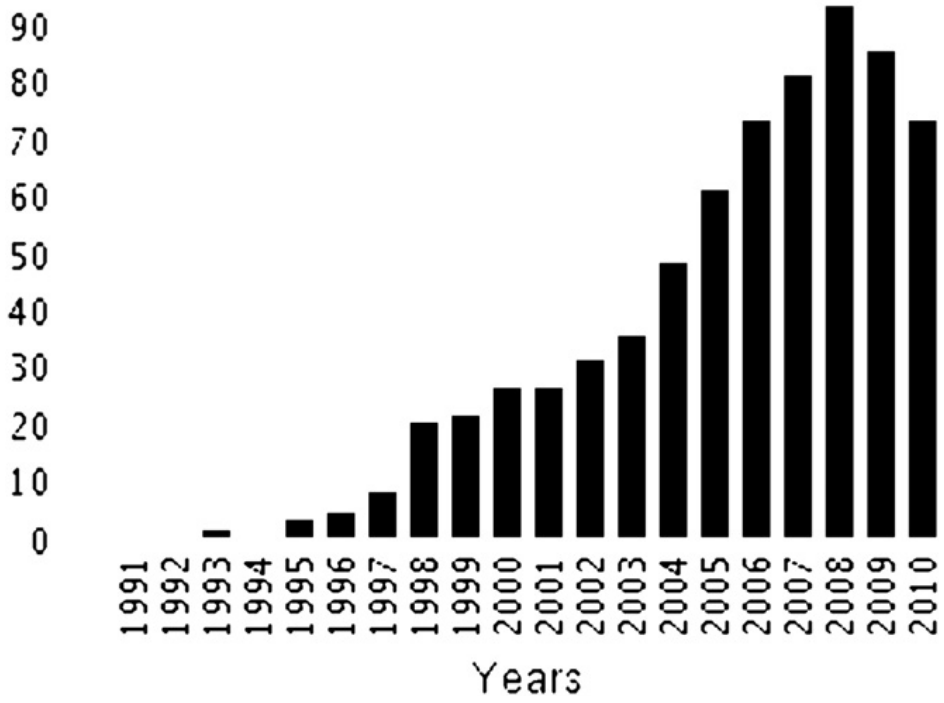

Figure 2.1 Number of publications in ISI Web of Science for "PLFA AND Soil" 2010-11-16

2.4. BENTHIC MICROBIAL COMMUNITY

Sedimentary microorganisms play an important role in a number of rate processes including particulate carbon export, nutrient regeneration, biochemical cycling and as a food source in estuaries. Microbes therefore

are not only involved in the cycling of major and trace elements (Malcolm et al. 1990) but also are responsible for the degradation of recalcitrant chemical compounds in sediments which constitute the final repository for many chemicals in estuarine systems (Rowe et al. 1975; Fenchel and Jørgensen 1977; Klump and Martens 1981; Fuhrman and Azam 1982; Binnerup et al. 1992; Sinsabaugh and Findlay 1995; Strom et al. 1997; Azam 1998; Pinckney et al. 2001; Yokokawa and Nagata 2010). These processes almost always involve mixed microbial populations. Temperature, salinity, organic matter input etc. show marked seasonal variations in benthic ecosystems. Microbial communities are expected to react to the seasonal and pollution-induced changes that are taking place in sediments. When a microbial community changes in either size or structure, there may be associated geochemical changes within the system (Findlay et al. 1990a; Rajendran et al. 1994). Despite the obvious importance of the benthic microflora, many studies on benthic ecology have not included examination of the microbes.

The use of molecular markers (including PLFA) for assaying the structure and activity of microbial communities in natural environments, particularly aquatic habitats such as sediments, was pioneered by David White and has been used in ecological investigations for quantitative estimation of microbial biomass and community structure of sediments (Bobbie and White 1980; Cunha et al. 2000). PLFA technique delivers relatively sensitive and reliable information through a reasonably straightforward methodology (Findlay et al. 1989; Rajendran et al. 1992b; Bååth et al. 1998). Recent investigations have indicated that the different physiological groups of prokaryotes could be quantitatively estimated by

PLFA analysis. It has been also used as tracers by Meziane and Tsuchiya (2000) to detect bacteria, diatoms and macroalgae in the tissues of macrozoobenthic species, fiddler crabs and gastropods.

2.4.1. International Scenario

PLFA has been successfully used in the characterization of the microbial community structure of sediments and its variations in estuaries, coastal bays, deep-sea and organically contaminated sediments (Bobbie and White 1980; Baird and White 1985; Smith et al. 1986; Balkwill et al. 1988; Mancuso et al. 1990; Rajendran et al. 1992a-b; Rajendran et al. 1992b; Rajendran et al. 1993b). Groups of PLFA, each associated with a group of microorganisms, have also been used to describe the relative abundances of the different microbial groups in sediments (Findlay et al. 1990a; Findlay et al. 1990b).

Estuarine sediments contained significantly abundance of SRB and microeukaryotes, whereas deep-sea sediments contained both aerobic as well as anaerobic bacteria (CranwellL, 1982; Taylor and Parkes, 1983; Perry *et al.,* 1979). Prokaryotic organisms dominated the microbial community and anaerobic organisms were present in greater proportions in Venezuela Basin and Puerto Rico Trench sediments than in either the North Atlantic abyssal sediments or shallow-water estuarine sediments (Baird and White 1985). Bacteria were the dominant group and microeukaryotes showed an irregular distribution in the estuarine mud flat sediments and there was station to station variability in their distributions (Federle et al. 1983). In subtropical Pearl River estuary and adjacent shelf at northern part of South China Sea bacterial fatty acids were significantly high and terrigenous fatty acids was

low (PUFA 0.2 - 4.8% of total fatty acids) as algal carbon was effectively recycled during the whole settling and depositing process (Hu et al. 2006). Analysis of the origin and transport of organic matter between the fresh water and marine end-members of two sub-tropical estuaries of South Florida, USA clearly reflected the differences between estuaries from a common geographical area, but characterized by different hydrology conditions, tidal influences and OM inputs (Jaffé et al. 2001).

The community of Intertidal sandy - bottom site adjacent to the Florida state, USA, comprised of microeukaryotes, Fungi and G-ve Proteobacteria and SRB group. Twenty five PLFAs of microbial biomass and community were controlled by the biotic (caused by biological agents) and abiotic (physical processes, such as wave action or tidal flow increased oxygen penetration into the sediments) variables, whereas total microbial biomass in the sediments of Damariscotta ranged from 45 to 150 nmol PLP g^{-1}. The 37 PLFAs identified were grouped into phototrophic microeukaryotes and two groups of anaerobic bacteria (Findlay et al. 1990a; Findlay and Watling 1998). Rusch et al (2003) studied the spatio-temporal variation and metabolic activity of the microbial community in coarse-grained sediment and found that members of different aerobic and anaerobic bacteria generally had a low abundance, but high organic matter turnover rates. Bühring et al. (2005) studied depth profiles of bacterial fatty acid relative abundances and found elevated subsurface peaks for the fine sediment, whereas at the sandy sediment stations the concentrations were less variable with depth. Although oxygen penetrates deeper into the coarser and more permeable sediments, the SRB biomarkers are similarly abundant,

indicating suboxic to anoxic niches in these environments. They detected SRB in all sediment types as well as in the surface and at greater depth, which suggests that SRB play a more important role in oxygenated marine sediments than previously thought. Meziane and Tsuchiya (2000) found that bacteria, diatoms and macroalgae were the dominant community in surface sediments from 2 intertidal flats on Okinawa Island.

In Acton Lake southwestern Ohio, the microbial biomass was 225 – 450 nmol PLP g^{-1} in riverine and 500 – 1500 PLP g^{-1} in lacustrine zone sediment. The major functional groups were microeukaryotes, G+ve, G-ve, anaerobic and sulfate reducing bacteria. Principal component analysis of phospholipid fatty acid (PLFA) profiles indicated that the sedimentary microbial communities at all three stations displayed a seasonal pattern of shift from aerobic microorganisms during times of cold water to anaerobic microorganisms during times of warm water. There was also difference in the community, River and Middle stations had disproportionately more bacterial fatty acids whereas Dam station had disproportionately more microeukaryotic phytoplankton imported from the overlying water column (Smoot et al 2001). Spatial variability trend was seen in the intertidal sediments of the shallow, semi-urbanized Coombabah Lake in southern Moreton Bay, Australia (Dunn et al. 2008).

Extensive studies have been carried out on microbial communities in the sediment bays of Japan. Surface sediment samples from the Hiroshima Bay and its adjacent bays of Seto Inland Sea of Japan recorded thirty-one individual fatty acids which comprised of saturated, monounsaturated, polyunsaturated and branched fatty acids. A majority of these fatty acids

were characteristic of aerobic and anaerobic bacteria especially sulphate-reducing bacteria and the Microeukaryotes were less because of high pollution impact on the Bays. Significant seasonal differences in sediment parameters, PLFA groups and microbial groups were observed in Hiroshima Bay. The factor influencing the community was redox potential and poor circulation (Rajendran et al. 1992a). In Suo Nada total of 50 PLFA were identified and were classified into saturated, monounsaturated, branched, PLFA. Microbial biomass was high in autumn and low in spring in Hiroshima Bay but no such significant seasonal difference of PLFA concentration was observed in Suo Nada. The aerobic prokaryotes and eukaryotes group were predominant in spring, whereas in autumn, the Gram-positive bacteria group was predominant. In Suo Nada, these parameters showed similar patterns to Hiroshima Bay but the differences of most of the parameters were insignificant. Microeukaryotes were less in number and prokaryotes were predominant (Rajendran and Nagatomo 1999). In the case of polluted bay (Ise Bay, Honshu Island, Japan), 31 individual PLFA were obtained representing the straight chain saturated, monounsaturated and branched fatty acids. The PLFA pattern in sediments revealed the presence of aerobic and anaerobic bacteria especially sulphate reducing bacteria, the composition of which differed among the stations. Like Hiroshima Bay microeukaryotes were absent and the relatively high proportions of bacterial biomarkers indicate the prokaryotic consortium responsive to organic contamination (Rajendran et al. 1992a). An extensive study was also carried out in this Bay. Prokaryotes were present and micro eukaryotic were absent. A wide distribution of aerobic bacteria, Gram-positive bacteria, anaerobic

bacteria and sulfate-reducing bacteria formed the microbial community structure. The trans/cis ratio for 16:1ω9 in Ise Bay was 0.2, showed that the bacterial population was likely to be exposed to some physiological stress as a result of pollution (Rajendran et al. 1993b). The regional differences in microbial community structure of Osaka Bay, Japan found the predominance of anaerobic bacteria and gram-positive prokaryotes, in the eastern and northeastern sides of the bay, where the reported concentrations of pollutants were also high. Total PLFA concentrations (0.56 to 2.97µg/g [dry weight] of the sediment) in sediments showed marked variation among the stations. 65 PLFA were identified. The dominant groups were *Desulfovibrio*, *Desulfobacter,* aerobic prokaryotes, etc.,. There was predominance of aerobic prokaryotes and eukaryotes, except for a few stations, in the western and southwestern sides of the bay (Rajendran et al. 1994). A comparative microbial community structure in surface sediments of Saroma KO lagoon, with eutrophic bays (Osaka Bay and Hiroshima Bay) and a lake (Lake Kojima) in the Seto Inland Sea of Japan during winter showed that the prokaryotes were a significant component of sediment organic matter. SRB were also detected. The aerobic prokaryotes and eukaryotes were dominantly present in Saroma KO lagoon, whereas microeukaryotes, and gram-positive bacteria and anaerobic bacteria were abundantly present in Hiroshima Bay and Osaka Bay. SRB and other anaerobic bacteria in Lake Kojima were higher than in other areas (Rajendran et al. 1995). In Osaka Bay and Hiroshima Bay the factor affecting the community was organic pollution, whereas in Lake Kojima it was influenced by oxidation – reduction potentials and in Saroma Ko lagoon it was influenced by low temperature. In

polluted sediments, microeukaryotes were absent (Smith et al. 1986; Rajendran et al. 1992b; Rajendran et al. 1993a). These results have revealed significant differences in microbial community structure within the bay as well as among bays.

2.4.2. National Scenario

Marine microbiology studies in India were initiated only in 70's and most of the studies were on identification, abundance, distribution and factors influencing the distribution of heterotrophic bacteria and pathogens. Few studies were also carried on the physiology, biochemistry and effect of pollutants on bacteria. The habitats studied were mostly close to the east and west coasts of India.

In the east coast, microbiological studies were carried out on the different enzyme producing bacteria such as arylsulfatase producing bacteria, chitinoclastic bacteria, luminous bacteria, phosphate solubilzing bacteria, nitrifying bacteria etc. in Vellar estuary and its adjoining Pitchavaram mangrove (Lakshmanaperumalsamy 1983; Rajendran et al. 1997). The distribution and role of methanogenic bacteria were reported from this area (Mohanraju and Natarajan 1992). Enzymatic study was carried out in the sediments of Coral reef of Gulf Mannar. The study showed the presence of six genera of phosphate mineralizing bacteria and Bacillus was found to be dominant (Kannapiran and Ravindran 2012). Recently, Nithya and Pandian (2010) studied the effect of metal and antibiotic on the benthic bacteria of Gulf of Mannar. Recently Jadhav et al. (2012) has studied on the effect of phenyl tins on total bacterial count of bacteria in surface sediment of the Visakhapatnam harbour, India.

In the West coast regions studies were on diverse groups of organisms such as bacteria, actinomycetes, yeasts, fungi, marine protists namely, Thraustochytrids and on the phylogenetic diversity of carbohydrate degrading culturable bacteria etc. (Karanth et al. 1977; Nair and Lokabharathi 1977; Nair 1979; Nair et al. 1980; Nair and LokaBharathi 1982; Raghukumar 2002; Khandeparker et al. 2011) from non estuarine areas. Studies have been carried out on the distribution of total heterotrophic and coliform bacteria, phenol-degrading bacteria, luminescence bacteria, associations of SRB with chemosynthetic sulphur oxidizers like *Thiobacillus* sp. and photosynthetic forms like *Chromatium* sp. phosphate solubilizers, various physiological groups like sulphate-reducing bacteria (SRB), sulphide oxidizers (SO) and denitrifying bacteria in Mandovi-Zuari estuary (Rowe 1981; Gomes and Mavinkurve 1982; LokaBharathi 1989; LokaBharathi et al. 1991; Ramaiah and Chandramohan 1993; DeSouza et al. 2000) and some activities of certain groups viz. dehydrogenase activity, sulphate-reducing activity, and nitrate reducing activity. Rodrigues et al (2011a) studied the long-term variations in abundance and distribution of sewage pollution indicator and human pathogenic bacteria along the central west coast of India and reported the occurrence of pollution indicator bacteria (total coliforms, fecal coliforms and *Escherichia coli*) and potential pathogens (*Vibrio cholerae, Shigella, and Salmonella* sp.). De Souza (2000) and Ram et al. (2002) studies the variability in particle associated bacteria and bacterioplankton and their role in the geochemical cycle of the estuary.

Compare to above two estuaries microbiological studies are very limited in Cochin estuary. Few studies that were carried on in the water

column were (1) on health indicator bacteria like *V. cholerae's* and *Bacillus pumilus*, using traditional and molecular approaches (Thomas et al. 2006; Parvathi et al. 2009; Parvathi et al. 2011) and (2) on bacterioplankton dynamics of the estuary (Thottathil et al. 2008a; Thottathil et al. 2008b). There is only one report available on the benthic bacteria from CE estuary. Jose et. al. (2011) has reported the reduction/adaptation in the diversity of benthic heterotrophic bacteria and enzyme expression to heavy metal pollution. In general, there is a lacuna on the microbial community studies from Indian region and few studies have been reported are based on the culturable fraction of the community. Though PLFA has been recognized as a relatively sensitive, straightforward and reliable method for community study, hardly this approach has been attempted except for a recent report by Harji et al. (2010) on the distribution of different markers in the sediments of Vishakapatanam harbour, east coast of India.

2.5. Scope and objective

Tropical estuaries are highly productive and rich in biodiversity. Microbial community is a central paradigm of the estuarine ecosystems because these organisms are responsible for the bulk of organic carbon utilization and respiration. Therefore, knowing the microbial community is a pre-requisite for the systematic study of microbial biogeography and community assembly. Moreover, cultural eutrophication (anthropogenically induced increases in primary productivity conditions) is cited as amongst the most important environmental problems presently requiring scientific and political attention (Diaz and Rosenberg 1995; Nixon 1995). In addition to their harmful effects on organisms and ecosystems, it could have broader

implications in global carbon cycling (Henrichs 1992). Benthic microbiology is field that has drawn very little attention among marine microbial ecologists. In Cochin estuary extensively studied have been carried out on the macro benthic community. There is an obvious lacuna in benthic microbial study and the microbial community remains poorly characterized in Cochin estuary. Therefore, the present study focuses on identifying spatial and temporal variation in sediment microbial community structure and its relationship to sediment characteristics in Cochin estuary.

Objectives: -

1. To establish the spatial and temporal distribution of the benthic microbial community structure based on culturable dependant and independent ester linked PLFA approach.
2. To establish the interrelationship between bacterial abundance and diversity.
3. To study the interrelationship between the environmental parameters and community structure.

CHAPTER 3

MATERIALS AND METHODS

3.1. STUDY AREA

Cochin Estuary (study area) is located (Figure 3.1) along the south west coast of India (Lat 09°40' – 10°10' N, Long 76°15' – 76°30' E). It spreads over 256 km^2 (Gopalan et al. 1983) and has an estimated length of ~60 km. The average depth of the estuary is 5±2 m, except in the mouth region where the depth varies from 10 to 13 m and this is maintained for the navigation purpose. The annual rainfall in Cochin is around 3200 mm of which nearly 75% occurs during summer monsoon period (Qasim 2003). The onset of summer monsoon and its duration vary from year to year. Normally, it occurs from June to September. During the peak monsoon (July/August), heavy rains (400 - 500 mm) can occur within a few hours (Qasim 2003). During this period, salinity remains near zero over a large part of the estuary. The freshwater discharge has a major influence on the salinity distribution which in turn plays an important role in the ecology of the estuary (Madhupratap 1987). During the post-monsoon period (October-January), as the river discharge gradually diminishes and tidal influence gains momentum, the estuary changes into partially mixed and weakly stratified ecosystem (Menon et al. 2000). During pre-monsoon period (February - May), the freshwater input is the lowest, when the estuary behaves as an extension of the Arabian Sea (Madhupratap 1987; Menon et al. 2000). Since the backwater system is geographically located in the tropical region, the mean temperature of surface water remains ~ 28°C during monsoon and 30°C during pre-monsoon periods (Madhupratap 1987). As it is influenced by tides and fresh water influx, wide gradients result in the limnological characteristics. It is a

very productive ecosystem. It also receives substantial input of untreated effluents (10^4 million Ld^{-1}) from major industries [e.g., like Hindustan Insecticide (HIL), Cochin Refineries Ltd, United Catalysts India (UCI), Periyar Chemicals, Indian Rare Earths (IRE), Fertilizers & Chemicals Travancore (FACT) and Travancore Chemical Manufacturing (TCMC)] and domestic sectors (260 t d^{-1}) (Joy et al. 1990).

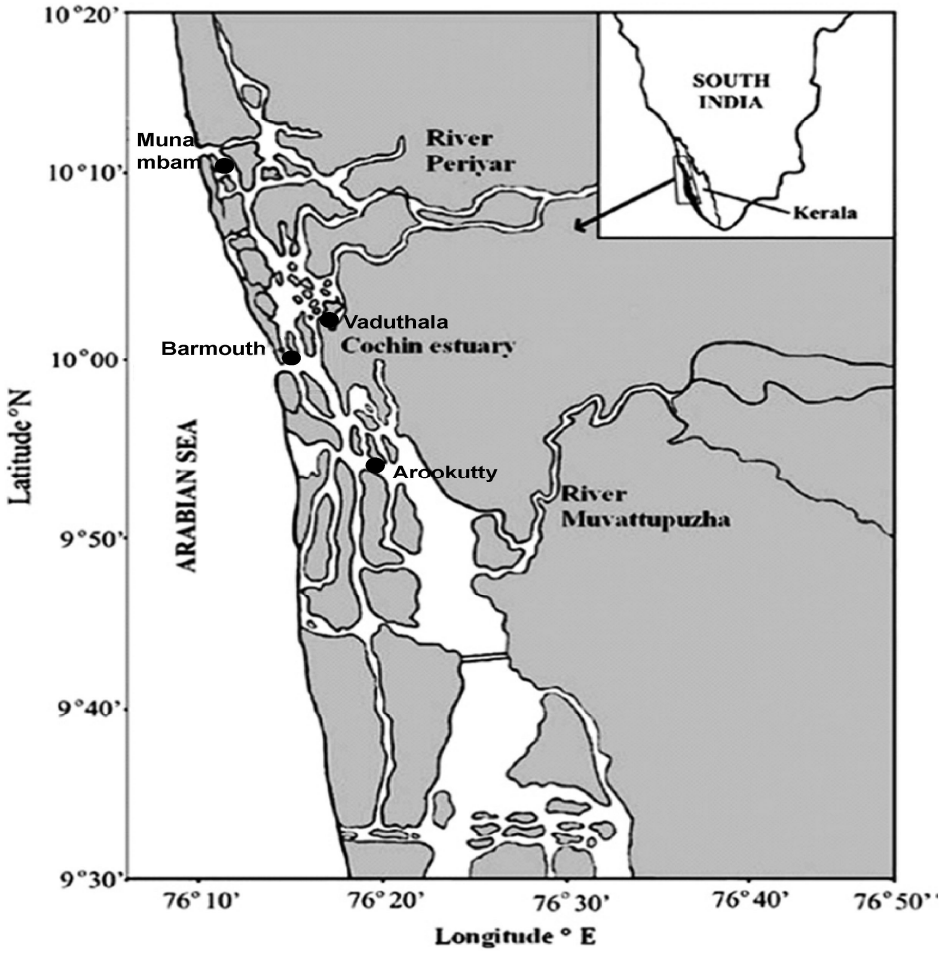

Figure 3.1 Map showing the station locations

3.1.1. Sampling strategy

For this study, 4 stations were selected based on the source of inputs. Organic matter and hazardous substances are known to accumulate in

estuarine sediment. Hence, a study on the influence of the riverine inputs in general and the bacterial community in particular will be useful for a better understanding and efficient management of this estuarine ecosystem. Stations were fixed using the Global Positioning System (Magellan NAV DLX 10, USA) as shown in Figure 3.1.

3.1.2. Description of the stations

STATION 1: Barmouth (9°58'N and 76°15'E) is the major opening through which Arabian Sea waters enters the estuary. This location is relatively less polluted and is considered as the reference station in this study. Average depth is 10 m and is influenced by intense tidal activity (Srinivas et al. 2003).

STATION 2: Vaduthala (10°02'N and 76°18'E) is located in the northern part of the estuary and is the entry point of River Periyar. Sixteen major and several minor industries (Chemical, Fertilizer, Metallurgical and Insecticides etc.) are situated on the banks of this river, discharging large quantities of effluents into the estuary. These effluents are either untreated or partially treated wastes (acids, nutrients and heavy metals) (Balachandran et al. 2003). The average depth in this location is 5.5 m.

STATION 3: Munambam (10°10'N and 76°11'E) is located in the northern end of the estuary nearer to the northern Azhikode inlet and has a fishing harbor. It also has sewage input and is categorized as a saline area. The average depth at this station is 4 m and lies close to the Munambam fishing harbor.

STATION 4: Arookutty (09°52'N and 76°20'E) is located in south side estuary, closer to the Muvattupuzha River entrance and receives wastes and nutrients from a paper mill and agriculture fields. The average depth at this station is 5 m.

3.1.3. Period of sampling

Sampling was done for one year (2009) covering three different season:– pre-monsoon (February – April), monsoon (June – August) and post-monsoon (October – December) at the above four locations using a hired boat. Sampling was done as a part of the Supra Institutional Project (SIP 1302) "Ecobiogeography of the southwest and southeast coasts of India".

3.1.4. Collection of samples

Surface water samples were collected using a clean plastic bucket while near bottom water samples were collected using 5L Niskin sampler (Hydrobios). The sampler was closed at the desired depth with the help of a messenger and hauled to the boat without contamination. Sediment samples were collected in duplicate using a Van Veen grab (mouth area 0.048 m^2). All samples were transported to the laboratory in ice for further analysis. Samples for microbiological studies were stored at -20°C till analysis.

3.2. ANALYSIS OF ENVIRONMENT PARAMETERS

3.2.1. WATER

The flow chart of the parameters measured for water column is given in Figure 3.2 and the methodologies, abbreviations and units used are given in Table 3.1.

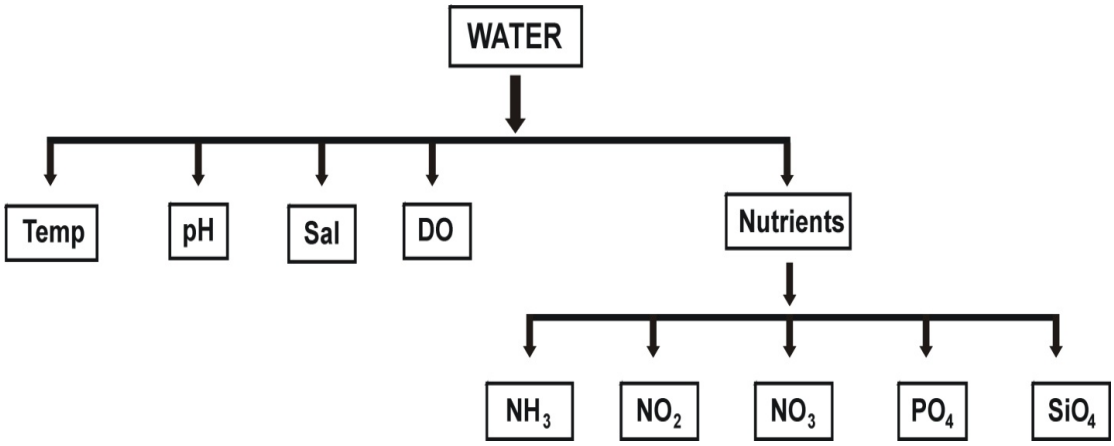

Figure 3.2 Flow chart of environment parameters measured for water sample

Table 3.1 Parameters, methodologies and units used for water sample

Sl. No	Parameter	Abbreviation	Techniques/Instruments	Unit
1	Temperature	Temp.	CTD (SeaBird)	ºC
2	Salinity	Sal.	CTD (SeaBird)	psu
3	pH	pH	pH electrode (ELICO)	
4	Dissolved Oxygen	DO	Titration method (Strickland and Parsons, 1972)	ml l^{-1}
5	Ammonia-N	NH_4	Spectrophotometer (Grasshoff et al. 1983)	µmol l^{-1}
6	Nitrite-N	NO_2	do	µmol l^{-1}
7	Nitrate-N	NO_3	do	µmol l^{-1}
8	Phosphate-P	PO_4	do	µmol l^{-1}
9	Silicate-Si	SiO_4	do	µmol l^{-1}

3.2.1.1. Salinity, Temperature and pH

Salinity and temperature were measured onboard with the aid of a portable Conductivity-Temperature-Depth (CTD) probe (Sea Bird Electronics, Inc., USA). A digital pH meter (ELICO L I610, accuracy ±0.01) was used for measuring pH after calibrating with standard pH buffers 4, 7, and 9.2. The DO samples were analyzed following the Winkler's method (Strickland and Parsons, 1972).

3.2.1.2. Dissolved oxygen (DO)

Dissolved oxygen was determined by the Winkler's method by Strickland and Parsons (1972) with standard iodimetric titration. The principle of the determination and the possible sources of systematic errors are discussed by Grasshoff et al. (1983).

Water samples were collected in 125 ml acid washed (10% HCl) glass-stoppered bottles taking care that no air bubbles were trapped inside and fixed immediately on board with 1 ml of manganous chloride and 1 ml of alkaline iodide solution (Winkler's reagents A & B respectively). The samples were mixed and the precipitate was allowed to settle. The water samples were transported for analysis to the laboratory. 1 ml of sulphuric acid (10N) was added and the sample was shaken thoroughly for dissolving the precipitate and was titrated against 0.01N sodium thiosulphate using starch as indicator. The procedure was standardized using potassium dichromate.

3.2.1.3. Ammonia-N (NH_4)

Ammonia-N was determined according to the indophenol blue method of Koroleff (1983). The measurement of ammonia included both free dissolved ammonia gas and the ammonium ions. This method estimates the sum of NH_4^+ and NH_3^- and is denoted here as NH_4-N. In a moderately alkaline medium, ammonia reacts with hypochlorite to form monochloramine, which in presence of phenol, catalytic amount of nitroprusside ions and excess of hypochlorite forms indophenols blue. The formation of monochloramine requires a pH between 8 and 11.5. At higher pH, ammonia is incompletely oxidized to nitrite. Calcium and magnesium ions in seawater precipitate as hydroxide and carbonate above pH 9.6. However, their precipitation can be prevented by complexing them with citrate buffer. Great care was taken to ensure that samples, blanks and standards are not contaminated during the course of analysis. The samples were 'fixed' by the addition of reagents immediately after collection and the absorbance, after the colour development (about 6 hours) were measured at 630 nm. The Standard graph is in the Appendix section

3.2.1.4. Nitrite-N (NO_2)

In this method, nitrite in the water sample when treated with sulphanilamide in acid solution results in a diazo compound, which reacts with N-l-naphthyl ethylene diamine dihydrochloride to form an azo dye (Grasshoff 1983). The absorbance of this colour complex was measured at 543 nm. Standards were run with analytical reagent quality sodium nitrite (Standard curve is in the Appendix section).

3.2.1.5. Nitrate-N (NO_3)

Nitrate-N in the water sample was quantitatively reduced to nitrite by passing through a reduction column filled with copper coated cadmium granules and measured as nitrite. During the reduction stage, ammonium chloride buffer was added to the sample to maintain a stable pH (Grasshoff 1983). The sample after reduction was analyzed for nitrite-N as described in section 3.2.1.4.

3.2.1.6. Phosphate-P (PO_4)

Phosphate-P was determined as inorganic phosphate by the formation of a reduced phosphomolybdenum blue complex in an acid solution containing molybdic acid, ascorbic acid and trivalent antimony. The method was developed by Murphy and Riley (1962) and a variation of this method described by Grasshoff et al. (1983) was adopted in the present work. Instead of single solution reagent as in the Murphy and Riley procedure, two stable reagent solutions were used here. A mixed reagent of 0.5 ml containing molybdic acid and antimony tartrate followed by 0.5 ml of ascorbic acid reagent were added to 25 ml aliquots of the samples. The absorbance was measured at 882 nm within 30 minutes to reduce any possible interference from arsenite. Potassium dihydrogen orthophosphate was used as standard (Standard curve in Appendix section)

3.2.1.7. Silicate -Si (SiO_4)

The determination of dissolved silicate in seawater is based on the formation of a yellow silicomolybdic acid when an acid sample is treated with a

molybdate solution (Grasshoff 1983). This is further reduced by ascorbic acid in presence of oxalic acid (to prevent interference from phosphate) to a blue coloured complex (molybdenum blue). The blue color was measured at 810 nm. The standard graph is in the Appendix section.

3.2.2. SEDIMENT

The flow chart of the parameters measured for sediment is given in Figure 3.3 and the methodologies, abbreviations and units used is given in Table 3.2.

Table 3.2 Techniques used for determination of geo-chemical parameter

Sl. No	Parameters	Abbreviation	Techniques/Instruments	Unit
1	Total Organic Carbon	TOC	CHN elemental analyzer (Thermo Finnigan, Flash EA1112) (Hedges and Keil, 1995)	%
2	Total Organic Nitrogen	TON	CHN elemental analyzer (Thermo Finnigan, Flash EA1112) (Hedges and Keil, 1995)	%
3	Protein	PRO	Spectrophotometer (Lowry et al. 1951)	$mg.g^{-1}$
4	Carbohydrate	CHO	Spectrophotometer (Kochert et al. 1978)	$mg.g^{-1}$
5	Lipid	LIP	Spectrophotometer (Parsons et al. 1984)	$mg.g^{-1}$

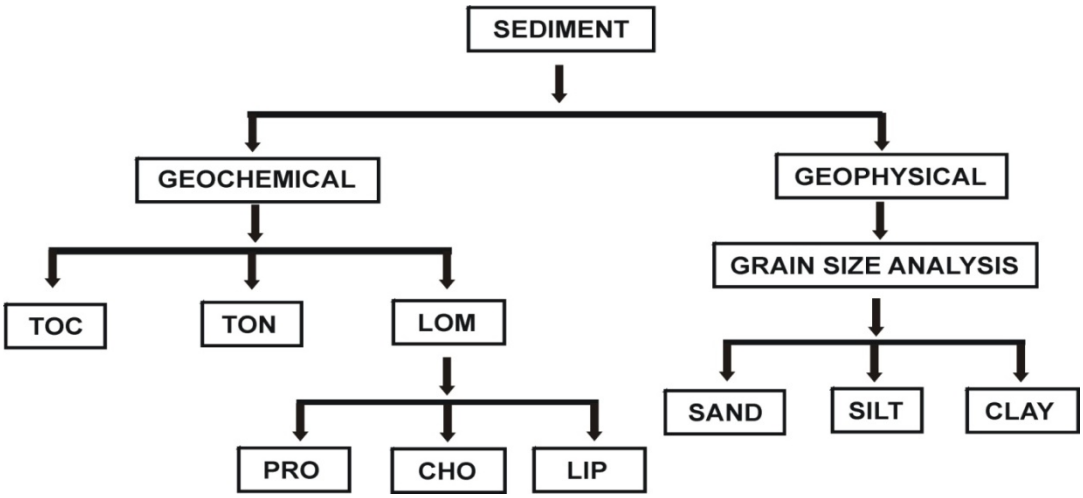

Figure 3.3 Flow chart for analysis of sediment sample

3.2.2.1. Sediment Texture

The sediment was dried in a hot air oven at 95°C. One portion of sediment was subjected to textural characteristics following pipette analysis (Krumbein. and Petti John 1938). The sand, silt and clay fractions were identified.

3.2.2.2. Total Organic Carbon (TOC) and Total Organic Nitrogen (TON)

Samples used for analysis were kept at 40°C and later ground into fine powder. TOC content was determined after acidification of the dry sediment following standard procedure (Hedges and Keil 1995). Sulfurous acid was added until cessation of effervescence, which indicates a complete removal of inorganic carbon. TOC and TON were subsequently measured using a CHN elemental analyzer (Thermo Finnigan, Flash EA1112) using L-Cystine as standard.

3.2.2.3. Protein (PRO)

Labile Organic Matter (LOM) is referred to the total of protein, carbohydrate and lipid compounds. Total protein in lyophilized sediments was extracted using Folin–Phenol reagent (Lowry et al. 1951). The principle involves a two step reaction: The Cu-protein complex formed in alkaline solution (NaOH) is reduced to phosphomolybdate phosphotungstate reagent to yield blue colour. The Folin's reagent is stable in acidic condition but, is reduced at pH > 10. Hence, Folin's reagent was added to alkaline copper-protein solution. To 500 mg of sediment (in triplicates), 2 ml of 1 N NaOH was added and boiled at 100^{o}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). (Bovine serum albumin was used as standard and the standard curve in Appendix section).

The amount of protein in the sediment was calculated by the formula:

$$\text{Protein mg per gm} = \frac{(\text{Sample absorbance} - \text{blank}) \times K}{\text{Volume of aliquot taken}}$$

Where K = constant from standard graph. The standard graph is given in the appendix.

3.2.2.4. Carbohydrate (CHO)

Total hydrolysable carbohydrates (CHO) were analyzed according to phenol sulphuric acid method, wherein glucose is used as the standard (Kochert 1978). The test is based on the formation of yellow colour when concentrated sulphuric acid is added to the sample mixed with phenol solution. Carbohydrates are dehydrated by concentrated sulphuric acid to form furfural and variety of other degradation products. 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^{o}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). Standard curve is in the Appendix section.

3.2.2.5. Lipid (LIP)

Lipids in the sediment samples were estimated using acid dichromate oxidation (micro or ultra method) as outlined by Parsons et al. (1984) using stearic acid. The test is based on the oxidation of acid dichromate which is followed by a decrease in the dichromate colour. The extinction of the reaction mixture has an inverse relationship with the lipid concentration based on the decrease of dichromate colour. To 3.8 ml of organic solvent, 0.5 g of sediment sample 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 and 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 is in Appendix section.

3.3. ANALYSIS OF MICROBIOLOGICAL PARAMETERS

The flow chart of microbiological analysis is given in Figure 3.4

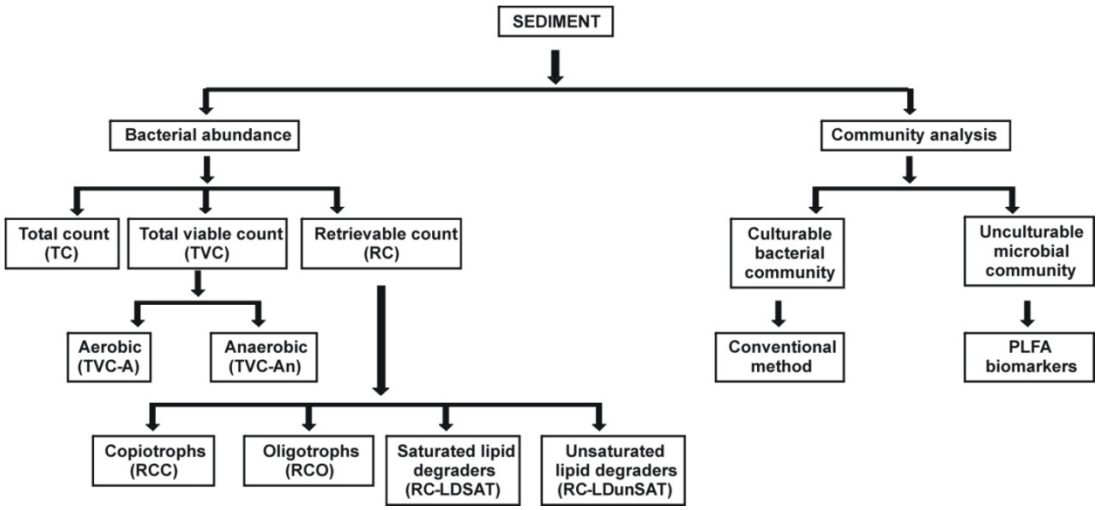

Figure 3.4 The flow chart of microbiological analysis of sediment samples

3.3.1. ABUNDANCE

Three distinct fractions of the benthic bacteria viz, total, total direct viable (aerobe and anaerobe) and retrievable counts were enumerated. 5.0 g of

sediment (wet weight) samples were dispersed in 45 ml autoclaved filter sterilized sea water with 0.5% Tween 80 and sonicated for 3 minutes. The sediment was allowed to settle for 2 minutes and the supernatant was serially diluted up to 10^{-6} dilution. CFU counts were also observed from this sample.

3.3.1.1. Total bacterial counts (TC)

Total bacterial counts were determined by Acridine Orange Direct Count (AODC) method (Hobbie et al. 1977). Samples for total bacterial counts were fixed immediately with buffered formalin and stored at 4°C. Duplicate samples (1 ml) preserved with 2% (final concentration) buffered formalin were stained with 100 µl of acridine orange stain (Hi-Media, Mumbai) (final concentration 0.01% w/v) and incubated in dark for 2 minutes before filtering through 0.22 µm black stained Nuclepore polycarbonate membrane filter (Whatman Asia Pacific, Singapore). The slide was then viewed under oil immersion lens using Olympus epifluorescence microscope (Olympus Corporation, Japan), equipped with HbO lamp and U-MWB2 mirror unit having excitation filter of 460–490 nm and emission filter of 520 nm. About 10-12 fields of >30 bacteria per field were counted per filter and the average field count was used to calculate the bacterial abundance. Bacterial abundance is expressed as numbers g^{-1} (Dry weight)

3.3.1.2. Total aerobic viable counts (TVC-A)

Total viable count was enumerated following the method of Kogure et al. (1987). Autoclaved yeast extract was added to the water sample to a final

concentration of 0.01% (wt/vol) followed by addition of filter sterilized antibiotic cocktail containing nalidixic acid (final concentration 0.002%, wt/vol), piromidic acid, (final concentration of 0.001%, wt/vol) and pipemidic acid (final concentration of 0.001%, wt/vol). The samples were incubated at room temperature (28±2°C) in dark for 6 hours and followed by fixation with formalin to a final concentration of 2%. Swollen and elongated cells were enumerated as viable bacterial cells. Counting procedure was the same as mentioned in the above section 3.3.1.1.

3.3.1.3. Total anaerobic viable counts (TVC-An)

Total viable counts for anaerobes were determined by adding nalidixic acid (0.002% w/v), piromidic acid (0.001% w/v), pipemidic acid (0.001% w/v) (Sigma, USA) and yeast extract (0.01%) followed by sodium sulphide (Na_2S) (0.05% concentration) in each vial. Samples were incubated for 6 hours in dark and fixation and counting were done as above.

3.3.1.4. Retrievable Count - Copiotrophs (RCC)

Retrievable counts of copiotrophs were estimated by surface plated on duplicate spread plate method on 100% Nutrient agar plates (HiMedia Laboratories Pvt. Ltd., Mumbai, India) after appropriate serial dilution. Nutrient agar (NA) was prepared in 50% filter- sterilized aged seawater. Colonies were counted after 48 hr of incubation at room temperature (28±2°C) and expressed as CFU g^{-1}.

3.3.1.5. Retrievable Count - Oligotrophs (RCO)

Retrievable counts of oligotrophs were estimated by spread plate method on 10% NA plates which were prepared in 50% filter- sterilized aged seawater, after appropriate serial dilution. The Colonies were counted after 48 hr of incubation at room temperature (28±2°C) and expressed as CFU g^{-1}.

3.3.1.6. Retrievable Saturated Lipid degrader (RC-LD_{SAT})

Saturated lipid degrading bacteria were enumerated on nutrient agar plates incorporated with Tween 40 (16:0, palmitic acid). Bacterial colonies with clear zone around it were counted.

3.3.1.7. Retrievable Unsaturated Lipid degrader (RC-LD_{UNSAT})

Unsaturated lipid degrading bacteria were enumerated on nutrient agar plates incorporated with Tween 80 (18:0, Oleic acid). Bacterial colonies with clear zone around it were counted.

3.3.2. COMMUNITY

Culture Independent

3.3.2.1. Ester Linked Microbial Community (EL-PLFA)

Phospholipid fatty acid analysis (PLFA ester linked fatty acid) was used to obtain the active microbial community structure of the sediment sample (White et al. 1979; Borga et al. 1994; White et al. 1996; Häggblom et al. 2000). Sediment samples were lyophilized (Heto Freeze dryer, FD 8) and ground to a fine powder and kept at -20°C.

Solid phase extraction (SPE)

Lyophilized and powdered samples (5g) were placed into 50 ml Pyrex centrifuge tubes with Teflon-lined caps. The first extraction phase consisted of addition of 10 ml methanol, 5 ml chloroform and 4 ml phosphate buffer (50 mM K_2HPO_4, pH 7.4). After sonication for 10 minutes in water bath sonicator, the tubes were incubated with intermittent shaking for 2 hours. The tubes were subsequently centrifuged for 10 minutes at 2500 rpm (25°C). The supernatant was poured into a 30 ml test tube with teflon lined screw cap. Separation of the phases was achieved by addition of 5 ml each of chloroform and water. The test tube was vigorously shaken and kept overnight for the phases to be separated. Top aqueous phase was removed and the bottom organic phase was evaporated under a stream of N_2 (Kehrmeyer et al. 1996).

SPE fractionation

The total lipid contained in the organic phase was fractionated using silica gel chromatography to separate the polar lipids from the other lipid fractions. The polar lipid fraction containing the phospholipids was separated from the lipid extracts by column chromatography with silica gel as the adsorbent. The lipid extract was evaporated under N_2 and re-dissolved in 250 µl of chloroform and then applied to the top of a 500 mg silica gel solid phase extraction (SPE) chromatography column (100±200 mesh, Agilent Technologies) rinsed with chloroform. Neutral lipids and glycolipids were eluted from the silica gel with 10 ml chloroform and 10 ml acetone, respectively. Polar lipids (mainly phospholipids) were then eluted with 10 ml

methanol into a 13x100 Pyrex test tube with a Teflon-lined screw cap. Methanol was evaporated under stream of N_2 (King et al. 1977; Guckert et al. 1985).

Trans esterification

The polar lipid fraction containing the phospholipids was then subjected to a mild alkaline methanolysis. This resulted in cleavage of the fatty acids from the phosphoglyceride backbones, creating fatty acid methyl esters (PL-FAME). One milliliter of methanol-toluene (1:1 [vol/vol]) and 1 ml of 0.2 M methanolic KOH were added to each Pyrex tube, which was capped, mixed, and placed in a water bath at 37°C for 15 minutes. Then, 2 ml of hexane, 0.3 ml of 1 M acetic acid, and 2 ml of de-mineralized water were added. After thorough mixing, samples were centrifuged and the organic phase was transferred to a clean Pyrex tube. After a second wash with 2 ml of hexane, the combined organic phase was dried under N_2 (Dowling et al. 1986; Petersen and Klug 1994).

Clean up

The final extract was SPE fractionated with NH_2 cartridge. The dried FAME extract was re-dissolved in chloroform (four times, 100 µl) and drained the sample into 100 mg Amino (NH_2) SPE chromatography column (Agilent Technologies) which was rinsed with 1 ml chloroform. The rest of the sample was eluted out into a clean test tube with 2 ml chloroform and dried under a stream of nitrogen.

Gas Chromatography analysis

The temperatures used were 250°C for the injection port and 300°C for the detector. FAME extract was re-dissolved in 200 µl MIDI reagent 3 and transferred to limited-volume GC vial. PL-FAMEs were separated by capillary gas chromatography on a Hewlett Packard 6890 N gas chromatograph in split mode (1:50) housing a cross-linked phenyl – methyl siloxane capillary (25 m, 0.2 mm) with flame – ionization detector. Hydrogen was used as the flame gas. The gas flow rates were approximately 400 $ml.min^{-1}$ for air, 30 $ml.min^{-1}$ for hydrogen and 30 $ml.min^{-1}$ for nitrogen. Temperature of the oven was increased from 170°C to 300°C at a rate of 5°C $min^{-1,}$ and held at 300°C for 12 min, and reduced to 170°C before the next sample was injected. The injector and Flame ionization detector (FID) temperatures were 250°C and 300°C, respectively. Since each fatty acid is sensitive to a particular temperature it gets volatilized at a particular retention time and gets detected by FID. Individual fatty acids (upto C30) is quantified as percentage of the total fatty acids recovered from the sample. Identification of the FAMEs was by comparison of retention time and equivalent chain length with known standards like Eukary calibration mixture – 1201A (Eukary6 method, Version: 3.7) and MIDI peak identification software (MIDI Inc., Newark, DE) (Drijber et al. 2000). Fatty acid nomenclature used was as follows: total bond from the methyl end of the molecule; cis and trans geometry are indicated by the suffixes c and t. The prefixes a and i refer to anteiso- and iso-branching; 10me indicates a methyl group on the tenth C atom from the carboxyl end of the molecule; position of

the hydroxy (OH) groups are noted; and cy indicates cyclopropane fatty acids.

3.3.3. Diversity of Culturable Bacteria

Fatty Acid Methyl Ester (FAME) based

Benthic culturable fraction of bacteria was identified based on Fatty acid finger printing using MIDI Inc. USA.

Bacterial Isolation and purification

After enumeration, single well isolated colonies of different morphotypes were isolated randomly from each media to identify the organisms from each group (Copiotrophs, oligotrophs, and saturated and unsaturated lipid degraders). Nearly 456 isolates were stored in NA slant at 4°C till further analysis. These isolates were purified by quadrant streaking on agar plate in a series of parallel streaks (Starr et al. 1981). These isolates were identified using fatty acid profiling. All the reagents and media used for fatty acid profiling are listed in Appendix.

Table 3.3. Number of different groups of culturable isolates.

Stations	RCC	RCO	RC-LD_{SAT}	RC-LD_{UNSAT}	Total
Barmouth	30	17	22	21	90
Vaduthala	30	24	28	26	108
Munambam	34	28	37	33	132
Arookutty	35	26	35	30	126
Total	129	95	122	110	456

Identification by Fatty acid Finger Printing

Fatty acid profile analysis of the isolates was performed using the standard protocols developed for aerobic bacteria by MIDI Inc. USA (Sasser 1990). Extracted fatty acid profile of the unknown bacterial isolates is compared to that of known isolates present in the inbuilt library.

Harvesting the cells

The isolates were grown in Soyabean Casein Digest Agar (SCDA) or Trypticase soy broth agar (TSBA, HiMedia) and incubated at room temperature (28±2^{o}C) for 24 hours. The cells were harvested from the culture media by gently scraping from the surface of the culture medium with a sterile 4 mm inoculating loop into an extraction tube with Teflon lined screw cap.

Saponification and Extraction

Before extracting the FAME, fatty acid was saponificated. First was the addition of a strong methanol base (1.0±0.1 ml of Reagent 1) and heating at 100°C for 30 minutes. Fatty acids were cleaved from the cell lipids and were converted to their sodium salts. The methylation step involves the addition of methylation reagent (2.0±0.1ml of Reagent 2) which converted the fatty acids (as sodium salts) to fatty acid methyl esters by keeping the tubes in 80°C for 10 minutes. Fatty acid methyl esters were extracted using the extraction solvent (1.25±0.1 ml of Reagent 3). A dilute base solution was added to remove free fatty acids and residual reagents from the organic

extract. The clear phase obtained was transferred to GC vials and kept at 4.0°C until the chromatographic analysis was carried out.

Gas Chromatography Analysis

FAME sample (2 µl) was injected into the column (25 m by 0.2 mm phenyl – methyl siloxane capillary) using a HP 6890N gas chromatograph equipped with flame – ionization detector. The gas flow rates were approximately 400 $ml.min^{-1}$ for air, 30 $ml.min^{-1}$ for hydrogen and 30 $ml.min^{-1}$ for nitrogen. The temperatures used were 250°C for the injection port and 300°C for the detector. The oven temperature was increased from 170 to 270°C at a rate of 5°C min^{-1}, and 270 to 310°C at a rate of 40°C min^{-1} and held at 310°C for 2 minutes, and then returned to 170°C before the next sample was injected. Since each fatty acid is sensitive to a particular temperature it gets volatilized at a particular retention time and gets detected by flame ionization detector (FID). Individual fatty acids were quantified as a percentage of the total fatty acids recovered from the sample. The chromatogram was visualized with the CHEMSTATION software and transferred to software SHERLOCK for matching the fatty acid profile of the unknown organism.

3.4. STATISTICAL ANALYSIS

Data on environmental parameters of water and sediment, abundance, community structure of culturable and non-culturable bacteria were subjected to various statistical analysis for making inferences (Snedecor and Cochran 1967). Depending on sample size and distribution, the data were normalized. The analysis carried out were correlation, two way ANOVA,

multiple regression in XLSTAT 2012 software. Diversity index (Shannon-Wiener) for culturable community (Shannon and Weaver 1963) and Cluster Analysis and PCA for PLFA community were calculated using primer 6 software. The PLFA data were calculated in moles percentages of total fatty acids, arcsine transformed, and extracted into a correlation matrix. Principal component analysis (PCA) was used to discern patterns within the data. The PCA loading scores indicated the relative importance of individual fatty acids in the weighting along each principal component. Redundancy discriminant analysis (RDA) was carried out in CANOCO 4.5 software.

CHAPTER 4

RESULTS

4.1. Environment Parameters

WATER CHEMISTRY

4.1.1. Temperature

Water column temperature at the 4 stations in CE ranged from 24 to 33.6°C with mean seasonal temperature of 29.1±1.9°C. Temperature of surface and near bottom waters ranged from 24.9 to 33.6°C and from 24.0 to 33.3°C respectively (Table 4.1). Analysis of variance showed significant spatial variation in the surface and near bottom water (F= 14.9 and 10.62 respectively, df = 3,5; p=0.001). During the study period the water column temperature generally decreased during the monsoon season at all the stations, but the variation was not significant. The surface and near bottom water temperatures also showed only marginal variation for any given month at all the locations.

Table 4.1 Seasonal variations in temperature of the surface and near bottom waters at the 4 locations

Surface water	**Pre-monsoon**			**Monsoon**			**Post-monsoon**		
	Feb	Apr	Avg	Jun	Aug	Avg	Oct	Dec	Avg
Barmouth	27.3	30.6	29.0	26.5	28.1	27.3	30.5	29.0	29.7
Vaduthala	29.5	32.3	30.9	24.9	28.0	26.6	28.5	29.5	29.0
Munambam	29.4	33.6	31.5	26.5	28.9	27.7	29.7	29.0	29.3
Arookutty	30.4	31.3	30.8	27.7	29.0	28.3	31.0	29.0	30.0
Near bottom water	**Pre-monsoon**			**Monsoon**			**Post- monsoon**		
	Feb	Apr	Avg	Jun	Aug	Avg	Oct	Dec	Avg
Barmouth	27.6	30.8	29.2	27.0	25.2	26.1	30.0	29.0	29.5
Vaduthala	29.7	32.4	31.1	27.7	27.7	27.7	28.9	29.0	28.9
Munambam	28.7	33.3	31.1	27.5	24.0	25.7	30.1	29.0	29.5
Arookutty	30.4	31.5	31.0	28.3	29.4	28.8	30.0	29.5	29.7

4.1.2. pH

The average seasonal pH of water column in CE during the study period was 7.5±0.52. Surface and near bottom water pH ranged from 6.33 to 8.04 and 6.2 to 8.12, respectively. pH higher than 8 was recorded in the month of December at Barmouth and Arookutty (Table 4.2). Analysis of variance showed significant spatial variation in pH for surface and near bottom water ($F = 6.35$ and 7.49 respectively, $df = 3,5$; $p = 0.001$). Unlike temperature, pH showed temporal variation at the surface and near bottom water ($F = 5.06$ and 6.61 respectively, $df = 3, 5$; $p = \geq 0.01$).

Table 4.2 Seasonal variations in pH of the surface and near bottom waters at the 4 locations

Surface water	**Pre-monsoon**			**Monsoon**			**Post-monsoon**		
	Feb	Apr	Avg	Jun	Aug	Avg	Oct	Dec	Avg
Barmouth	7.9	7.6	7.8	7.7	6.8	7.3	7.4	8.0	7.7
Vaduthala	7.4	7.6	7.5	6.9	6.9	6.9	6.4	7.5	6.9
Munambam	7.9	8.0	7.9	7.3	7.9	7.6	7.5	8.0	7.7
Arookutty	7.6	7.4	7.5	7.5	6.8	7.1	6.3	8.0	7.2
Near bottom water	**Pre-monsoon**			**Monsoon**			**Post- monsoon**		
	Feb	Apr	Avg	Jun	Aug	Avg	Oct	Dec	Avg
Barmouth	8.0	7.7	7.9	7.7	7.2	7.5	7.5	8.0	7.8
Vaduthala	7.8	7.4	7.6	7.0	6.8	6.9	6.2	7.6	6.9
Munambam	7.9	8.0	8.0	7.4	7.6	7.5	7.8	8.1	8.0
Arookutty	7.9	7.4	7.7	7.4	6.8	7.1	6.2	8.1	7.2

4.1.3. Salinity

The average salinity of the CE during the study period was 18.67±12.36 psu. Though Munambam and Barmouth recorded high salinity compared to Arookutty and Vaduthala, variation in salinity was less (Table 4.3). Surface and near bottom water salinity ranged from 0.2 to 34.53 psu and 0.11 to 34.55 psu, respectively. Maximum salinity was observed during the pre- monsoon followed by post-monsoon and minimum during the monsoon season. Analysis of variance showed significant spatial variation only in surface salinity ($F = 4.92$, $df = 3, 5$; $p=0.001$) whereas significant temporal variation was observed in surface and near bottom salinity ($F = 9.44$ and 23.41 respectively, $df = 3, 5$; $p= \leq 0.001$)

Table 4.3 Seasonal variations in salinity of the surface and near bottom waters at the 4 locations

Surface water	**Pre-monsoon**			**Monsoon**			**Post-monsoon**		
	Feb	Apr	Avg	Jun	Aug	Avg	Oct	Dec	Avg
Barmouth	32.4	23.4	27.9	11.3	4.3	7.8	32.2	31.4	31.8
Vaduthala	7.2	12.6	9.9	1.6	0.2	0.9	o.2	10.4	5.3
Munambam	29.1	34.5	31.8	3.5	29.1	16.3	12.5	30.1	21.3
Arookutty	20.2	18.2	19.2	2.8	0.4	1.6	11.5	17.1	14.3
Near bottom water	**Pre-monsoon**			**Monsoon**			**Post- monsoon**		
	Feb	Apr	Avg	Jun	Aug	Avg	Oct	Dec	Avg
Barmouth	33.4	27.2	30.3	24.6	27.6	26.1	32.8	33.4	33.1
Vaduthala	9.5	15.4	12.5	6.3	0.1	3.2	0.2	12.7	6.5
Munambam	30.7	34.6	32.6	32.8	34.2	33.5	30.9	33.2	32.1
Arookutty	22.5	18.9	20.7	3.9	0.4	2.2	23.0	26.9	25.0

4.1.4. Dissolved Oxygen (DO)

The average seasonal DO in the CE during the study period was 4± 0.98 ml.l^{-1}. The spatial and temporal variations in concentrations of DO at the four locations are shown in Figure 4.1. Maximum DO was observed at Vaduthala followed by Arookutty, Munambam and Barmouth. The surface and near bottom water DO ranged from 2.6 to 6.99 and 1.24 to 4.83 ml.l^{-1}, respectively. Maximum DO was observed during pre-monsoon followed by post-monsoon and monsoon. There was significant spatial and temporal variation in DO only of the surface water ($F = 4.40$ df = 3, 5; $p = \leq 0.01$ and $F = 4.19$ df = 3, 5; $p = \leq 0.01$).

4.1.5. Nutrients

The seasonal distribution of nutrients viz. Ammonia (NH_4-N), Nitrite (NO_2-N), Nitrate (NO_3-N), Phosphate (PO_4 – P) and Silicate (SiO_4 – Si) in the surface and near bottom waters are presented in Figure 4.2 A & B. The average concentrations of NH_4, NO_2, NO_3, PO_4 and SiO_4 in water column of CE during the study period were 10.81±11.66, 0.84±1.23, 15.99±9.17, 2.45±3.09 and 52.27±38.73 µmol.l^{-1}, respectively. Station wise variation was seen in the concentration of different nutrients. Silicate was the predominant nutrient at all the locations during the study period. Phosphate and nitrite concentrations were relatively very low. Maximum concentration of ammonia in the water column (surface and near bottom) was observed in Munambam followed by Vaduthala, Barmouth and Arookutty whereas for nitrite the decreasing order was Arookutty, Vaduthala, Barmouth and Munambam.

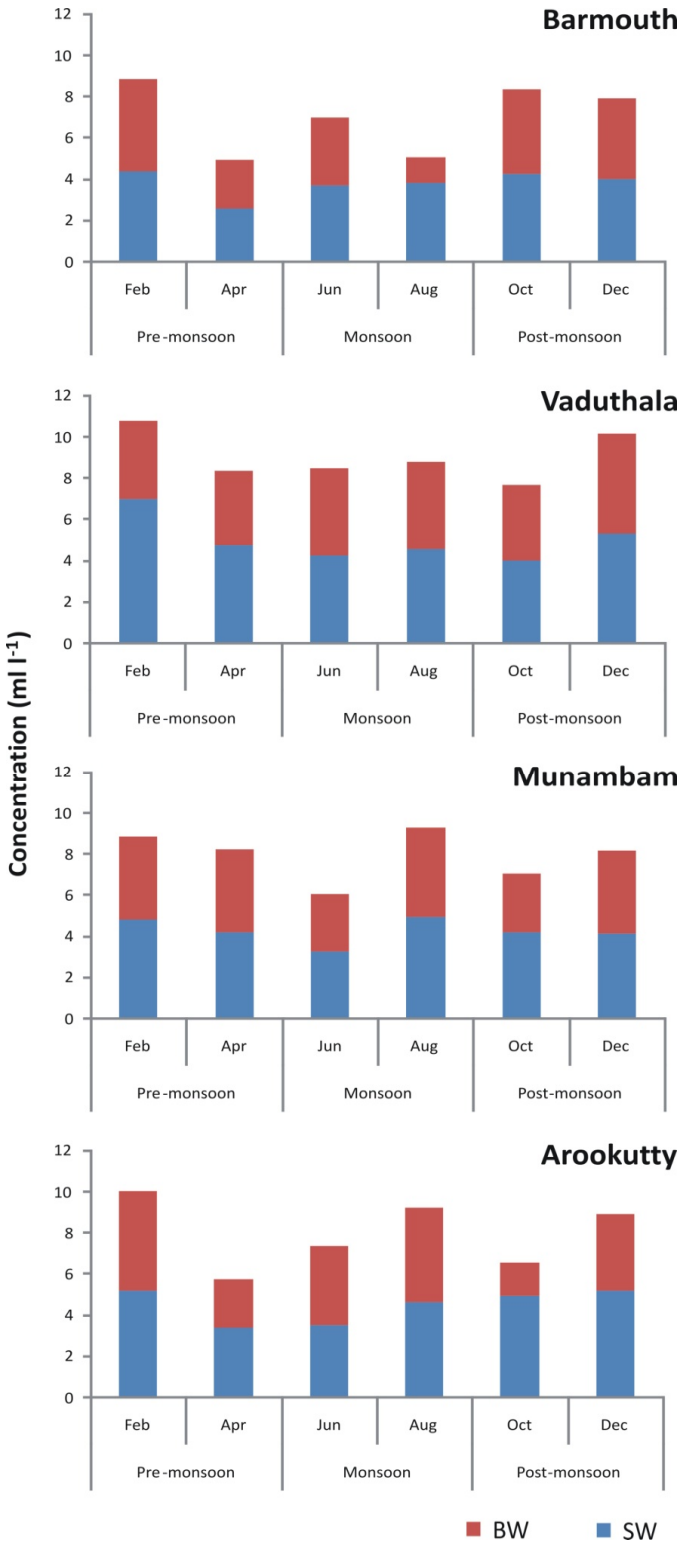

Figure 4.1 DO of surface (SW) and near bottom water (BW) at the study locations

The decreasing trend in phosphate concentration was Munambam >Arookutty >Barmouth >Vaduthala. The trend in the decreasing level for nitrate and silicate were similar (Barmouth >Munambam >Arookutty >Vaduthala). The range in concentrations of ammonia, nitrite, nitrate, phosphate and silicate in the surface and near bottom waters was 1.12 - 30.72 and 1.12 - 63.56; 0.13 - 3.9 and 0.15 - 7.86; 2.23 - 35.63 and 1.16 - 32.72; nd - 5.7 and 0.06 - 19.17; 8.33 - 151.51 and 7.37 - 118.22 $\mu mol.l^{-1}$, respectively (Figure 4.2 A & B). The concentration of the nutrients was more at the near bottom water at all the locations than in the surface water. Seasonal fluctuation was observed in the nutrient concentrations at all stations, but irrespective of the station, all the nutrients recorded the highest concentration during the monsoon period. The concentration of nutrients generally decreased from post to pre-monsoon season. There was no significant spatial and temporal variation seen in ammonia and nitrite concentrations both in surface and bottom waters. Significant spatial variation was observed in nitrate and phosphate concentrations in only surface water ($F = 3.30$ and 3.90 respectively, $df = 3, 5$; $p = \leq 0.01$). In case of silicate, analysis of variance showed significant spatial variation both in surface and near bottom waters. However, temporal variation in silicate concentration was seen only in near bottom water ($F = 7.7$, $df = 3, 5$; $p = \leq 0.01$).

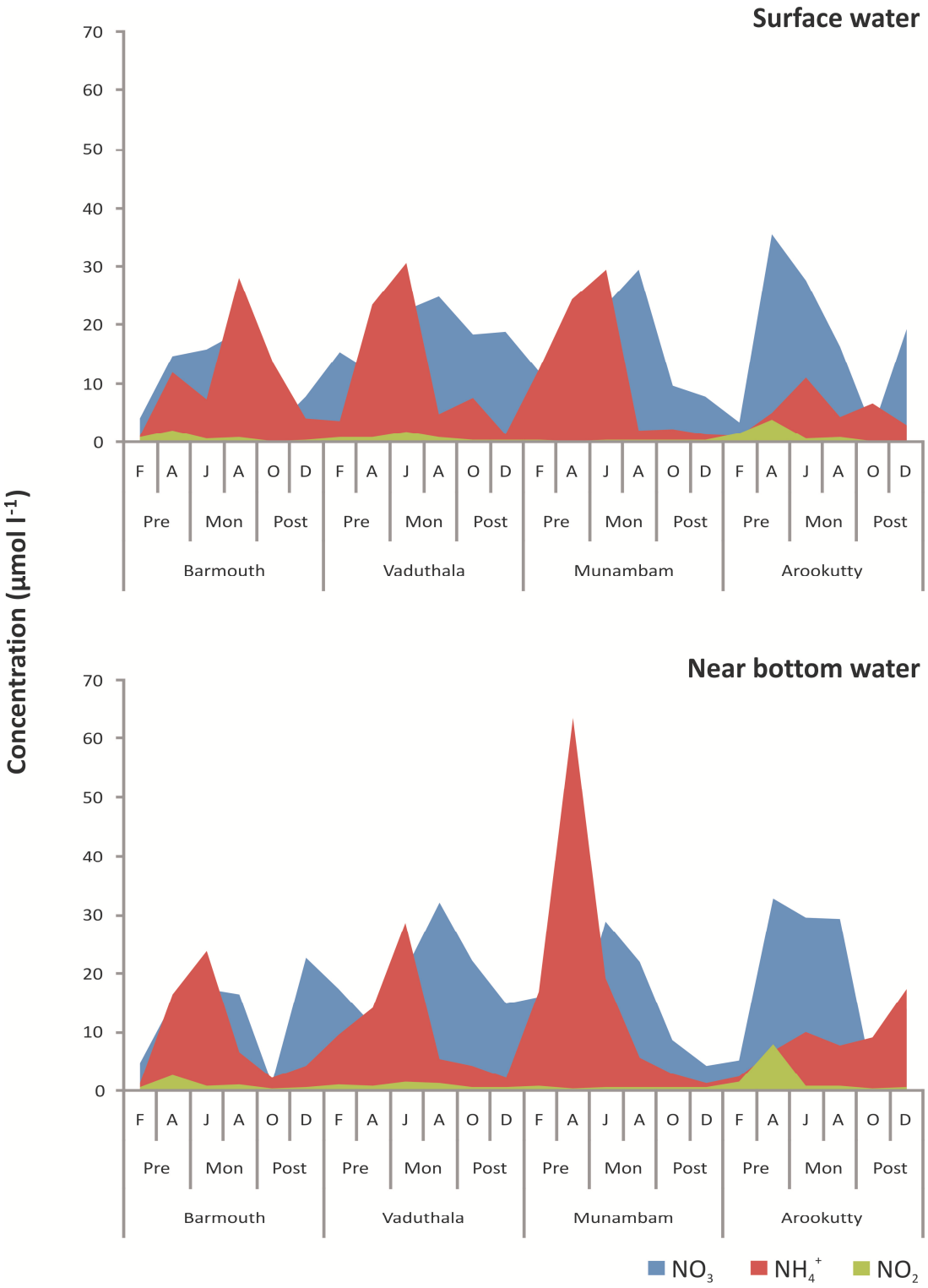

Figure 4.2A Distribution of Ammonia, Nitrate and Nitrite at the study locations

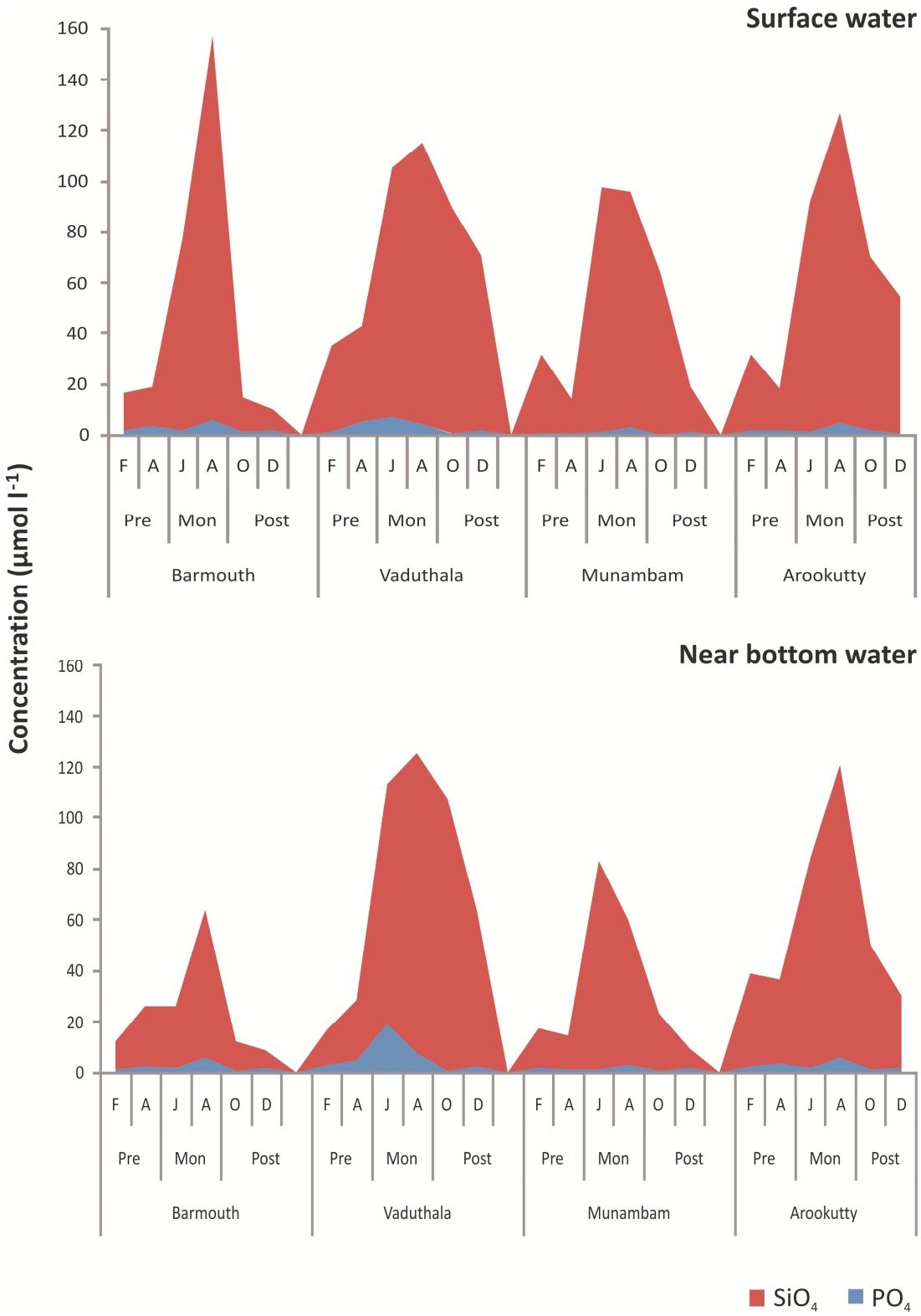

Figure 4.2B Distribution of Silicate and phosphate at the study locations

SEDIMENT CHARACTERISTICS

4.1.6 Sediment Texture

The texture of the surface sediment was dominated by sand (45±27%) followed by clay (32±14%) and silt (23±14%) in CE. The percentage of sand, silt and clay ranged from 4 to 81%, 3 to 46% and from 13 to 59% respectively (Figure 4.3). The percentage of sand was high at all the stations except Arookutty where the clay content was high during the study period.

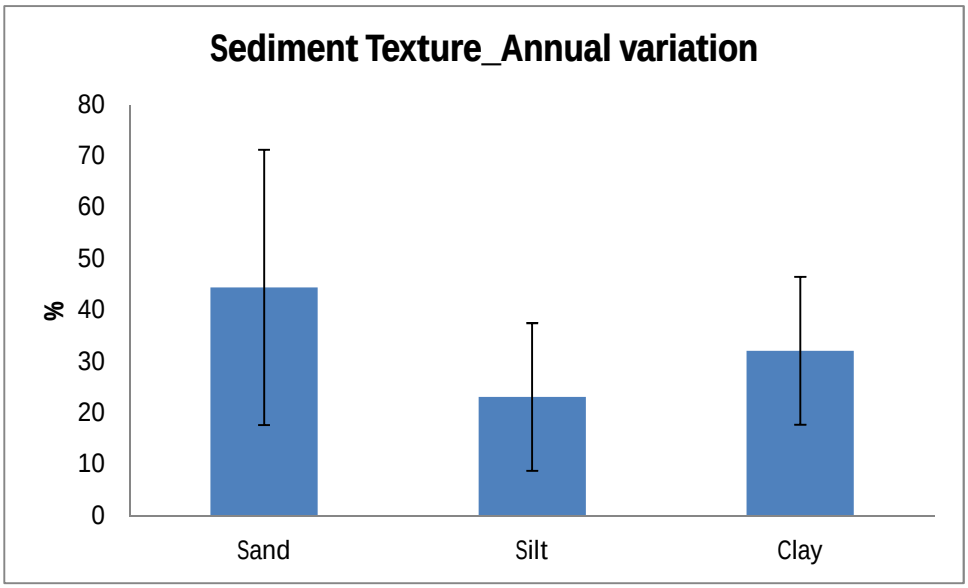

Figure 4.3 Percentage of sand, silt and clay in CE

The sediment texture showed seasonal variations at all the stations (Table 4.4). Significant temporal variation was observed in the percentage composition of sand, silt and clay ($F=16.62, 7.61, 34.62$; $df = 11$, $p \geq 0.001$).

Table 4.4 Seasonal average of sand, silt and clay at the 4 locations.

Sand	**Pre-monsoon**			**Monsoon**			**Post-monsoon**		
Station	Feb	Apr	Avg	Jun	Aug	Avg	Oct	Dec	Avg
Barmouth	26.15	46.50	36.33	55.70	22.45	39.08	39.60	62.35	50.98
Vaduthala	79.10	65.50	72.30	23.20	70.15	46.68	79.95	65.90	72.93
Munambam	51.60	65.40	58.50	66.30	81.15	73.73	69.50	50.15	59.83
Arookutty	4.10	6.70	5.40	8.05	10.60	9.33	12.00	7.05	9.53
Silt	**Pre-monsoon**			**Monsoon**			**Post-monsoon**		
Station	Feb	Apr	Avg	Jun	Aug	Avg	Oct	Dec	Avg
Barmouth	38.85	23.60	31.23	16.90	42.55	29.73	32.90	10.15	21.53
Vaduthala	3.25	11.60	7.43	42.70	10.05	26.38	7.55	11.60	9.58
Munambam	18.35	12.00	15.23	11.20	6.35	8.78	13.00	12.35	12.68
Arookutty	40.90	39.70	40.30	33.20	35.55	34.38	45.50	37.95	41.73
Clay	**Pre-monsoon**			**Monsoon**			**Post-monsoon**		
Station	Feb	Apr	Avg	Jun	Aug	Avg	Oct	Dec	Avg
Barmouth	35.00	30.00	32.50	27.50	35.00	31.25	27.50	27.50	27.50
Vaduthala	17.65	23.00	20.33	34.20	19.80	27.00	12.50	22.50	17.50
Munambam	30.05	22.50	26.28	22.50	12.50	17.50	17.50	37.50	27.50
Arookutty	55.00	53.60	54.30	58.80	53.85	56.33	42.50	55.00	48.75

4.1.7. Total Organic Carbon (TOC) and Total Organic Nitrogen (TON)

Overall, TOC concentration in the sediment in the CE during the study period varied from 0.79 to 3.52% (av.2.05±0.8%). Monthly distribution of TOC for each station with respect to the total of that station is illustrated in Figure 4.4. At Arookutty, the concentration was high and monthly variation was minimum. Clay content at this station was also high. Maximum concentration of TOC was observed during monsoon followed by post-

monsoon and pre-monsoon. The seasonal average of TON concentration in Cochin estuary throughout the study period was 0.23±0.11% and the concentration ranged from 0.05 to 0.42%. Maximum concentration was observed in Arookutty followed by Barmouth, Vaduthala and Munambam (Figure 4.4). Season wise variation of TON concentration in Cochin estuary was less but was significant. There was significant temporal variation in TOC and TON ($F= 5.19$ and 8.47 respectively, $df=11$, $p \leq 0.001$) in the sediment samples.

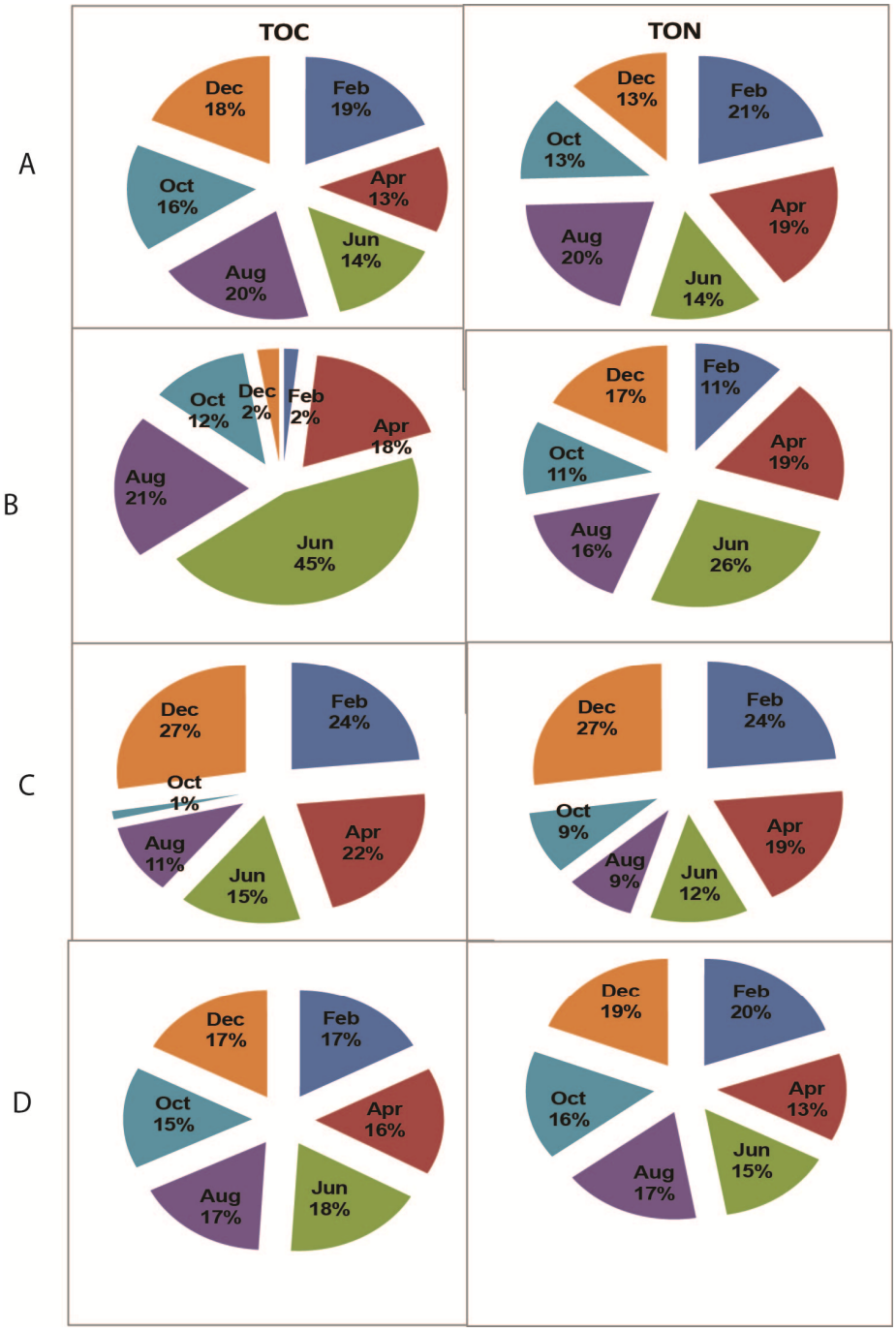

Figure 4.4 Percentage seasonal distribution of TOC and TON in A) Barmouth B) Vadutala C) Munambam D) Arookutty

4.1.8. Carbon: Nitrogen Ratio(C:N)

The average C:N ratio in CE was > 6. It varied from 6.58 to 16.20. Although seasonal variation was seen, no particular trend in C:N ratio was

observed (Table 4.5). Compared to the other 3 stations, at Arookutty, the C:N ratio was always < 10.

Table 4.5 Seasonal variation in the ratio of C:N at the 4 stations

Stations	Pre-monsoon		Monsoon		Post-monsoon	
	Feb	Apr	Jun	Aug	Oct	Dec
Barmouth	8.88	6.51	9.89	9.50	12.38	13.99
Vaduthala	16.20	6.58	11.47	8.75	7.10	8.56
Munambam	7.76	9.04	9.71	9.46	14.47	7.88
Arookutty	6.97	9.80	9.76	7.70	7.39	7.24

4.1.9. Labile Organic Matter (LOM)

LOM is the sum total of protein (PRO), carbohydrate (CHO) and lipid (LIP). It ranged from 0.78 to 2.22 $mg.g^{-1}$. Maximum concentration was observed at Vaduthala and the minimum at Barmouth (Figure 4.5). Average concentration of CHO was 0.15 ± 0.05 mg g^{-1} and the concentration ranged between 0.06 and 0.25 $mg.g^{-1}$. The average concentration of PRO in the CE during the study period was 0.92 ± 0.36 $mg.g^{-1}$ and ranged from 0.5 to 1.96 $mg.g^{-1}$ and the trend in distribution was more or less similar to that of LOM. LIP concentration varied from 0.01 to 0.24 $mg.g^{-1}$ with an average of 0.11 ± 0.08 $mg.g^{-1}$. High concentrations of PRO and CHO were observed in Vaduthala, Arookutty and Munambam, whereas high concentration of LIP was observed at Barmouth followed by Vaduthala. Seasonal variation in PRO and CHO followed the same trend, having the maximum concentration during monsoon and minimum during pre- monsoon. There was hardly any

difference in LIP concentration during different seasons. Significant temporal variation was observed in PRO and CHO concentrations in the sediment ($F= 5.72$ and 10.59 respectively, $df=11$, $p > 0.01$) whereas no such variation was seen in LIP. Overall, PRO was the dominant labile organic matter in all sediment samples when compared to CHO and LIP. CHO and LIP showed inverse trend at all the stations. The LOM content was more in clay and silt than sandy sediment and showed temporal variation.

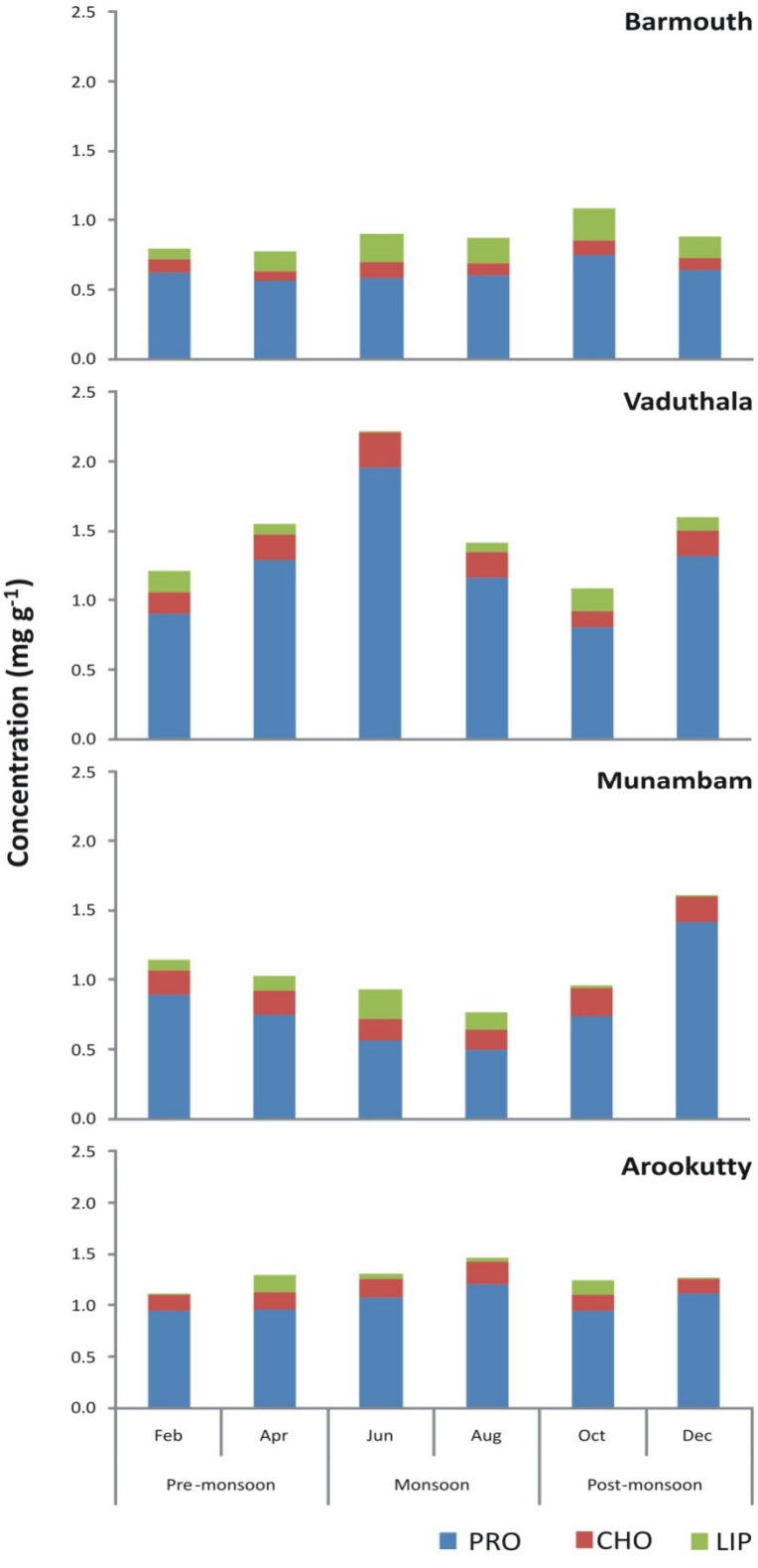

Figure 4.5 Distribution of LOM (PRO, CHO, LIP) at study locations

4.2. Microbiological parameters

4.2.1 Bacterial Abundance

4.2.1.1 Total Direct Count (TC)

The average total count in Cochin estuary was 3.99 ±11.48 x 10^9 cells g^{-1}. The bacterial abundance ranged from 7.33x10^8 to 3.04x10^9 cells g^{-1} in the sediment samples collected from the four different stations (Figure 4.6). There was one order variation in number except for station Arookutty where the number was constant throughout the study period (10^9 cells g^{-1}). Post-monsoon period recorded the highest number. There was no significant variation in total counts within and between stations. TC was positively correlated with CHO ($r= 0.46$, $p<0.05$) and negatively correlated with lipid ($r= -0.56$, $p<0.01$).

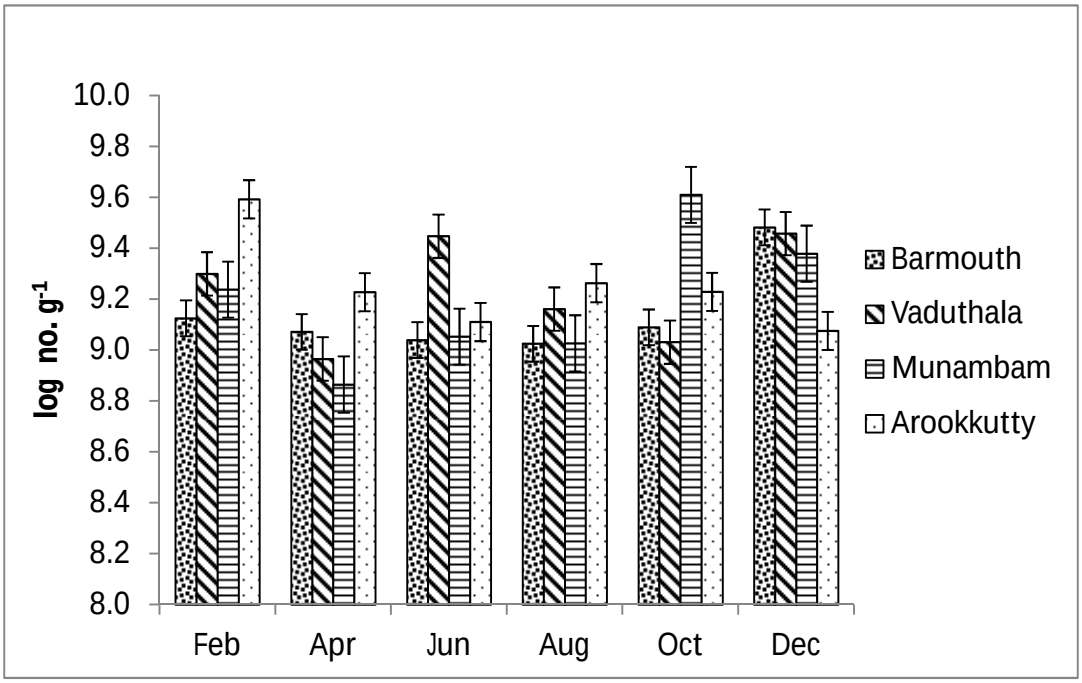

Figure 4.6 Distribution of total direct bacterial counts at the 4 study locations

4.2.1.2. Total Viable Counts of Aerobes (TVC-A)

The average TVC-A was 4.5 ±6.4 x 10^5 cells g^{-1} in CE and ranged from 3.33 x 10^4 to 2.3x10^6 cells g^{-1}. High count was observed during post-monsoon period. Though the counts varied by 2 orders, there was no significant spatial and temporal variation during the entire study period (Figure 4.7). TVC-A related negatively with nitrate concentration (r= -0.5 $p<0.01$).

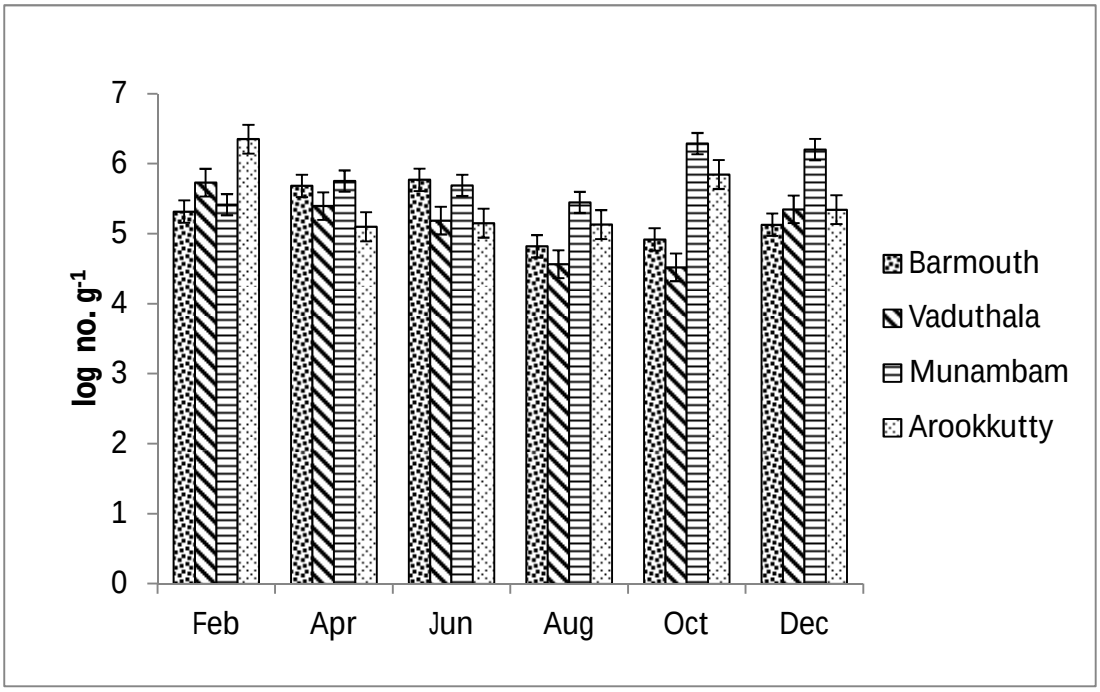

Figure 4.7. Distribution of TVC-A at the 4 study locations

4.2.1.3. Total Viable Counts of Anaerobes (TVC-An)

TVC-An ranged from 2.02 x 10^4 to 1.96 x10^6 cells $g^{-1}$and the average TVC-An in CE during the study period was 4 ± 5.7 X 10^5 cells g^{-1}. There was no significant spatial and temporal variation in counts. Post-monsoon period recorded high number (Figure 4.8). TVC-An showed significant negative

correlation with nitrate concentration of near bottom water ($r = -0.47$, $p<0.05$) and with LIP content of the sediment ($r = -0.41$, $p<0.05$).

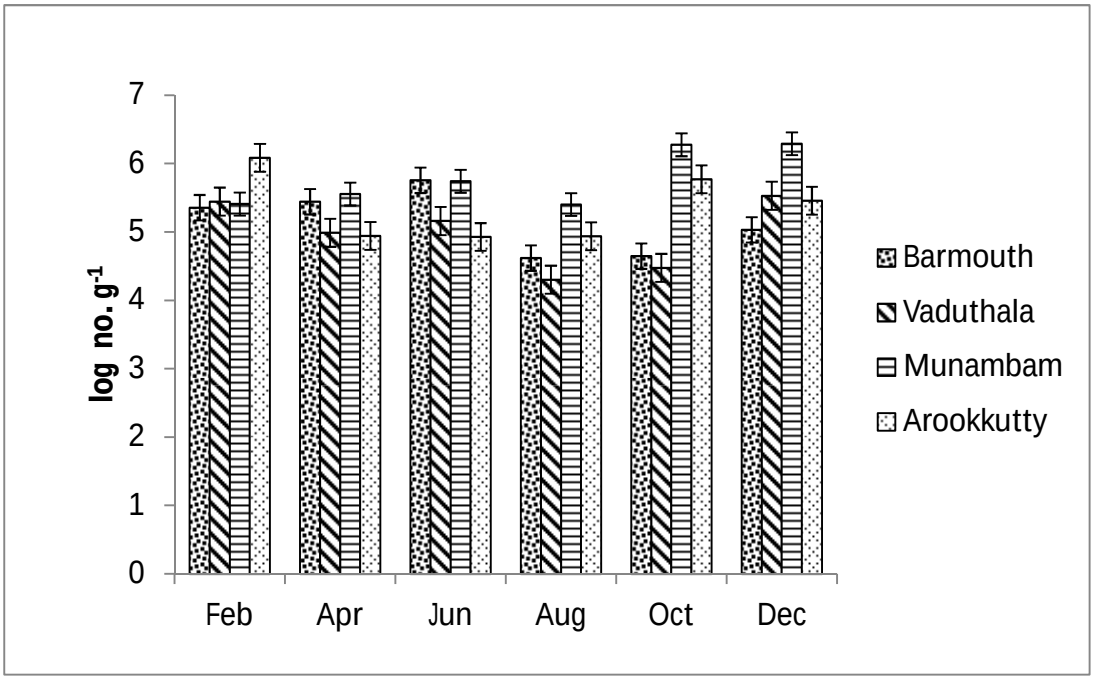

Figure 4.8 Distribution of TVC-An at the 4 study locations

4.2.1.4. Retrievable counts of Copiotrophs (RCC)

RCC ranged from 5.92×10^4 to 1.47×10^5 CFU g^{-1} and the average RCC in CE during the study period was $3.9 \pm 5.3 \times 10^5$ CFU g^{-1}. One order higher number was recorded in the month of June and August at Vaduthala and Munambam (Figure 4.9). There was no significant spatial and temporal variation in RCC. High count was observed during monsoon. There was significant positive correlation with PO_4 concentration of near bottom waters($r=0.53$, $p<0.01$) and with CHO ($r=0.50$, $p<0.01$).

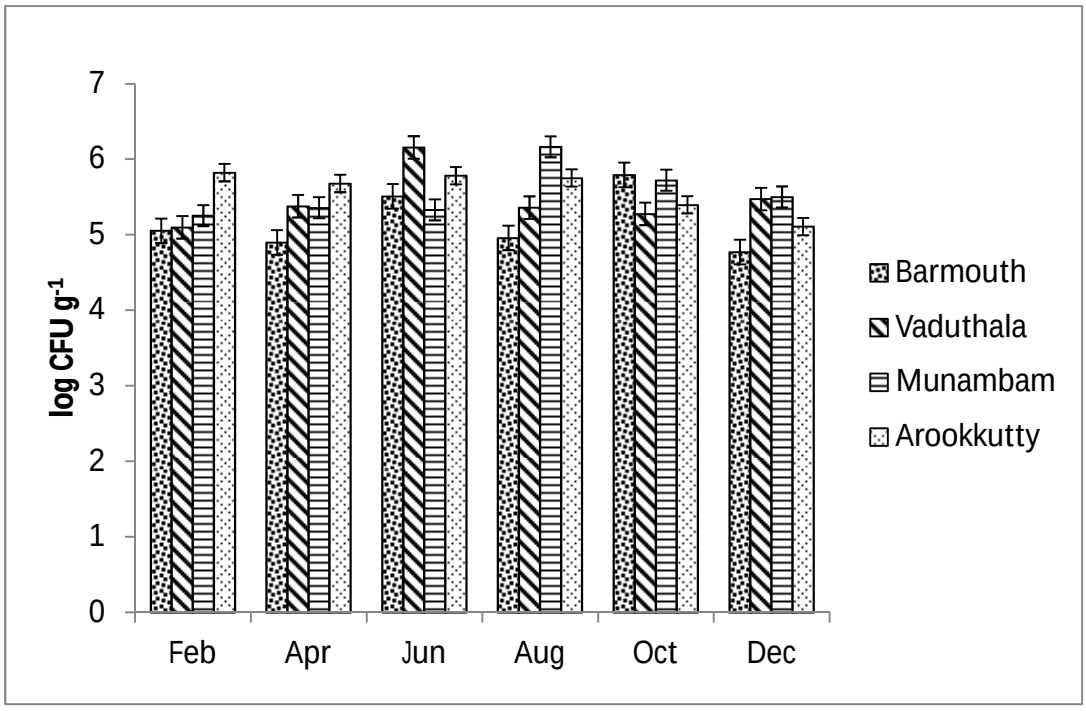

Figure 4.9 Distribution of RCC at the 4 study locations

4.2.1.5. Retrievable counts of Oligotrophs (RCO)

RCO in CE ranged from 5.71 x 10^4 to 1.04 x 10^6 CFU g^{-1} at the four stations with an average of 3.5 ± 3.97 x 10^5 CFU g^{-1} (Figure 4.10). Highest number was recorded in Arookutty and the least count was recorded at Barmouth. There was no significant spatial and temporal variation. High count was observed during pre-monsoon. Significant correlation was observed with physiochemical parameters of near bottom waters [temperature ($r=0.47$, $p<0.05$), NO_2 ($r= -0.55$, $p<0.01$) and NO_3 ($r= -0.47$, $p<0.05$)].

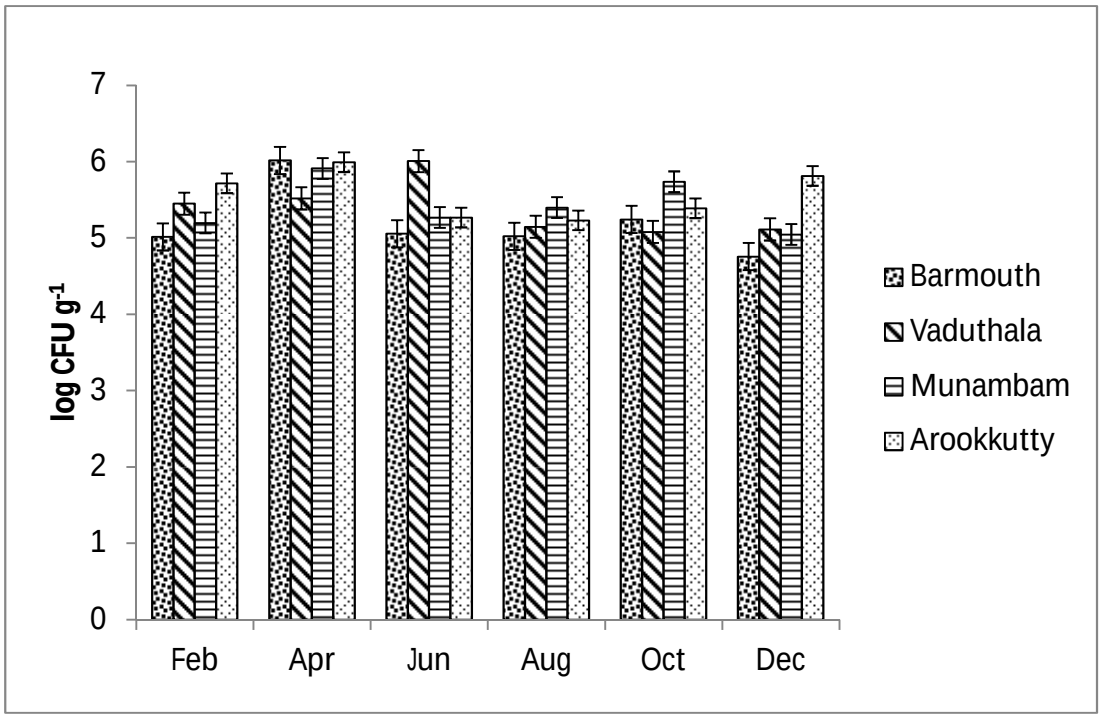

Figure 4.10 Distribution of RCO at the 4 study locations

4.2.1.6. Retrievable counts of lipid degraders Saturated (RC-LD_{SAT})

RC-LD_{SAT} in CE ranged from 4.70x 10^3 to 4.96 x 10^5 CFU g^{-1} and the average RC-LD_{SAT} was 6.3 ± 14 x 10^4 CFU g^{-1} during the study period. There was no significant spatial and temporal variation during the entire study period. Munambam station recorded high counts compared to the other 3 stations (Figure 4.11). High count was observed during monsoon. No significant correlation was observed with the geochemical variables but showed negative correlation with temperature of near bottom water ($r = -0.46$, $p<0.05$).

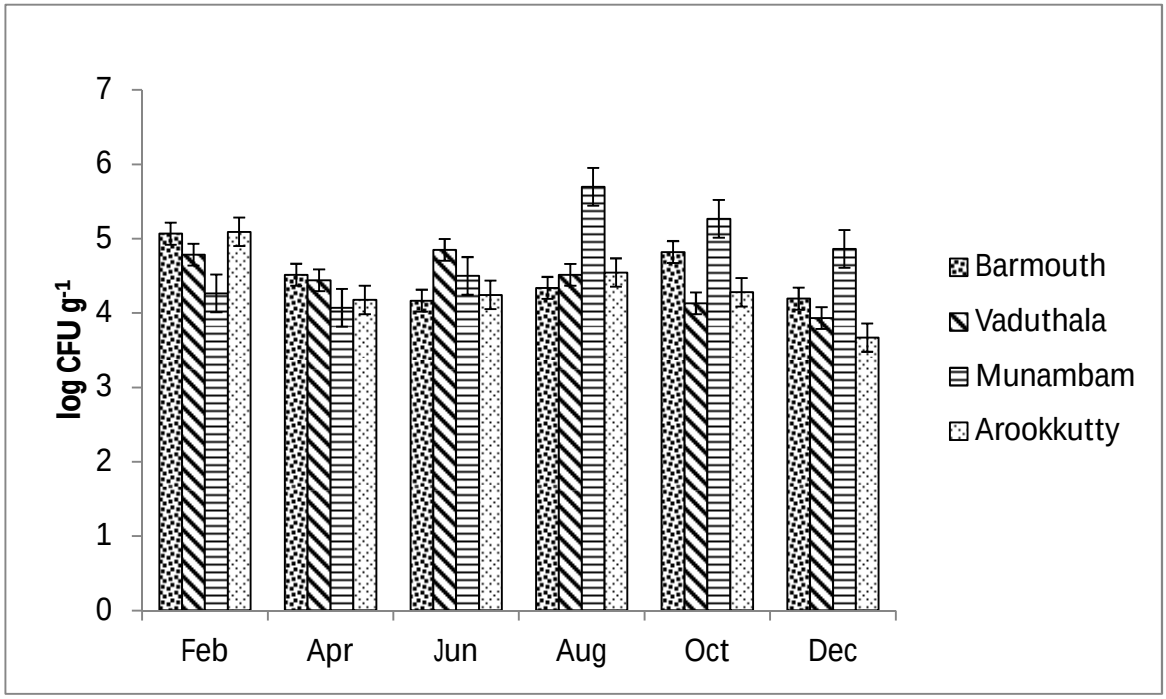

Figure 4.11 Distribution of RC-LD_{SAT} at the 4 study locations

4.2.1.7. Retrievable counts of unsaturated lipid degraders (RC-LD_{UNSAT})

The RC-LD_{UNSAT} in CE during the study period ranged from $1x10^4$ to $3.5x10^5$ CFU g^{-1} in the four stations with an average of 7.1 ± 11.8 x 10^4 CFU g^{-1}. High counts were recorded at Munambam (Figure 4.12). The counts were generally high during monsoon. Although no significant spatial variation was seen, the temporal variation was significant ($f=4.2$, $df = 5$, $p \geq 0.01$). RC-LD_{UNSAT} was positively correlated with sand content ($r=0.42$, $p<0.05$) and negatively correlated with TOC and TON values ($r= -0.52$, $p<0.001$ and $r = -0.48$, $p< 0.05$ respectively).

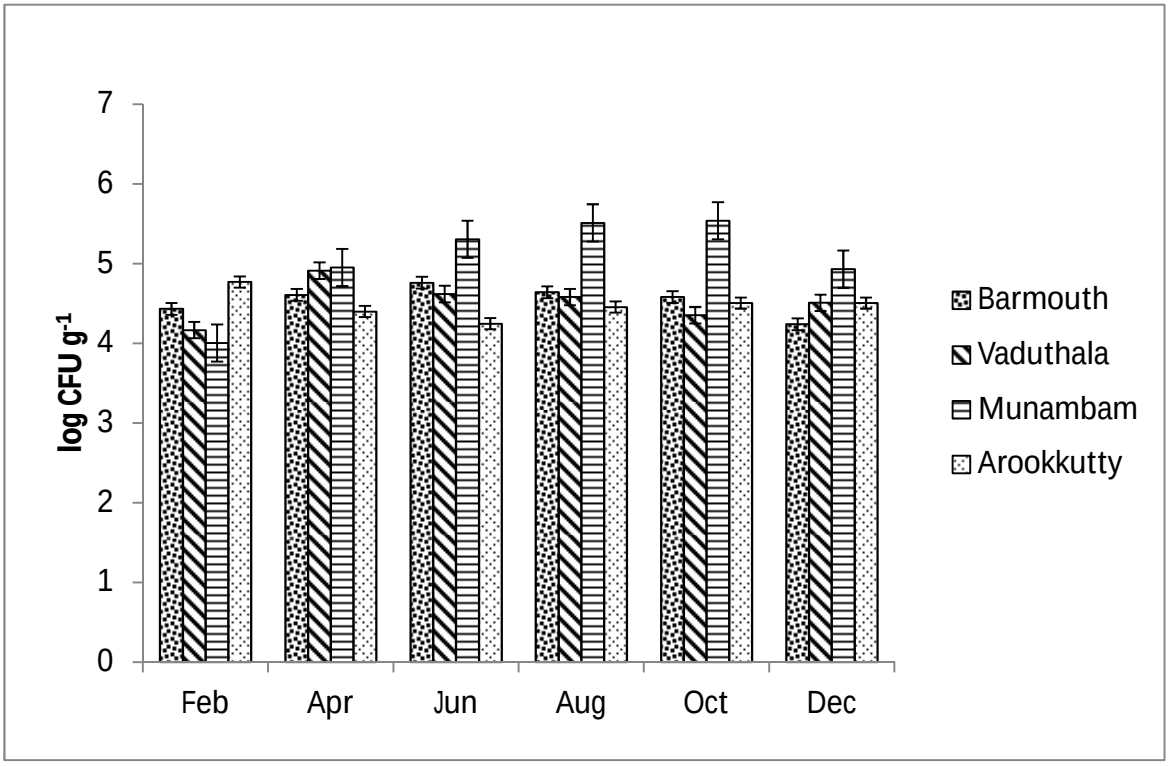

Figure 4.12 Distribution of RC-LD_{UNSAT} at 4 locations

4.2.1.8. Interrelationship between abundance and sediment characteristics

Though few geochemical variables showed influence on the biomass, the Redundancy analysis (RDA) showed a clear pattern in the abundance of TC, TVC-A and TVC-An, and RC bacterial groups (4) in the three seasons with respect to stations and the geochemical parameters. The relationship between geochemical factors with the abundance of different bacterial groups was shown in RDA triplot (Figure 4.13). In the figure, the bacterial count and geochemical characteristics are represented as (arrow) whereas locations and seasons are shown as points. The total variation in abundance

and geochemical variables were explained with cumulative percentage of 64.8. The first canonical axis accounted for 29.4% of the variance and second axis accounted for 26.9% of variance.

Among the bacterial abundance, TC count was more influenced by protein and to a lesser extent by TOC. The viable counts of aerobes and anaerobes were not influenced by any geochemical parameters. Carbohydrate was centrally placed and it had influence on all the other bacterial parameters, whereas the influence was to a lesser extent on RCC. The RCO counts were highly influenced by silt and clay texture, distantly influenced by TON and TOC. The RC-LD_{SAT} and RC-LD_{UNSAT} were highly influenced by lipid content of sediment and sandy texture.There was difference in influence of various geochemical and bacterial parameters based on stations and seasons depending on the canonical axis. At Barmouth, abundance of the bacterial groups where varying in different seasons. The bacterial assemblage where negatively loaded in both the axis.

During monsoon and post-monsoon season, Munambam station was influenced by the abundance of lipid degraders. Whereas in pre-monsoon, it was distantly influenced by TVC (A and An) and RCO. In Arookutty and Vaduthala during post-monsoon, RCO variability was strong compared to TC but during monsoon the variation of RCC was high. During pre-monsoon, RCO distantly influenced Arookutty, whereas Vaduthala was influenced by RCC. All pre-monsoon samples except Barmouth were placed very close to canonical axis 2. In general based on RDA analysis the four stations were

grouped into two clusters. The first axis brought about variation at Barmouth and Munambam stations located near the mouth of estuary which were influenced only by sand and LIP. Arookutty and Vaduthala were mainly influenced by TC, RCC and RCO and each of these was differently influenced by TOC, TON, CHO, PRO, silt and clay.

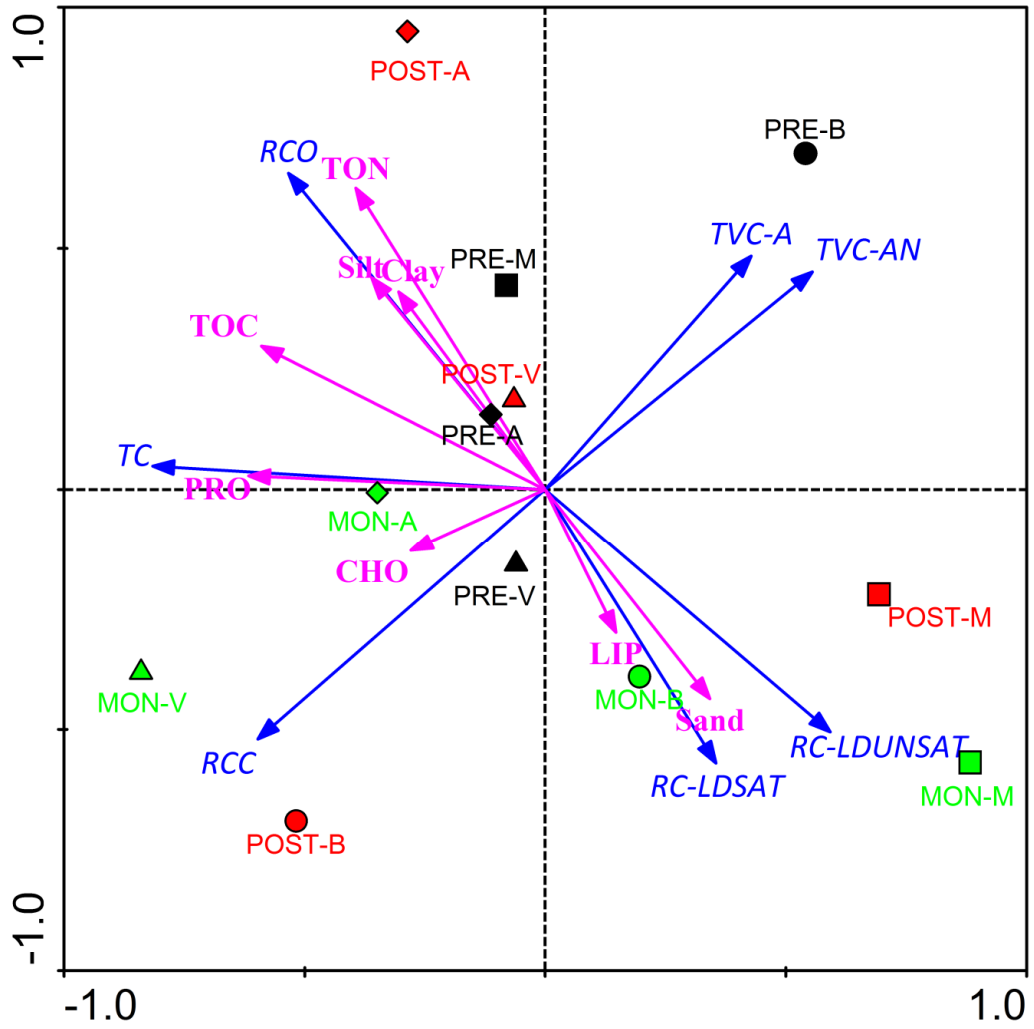

Figure 4.13 Redundancy analysis of TC, TVC (A and An) and RC (RCC, RCO, RC-LD_{SAT} and RC-LD_{UNSAT}) and geochemical parameters during the 3 seasons. The arrow points in the direction of maximum variation in the RC and the length is proportional to the rate of change. Abbreviations are the same as mentioned in the text.

4.2.2. Microbial community (EL- PLFA)

4.2.2.1. EL- PLFA distribution pattern

The amount of individual PLFA determined in the sediment samples is expressed as mol percentage of total PLFA. A total of 33 PLFAs could be identified in CE which ranged from C10 to C28. The current chromatographics cannot separate 18:1ω7c, 18:1ω9t, 18:1ω12t, 18:1 ω9t, 20:1 ω12c and 20:1 ω11c therefore their combination is designated as Sum in Feature 7, 8 and 12 respectively. For each unidentified fatty acid, an equivalent chain length was determined and the resulting values were used as interim values (for example, the unknown with an equivalent chain length of 21.252 and 22.267 were designated as unknown 21.252 "C and 22.267 "C respectively. The average range of total PLFA concentration is presented in the Table 4.6. The chromatogram and composition report of sediment sample is in Appendix section.

Table 4.6 Range of EL- PLFA in CE (average value is the repeat of each sample,* detected only in single sample)

Sl. No.	PLFA profile	Range (mol. %)
1	10:0	2.85*
2	12:0	2.22*
3	14:0 ISO	1.56 - 2.29
4	14:0	4.83 - 6.14
5	15:0 ISO	11.51 - 13.41
6	15:0 ANTEISO	8.13 - 11.31
7	15:0	2.47 - 4.02
8	16:0 ISO	3.48 - 4.38
9	16:1 ω7c	7.7 - 10.46
10	16:1 ω8c	27.76 – 34.19
11	16:1 ω5c	1.25 - 2.26
12	16:0	29.28 - 36.29

13	ISO 17:1 G	5.47 - 7.95
14	ISO 17:1 AT 9	28.32 – 31.56
15	17:0 ISO	2.4 - 2.84
16	17:0ANTEISO	1.54 - 2.5
17	17:0	1.89 - 2.58
18	18:2 ω6c	1.65*
19	18:1 ω9c	3.19 - 4.45
20	18:0	5.11 - 7.53
21	19:0	2.15*
22	19:0 cyclo c11-12	1.73*
23	20:0	3.43 - 4.4
24	20:4 ω6c	5.1*
25	20:5 ω3c	9.1*
26	Sum In Feature 12(20:1 ω12c, 20:1 ω11c)	4.95*
27	21:0	2.25*
28	22:0	3.35 - 4.9
29	22:4 ω6c	3.46*
30	22:6 ω3c	12.17*
31	23:0	2.32*
32	24:0	3.55 – 5.13
33	25:0	2.39*
34	28:0	4.49*
35	Unknown 21.252 "C"	1.57*
36	Unknown 22.267 "C"	6.46 - 9.31
37	Sum In Feature 7	37 – 100
38	Sum In Feature 8 (18:1 ω9t.)	11.75 - 14.32

The thirty three identified PLFAs were classified into seven major groups (Table 4.7).

Table 4.7 Major EL-PLFA groups observed during the study period

Sl. No.	Major group	PLFAs
1	Saturated PLFA (< 19)	10:0,12:0, 14:0, 15:0, 16:0, 17:0, 18:0 and 19:0
2	Saturated PLFA (>19)	20:0, 21:0, 22:0, 23:0, 24:0, 25:0 and 28:0
3	Monounsaturated PLFA (MUFA)	16:1ω7c, 16:1ω5c, ISO17:1G, 18:1ω9c, 18:1ω9t, 20:1ω12c, 20:1ω11c
4	Branched saturated PLFA	14:0 ISO, 15:0 ISO, 15:0 ANTEISO, 16:0 ISO, 17:0 ISO, 17:0 ANTEISO
5	Branched monounsaturated PLFA	ISO 17:1G
6	Polyunsaturated PLFA (PUFA)	18:2ω6c, 20:4ω6c, 20:5ω3c, 22:4ω6c, 22:6ω3c
7	Cyclopropane PLFA	19:0 cyclo C11 – 12

The percentage distribution of fatty acids identified in PLFA analyses of sediment samples from four different stations during the entire study period is shown in Table 4.8. The fatty acid composition revealed a complex suite of different fatty acids at the four stations. In general, the fatty acid composition appeared similar in all the four stations. However, there was minor difference in relative quantity and quality at each station. The most notable difference was observed in concentration of the common biomarker (16:0) during the study period. Munambam recorded maximum number of markers. The poly unsaturated fatty acids (PUFA) especially 18:2 ω6c, 20:4 ω6c, 20:5 ω3c, 22:4ω6c and 22:6ω3c were extracted from the sediment samples collected from Munambam station during the post-monsoon period.

Table 4.8 Mean percentage and standard deviations of EL-PLFA of the four stations (n=5)

Sl. No.	Fatty acids	Barmouth	Vaduthala	Munambam	Arookutty
1	10:0			2.85 ± 2.08	
2	12:0			2.22 ± 0.00	
3	14:0 ISO		2.29 ± 0.15	1.56 ± 0.21	1.70 ± 0.21
4	14:0	6.14 ± 1.16	5.41 ± 0.57	4.83 ± 0.80	5.34 ± 0.51
5	15:0 ISO	13.05 ± 1.38	13.41 ± 1.66	11.51 ± 3.00	12.29 ± 1.43
6	15:0 ANTEISO	8.93 ± 0.82	11.31 ± 2.22	8.13 ± 2.71	9.33 ± 1.46
7	15:0	4.02 ± 1.17	2.86 ± 0.41	2.67 ± 0.87	2.47 ± 0.17
8	16:0 ISO	3.92 ± 0.32	4.38 ± 0.56	3.48 ± 0.97	3.88 ± 0.29
9	16:1 ω7c	10.46 ± 2.01	10.14 ± 1.57	8.66 ± 1.39	7.70 ± 1.31
10	16:1 ω8c	34.19 ± 0.00			27.76 ± 0.00
11	16:1 ω5c	2.26 ± 0.53	1.89 ± 0.23	2.06 ± 0.57	1.25 ± 0.28
12	16:0	36.29 ± 4.24	31.92 ± 4.49	29.28 ± 6.12	29.77 ± 4.79
13	ISO 17:1 G	7.95 ± 1.86	5.66 ± 1.21	5.47 ± 1.96	7.39 ± 1.08
14	ISO 17:1 AT 9	28.32 ± 0.00			31.56 ± 0.00
15	17:0 ISO	2.49 ± 0.14	2.42 ± 0.07	2.40 ± 0.54	2.84 ± 0.20
16	17:0ANTEISO	2.31 ± 0.49	2.02 ± 0.00	1.54 ± 0.00	2.50 ± 0.05
17	17:0	2.58 ± 0.67	2.51 ± 0.43	1.89 ± 0.17	2.12 ± 0.27
18	18:2 ω6c			1.65 ± 0.32	
19	18:1 ω9c	3.19 ± 0.25	3.90 ± 0.62	4.45 ± 1.34	3.42 ± 0.62
20	18:0	6.12 ± 0.64	5.37 ± 0.71	7.53 ± 1.18	5.11 ± 0.51

21	19:0				2.15 ± 0.00
22	19:0 cyclo c11-12			1.73 ± 0.36	
23	20:0	4.40 ± 0.00	3.49 ± 0.00	3.43 ± 0.00	3.51 ± 0.00
24	20:4 ω6c			5.10 ± 1.47	
25	20:5 ω3c			9.10 ± 5.38	
26	Sum In Feature 12(20:1 ω12c, 20:1 ω11c)			4.95 ± 0.00	
27	21:0				2.25 ± 0.00
28	22:0	3.95 ± 0.00	3.35 ± 0.00	3.55 ± 0.00	4.90 ± 0.00
29	22:4 ω6c			3.46 ± 0.00	
30	22:6 ω3c			12.17 ± 0.00	
31	23:0				2.32 ± 0.00
32	24:0	3.78 ± 0.00	3.55 ± 0.00	4.14 ± 0.00	5.13 ± 0.00
33	25:0				2.39 ± 0.00
34	28:0				4.49 ± 0.00
35	Unknown 21.252 "C"			1.57 ± 0.00	
36	Unknown 22.267 "C"	9.01 ± 5.05	9.31 ± 3.23	7.95 ± 3.79	6.46 ± 4.45
37	Sum In Feature 7	37.50 ± 0.00	100.00 ± 0.00		40.68 ± 0.00
38	Sum In Feature 8 (18:1 ω9t.)	13.82 ± 2.66	11.75 ± 2.17	14.32 ± 2.54	12.55 ± 1.51

The mean percentages and standard deviations of major PLFA groups in the 4 stations are shown in Table 4.9. Saturated PLFAs (< 19) ranged from 40.76 to 46.07%, Saturated PLFA (>19) from 0.37 to 1.06%, Monounsaturated PLFA (MUFA) from 9.67 to 11.71%, Branched saturated PLFA from 22.45 to 29.2%, Branched monounsaturated PLFA from 3.31 to 7.92%, Polyunsaturated PLFA (PUFA) from 0 to 4.34% and Cyclopropane PLFA 1.73% of the total PLFA in Cochin estuary.

Table 4.9 Mean percentages and standard deviations of PLFA groups in sediments

PLFA profile	Barmouth	Vaduthala	Munambam	Arookutty
Saturated PLFA (< 19)	46.07 ± 5.99	42.16 ± 3.31	42.36 ± 5.80	40.76 ± 3.03
Saturated PLFA (>19)	00.42 ± 2.25	00.38 ± 2.00	00.37 ± 2.03	01.06 ± 5.30
Monounsaturated PLFA (MUFA)	11.71 ± 7.29	10.68 ± 4.25	11.50 ± 3.57	09.67 ± 5.64
Branched saturated PLFA	23.39 ± 4.36	29.20 ± 4.17	22.45 ± 6.03	28.69 ± 2.98
Branched monounsaturated PLFA	05.20 ± 6.87	03.85 ± 2.54	03.31 ± 3.06	07.92 ± 6.29
Polyunsaturated PLFA (PUFA)	00.00 ± 0.00	00.00 ± 0.00	04.34 ± 10.31	00.00 ± 0.00
Cyclopropane PLFA	00.00 ± 0.00	00.00 ± 0.00	01.73 ± 0.36	00.00 ± 0.00

4.2.2.2. Cluster analysis

Cluster analysis of PLFAs grouped the stations into three major clusters (Figure 4.14). There was no exclusive group based on season or station. Cluster 1 corresponded to the sampling site of the post-monsoon season of Munambam station (POSTM). The second cluster represented the pre-monsoon (PRE) and monsoon (MON) period of Arookutty (A) and Vaduthala (V). Cluster 3 was representative of pre-, post- and monsoon periods of Barmouth (B), post-monsoon of Vaduthala and Arookutty stations. This sampling sites array were better defined as navigational Barmouth and post monsoon riverine input stations. When the similarity criterion was increased from 60 to 85% more station specific, clusters emerged rather than season.

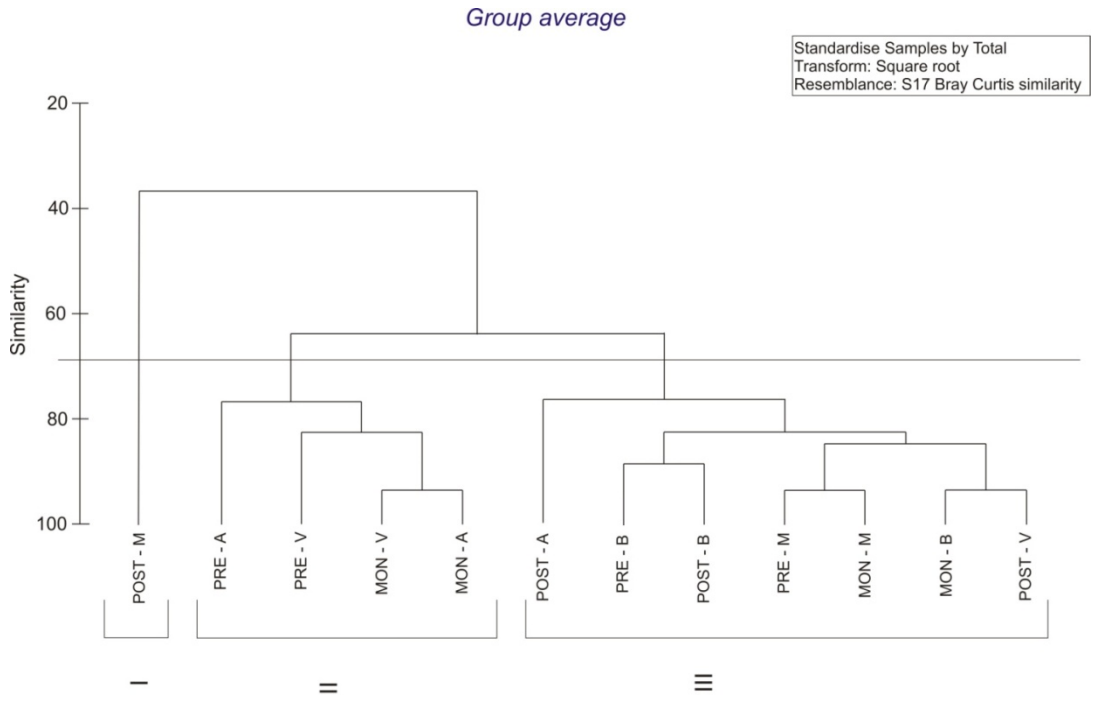

Figure 4.14 Cluster analysis of the sampling stations in each seasons. PLFA profiles were used to associate the sites. Clusters were obtained at 70% similarity threshold value

4.2.2.3. Principal Component Analysis

Cluster analysis showed that sampling locations and sampling seasons did not group exclusively. Further, principal component analysis (PCA) was carried out to understand which PLFA biomarkers were responsible for the variations at different study locations and seasons. The analysis aggregated the variables into three main components, which accounted for 71.39% of the total variability (Table 4.10). PC1 accounted for 39.5% of the variance with negative loadings (-0.55 to -0.88) on 12:0, 18:2 ω6c, 19:0 cyclo c11-12, 20:4 ω6c, 20:5 ω3c, SIF 12, 22:4 ω6c, 22:6 ω3c, unknown 21.252 "C", 14:0 ISO, 16:1 ω5c, 18:1 ω9c, 20:0, 22:0, 24:0 and with positive loadings (0.53 to 0.96) on 14:0, 15:0 ISO, 15:0 ANTEISO, 16:0 ISO, 16:1 ω7c, 16:0. Of these,

negatively loaded PUFA biomarkers like 18:2 ω6c, 20:4ω6c, 20:5 ω3c, 22:6 ω3c were represented by the microeukaryotes such as fungi, protozoa, diatoms and dinoflagellates respectively. The other important markers such as 19:0 cyclo c11-12, 14:0 ISO, 16:1 ω5c suggests the presence of acidophilic bacteria, SRB group, Arbuscular mycorrhizal fungi respectively. Among the positively loaded PLFAs, 15:0 ISO, 15:0 ANTEISO, 16:0 ISO, 16:1 ω7c represented G+ve Firmicutes and G-ve anaerobic bacteria such as SRB, especially *Desulfovibrio* and G-ve Proteobacteria. PC2 accounted for 20.81% of the total variance and was influenced by positive loadings (0.52 to 0.82) of 17:0 ANTEISO representing *Desulfovibrio desulfuricans* and saturated PLFAs >19 groups like 19:0, 21:0, 22:0, 23:0, 25:0 and 28:0 which are indicative of terrigenous input. The negatively loaded PLFA in PC2 was 18:0 (-0.61). PC3 showed only 11.09% of the total variance, and was influenced by positively loaded (0.65 to 0.77) PLFAs 14:0, 15:0, 17:0, 16:1 ω8c and ISO 17:1 AT9. Among these 16:1ω8c indicated Type I methanotrophs. The negatively loaded (-0.56 to -0.58) PLFAs are ISO 17:1 G and Unknown 22.267 "C". Among these ISO 17:1G represented sulphate reducers.

PCA *Eigenvalues*

Factor	Eigenvalue	Variability (%)	Cumulative %
F1	15.01	39.50	39.50
F2	7.91	20.81	60.31
F3	4.21	11.09	71.39

Table 4.10 Eigen values and factor loadings on the PLFA compounds using PCA in the sediments variables of four stations covering the three seasons. Values in bold are >0.5

Factor loadings

Variables	F1	F2	F3
10:0	-0.002	-0.284	-0.493
12:0	**-0.877**	-0.433	0.071
14:0 ISO	**-0.551**	0.391	0.145
14:0	**0.584**	0.024	**0.647**
15:0 ISO	**0.961**	-0.113	0.040
15:0 ANTEISO	**0.847**	-0.050	0.006
15:0	-0.202	0.249	**0.659**
16:0 ISO	**0.530**	0.211	0.221
16:1 ω7c	**0.637**	-0.336	0.204
16:1 ω8c	0.300	-0.067	**0.773**
16:1 ω5c	**-0.607**	0.164	-0.257
16:0	**0.836**	-0.272	-0.099
ISO 17:1 G	0.481	0.168	**-0.584**
ISO 17:1 AT 9	0.304	-0.056	**0.731**
17:0 ISO	-0.277	0.471	0.106
17:0ANTEISO	-0.047	**0.516**	0.207
17:0	-0.499	0.413	**0.670**
18:2 ω6c	**-0.877**	-0.433	0.071
18:1 ω9c	**-0.834**	0.085	-0.167
18:0	-0.438	**-0.600**	-0.329
19:0	-0.268	**0.862**	-0.151
19:0 cyclo c11-12	**-0.877**	-0.433	0.071
20:0	**-0.685**	0.392	0.018
20:4 ω6c	**-0.877**	-0.433	0.071
20:5 ω3c	**-0.877**	-0.433	0.071
Sum In Feature 12(20:1 ω12c, 20:1 ω11c)	**-0.877**	-0.433	0.071
21:0	-0.268	**0.862**	-0.151
22:0	**-0.704**	**0.524**	-0.016
22:4 ω6c	**-0.877**	-0.433	0.071
22:6 ω3c	**-0.877**	-0.433	0.071
23:0	-0.268	**0.862**	-0.151
24:0	**-0.749**	0.490	-0.012
25:0	-0.268	**0.862**	-0.151
28:0	-0.268	**0.862**	-0.151
Unknown 21.252 "C"	**-0.877**	-0.433	0.071
Unknown 22.267 "C"	0.274	-0.071	**-0.559**
Sum In Feature 7	0.428	-0.207	0.309
Sum In Feature 8 (18:1 ω9t.)	0.487	-0.394	-0.499

An ordination in reduced space procedure by principle component analysis was used to determine the relationship of the PLFAs from different stations and seasons, in the sediments (Figure 4.15A &B). Sample scores for individual PLFAs showed three clusters. Cluster I. was represented by 4 stations having different sampling time. Barmouth, Vaduthala and Arookutty stations were represented by monsoon and post-monsoon samples whereas Munambam was represented by monsoon and pre-monsoon period. This cluster seems to belong to “wet and transitional” group. Cluster II represented the dry period (less riverine input) of the 3 stations except Munambam. Cluster III solely represented the transitional period (post-monsoon) of Munambam. Cluster I was represented by 6 groups (G+ve Firmicutes and G-ve anaerobic bacteria, G-ve Proteobacteria, sulphate reducers, *Desulfovibrio* and Type I and II methanotrophs. Dry period cluster II was mostly dominated by Arbuscular mycorrhyzae (16:1 ω5c), sulphate reducers especially *Desulfovibrio desulfuricans* (ISO and ANTEISO 17:0). Cluster III was exclusively represented by microeukaryotes like diatoms, dinoflagellates, protozoa, fungi and acidophilic bacteria.

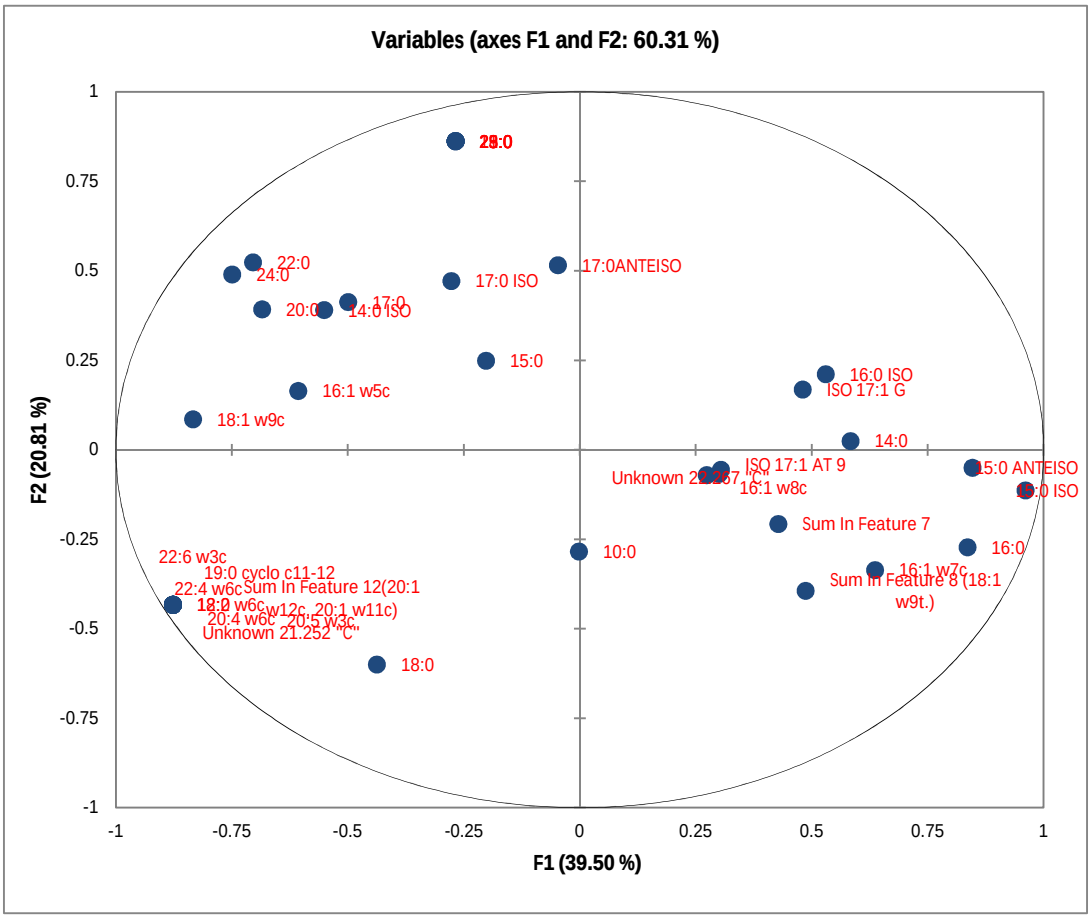

Figure 4.15A Principal Component Analysis of variables with Factor loadings >0.5 in the four stations

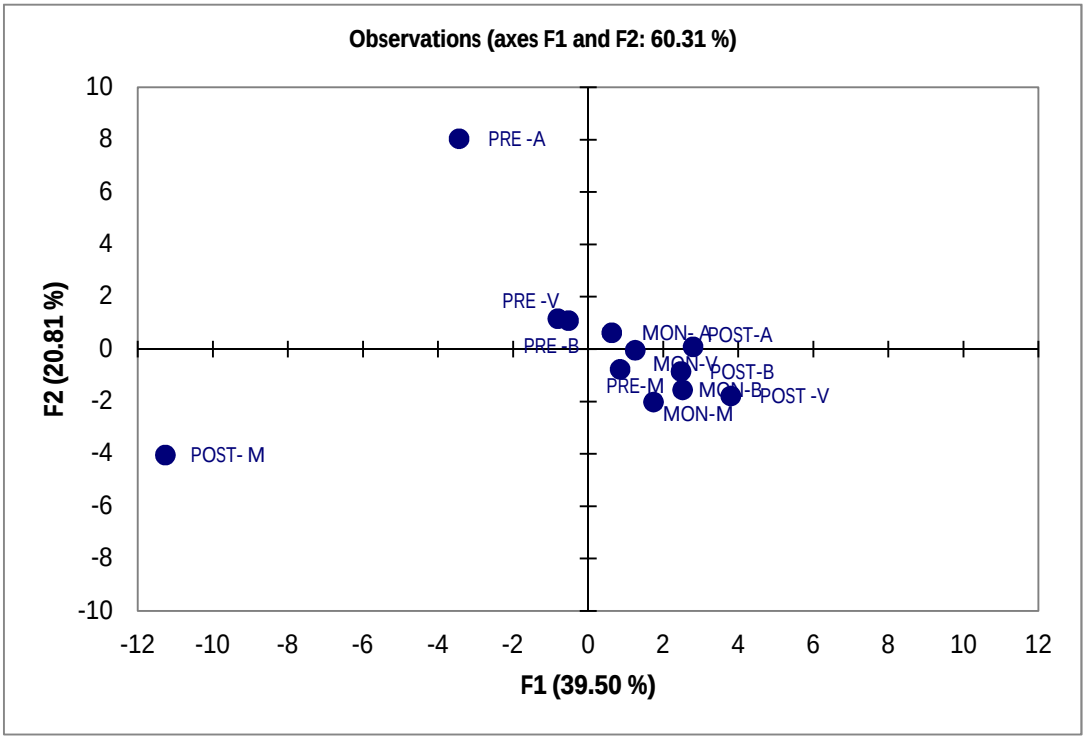

Figure 4.15B The scores of the first two principle components for the sampling sites (B-Barmouth, V-Vaduthala, M- Munambam and A-Arookutty during monsoon (MON), pre-monsoon (PRE) and post-monsoon (POST) for the PLFA compounds.

4.2.2.4. Structure and Percentage of Major Groups

Based on the EL-PLFA analysis, 16 biomarkers representative of prokaryotes and microeukaryotes were recorded in the CE. The prokaryotes represented by Gram negative proteobacteria, Type – I Methanotrophs, acid producing/acidophilic bacteria, G+ve firmicutes, Sulphate Reducing Bacteria (SRB) like *Desulfovibrio* sp., *Desulfovibrio desulfuricans* and microeukaryotes by Ectomycorrhizal Fungi, Arbuscular Mycorrhizal Fungi (AMF), Protozoa, Diatoms and Dinoflagellates along with the markers are

shown in Table 4.11. The relative abundance or weight percent values of the 13 important biomarkers are as follows: 27% of 16:1 ω8c (Type I methanotrophs), 19% of iso and anteiso 15:0 (G+ve Firmicutes and G-ve anaerobes), 11% of 22:6 ω3c (diatoms and dinoflagellates), 8% of 16:1 ω7c G-ve Proteobacteria, 8% of 20:5 ω3c (diatoms), 6% of ISO 17:1 G (sulphate reducers), 5% of 18:2 ω6c and 18:1 ω9c (Ectomycorhyzal fungi), 5% of 20:4 ω6c (protozoa), 4% of i and ai 17:0 (*Desulfovibrio desulfuricans*- SRB), 3% of 16:0 ISO (*Desulphovibrio*), 2% of 14:0 ISO (SRB groups), 1% of 16:1 ω5c (Arbuscular mycorrhizal fungi, 1% 19:0 cyclo c11-12 (Acidophilic bacteria). These results indicate that the dominant groups in microbial community of Cochin estuary during the study period were gram positive Firmicutes followed by Gram negative Proteobacteria and some anaerobic gram negative bacteria especially sulphate reducers indicating the predominance of prokaryotes.

Table 4.11 Major groups and percentage of representative biomarkers in CE.

Sl.No.	Biomarkers	Major groups	%
1	**16:1 ω7c**	G-ve proteobacteria	8
2	**16:1ω8c**	Type – I Methanotrophs	27
3	**16:1 ω5c**	Arbuscular mycorrhizae	1
4	**19:0 cyclo c11-12**	Acid producing/Acidophilic bacteria	1
5	**14:0 ISO**	SRB	2
6	**15:0 ISO**	G+ve firmicutes and G-ve anaerobes especially SRB	19
7	**15:0 ANTEISO**		
8	**16:0 ISO**	Desulfovibrio	3
9	**17:0 ISO**	*Desulfovibrio desulfuricans (SRB)*	*4*
10	**17:0ANTEISO**		
11	**ISO 17:1 G**	Sulphate reducers	6
12	**18:2 ω6c**	Ectomycoryzal Fungi	5
13	**18:1 ω9c**		
14	**20:4 ω6c**	Protozoa	5
15	**20:5 ω3c**	Diatoms	18
16	**22:6 ω3c**	Diatoms & Dinoflagellates	11

4.2.2.5. Spatial Structure of Major Groups

There was spatial variation in the number of groups observed in CE stations. At Munambam station, 11 groups were recorded out of the 13. Vaduthala and Arookutty recorded 8 groups and Barmouth station recorded only 5 groups. Figure 4.16 shows the mol % of the total PLFA at each station. The common groups present in all the four stations during the study period were Gram positive Firmicutes (26 - 45%), followed by Gram negative Proteobacteria (10 - 18%) and sulphate reducers (7 - 10%) mainly Desulfovibrio spp (4 – 8%). Among Desulfovibrio, *Desulfovibrio desulfuricans* (3 -7%) were isolated from all the stations except Barmouth (Figure 4.16). Few markers were recorded at a certain period and at a particular station.

Biomarker (i14:0) of SRB group (4%) was present only at Vaduthala station. Type I Methanotrophs (35 – 44%) were present only at Barmouth and Arookutty stations. Ectomycorrhizal fungi (4 – 6%) and Arbuscular mycorrhizal fungi (AMF) (2 – 3%) were present in all the stations except Barmouth. At Munambam station, markers specific for acidophilic bacteria (2%) and microeukaryotes especially protozoa (7%), diatoms (4%) and marker specific to both diatoms and dinoflagellates (20%) were also present. Overall, Munambam recorded maximum community diversity. The polyunsaturated fatty acids (PUFA) characteristic of microeukaryotes and fungi were generally present in minor proportions, indicating the predominance of prokaryotes at all the stations.

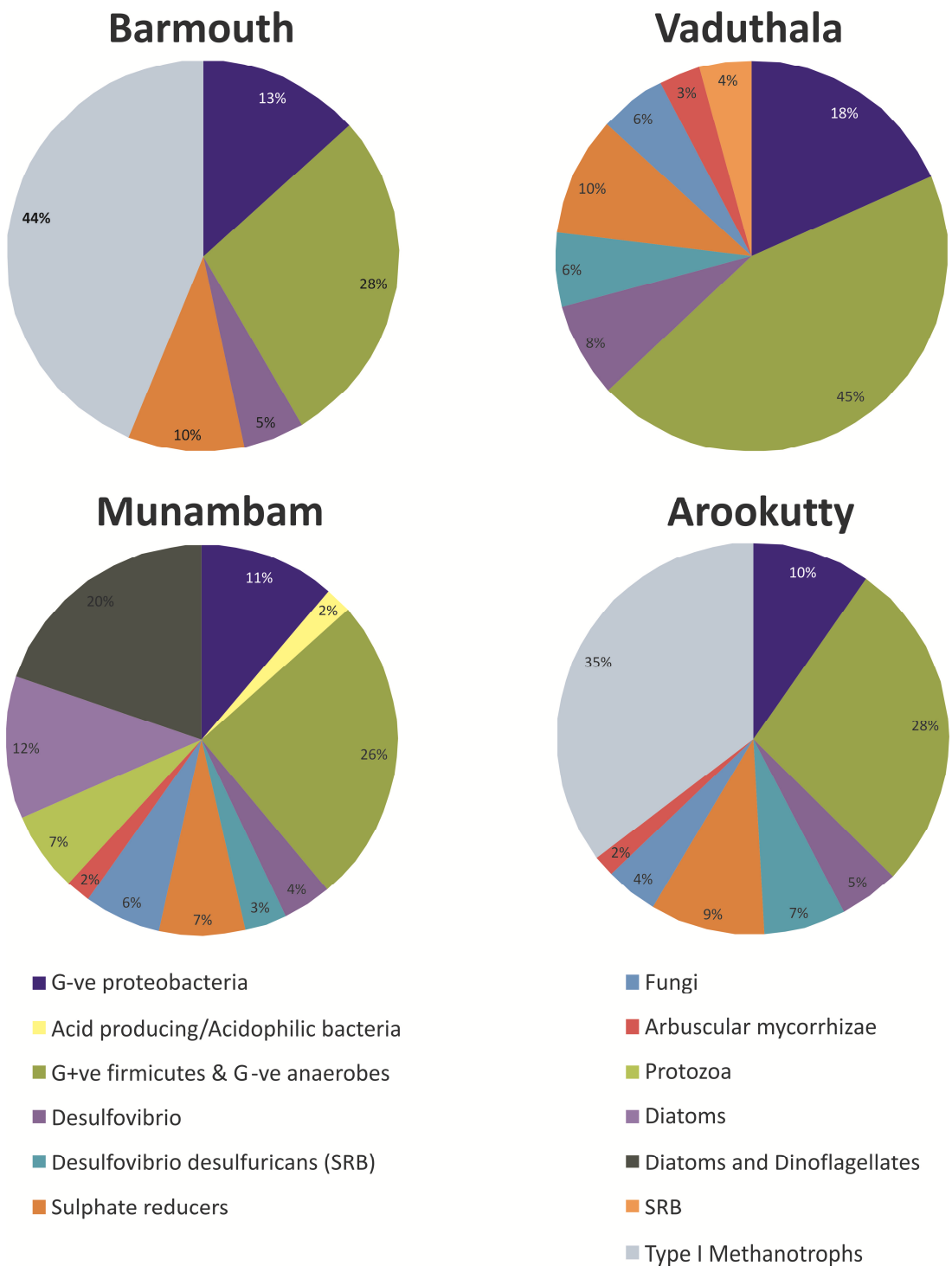

Figure 4.16 Spatial variation of major microbial groups (%) in 4 stations.

4.2.2.6. Temporal Structure of Major Groups

Major microbial groups showed temporal variation depending on the station. Number of major groups recorded was 9, 8 and 13 in CE during monsoon, pre-monsoon and post-monsoon, respectively. The difference in number between the pre- and monsoon season was due to presence of acidophilic bacteria (3%) during monsoon season. The seasonal variation was in the percentage of occurrence of the groups (Figure 4.17). Gram positive Firmicutes were 40% during pre- and monsoon which decreased to 20% during post–monsoon. Though the number of groups was more in the post-monsoon period the % of most of the groups were less than that of pre- and monsoon seasons. Unlike pre- and monsoon, in post monsoon, the most dominant group was Type I Methanotrophs (28%) and was recorded only in October at Barmouth and Arookutty stations. Certain additional groups (microeukaryotes – diatoms, dinoflagellates and protozoa) were recorded only at Munambam stations during this season.

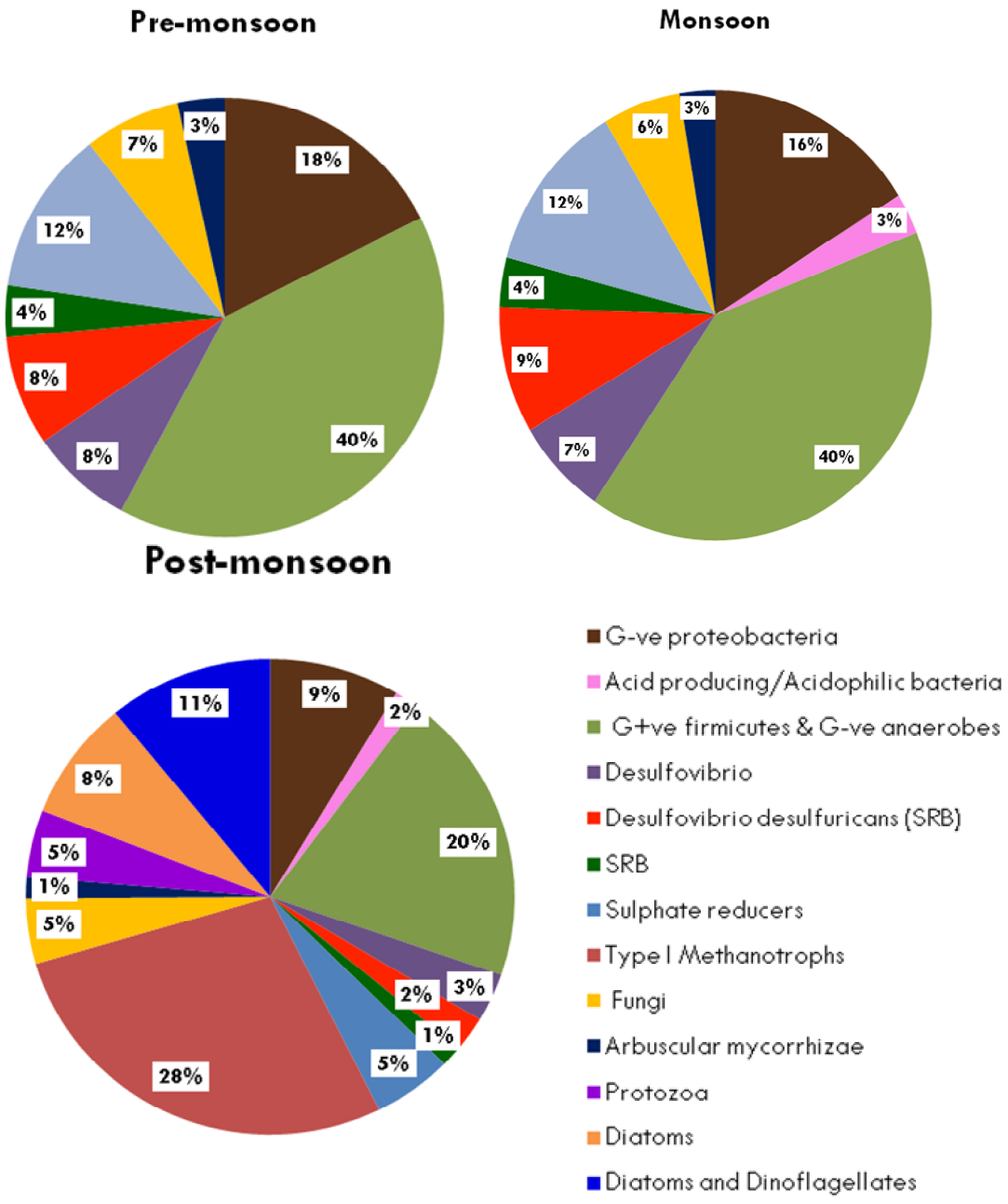

Figure 4.17 Temporal variation of the major groups pre-monsoon, monsoon and post-monsoon in CE

4.2.2.7. Factors influencing community (RDA)

The relationship between physiochemical factors of near bottom water, geochemical factors of sediment, bacterial parameters (abundance of TC,

TVC-A and TVC-An and retrievable count of 4 bacterial groups such as RCC, RCO, RC-LD_{SAT} and RC-LD_{UNSAT}) and the microbial community (PLFA biomarkers) of the 4 stations during three seasons is shown as RDA Triplot (Figure 4.18). In the figure the PLFA biomarkers, the bacterial count and environmental parameters are represented as (arrow) whereas locations and seasons are shown as points. The total variation in PLFA biomarkers, the bacterial count and environmental parameters were explained with cumulative percentage of 78.1%. The first canonical axis accounted for 37.7% of the variance and second axis accounted for 19.7% of variance.

During the pre-monsoon, the dominant groups were *Arbuscular mycorrhyzae* and Ectomycorrhyzal fungi at all the 4 stations. This community was influenced by carbohydrate content and percentage of sand in the sediment and to a lesser extent by protein and DO of near bottom water. This community was also influenced by the abundance of RC-$LD_{SAT,}$ RCC and RCO.

During the post-monsoon, the dominant groups were Type I Methanotrophs and *Desulfovibrio desulfuricans* at Barmouth, Vaduthala and Arookutty stations. This community was mainly influenced by near bottom water temperature, TOC and TON, silt and clay texture of sediment. However, at Munambam the community was microeukaryotes like diatoms, dinoflagellate, protozoa along with fungi and acidophilic bacteria and was mainly influenced by salinity and bacterial abundance of aerobic and anaerobic TVC and, unsaturated lipid degraders.

During the monsoon season, there was station wise difference in communities. The dominant groups at Vaduthala and Arookutty stations were G+ve firmicutes and G-ve anaerobes, SRB mainly *Desulfovibrio desulfuricans*, G-ve proteobacteria and Type II Methanotrophsin. These were by lipid content of sediment and to a lesser extent of protein of sediment and DO, nitrate, silicate and phosphate of near bottom water. At Munambam, the dominant community was the same as seen during the pre-monsoon season and the factors influencing was also the same. At Barmouth station, the dominant groups were the same as seen in the post-monsoon and the same factors also influenced the community.

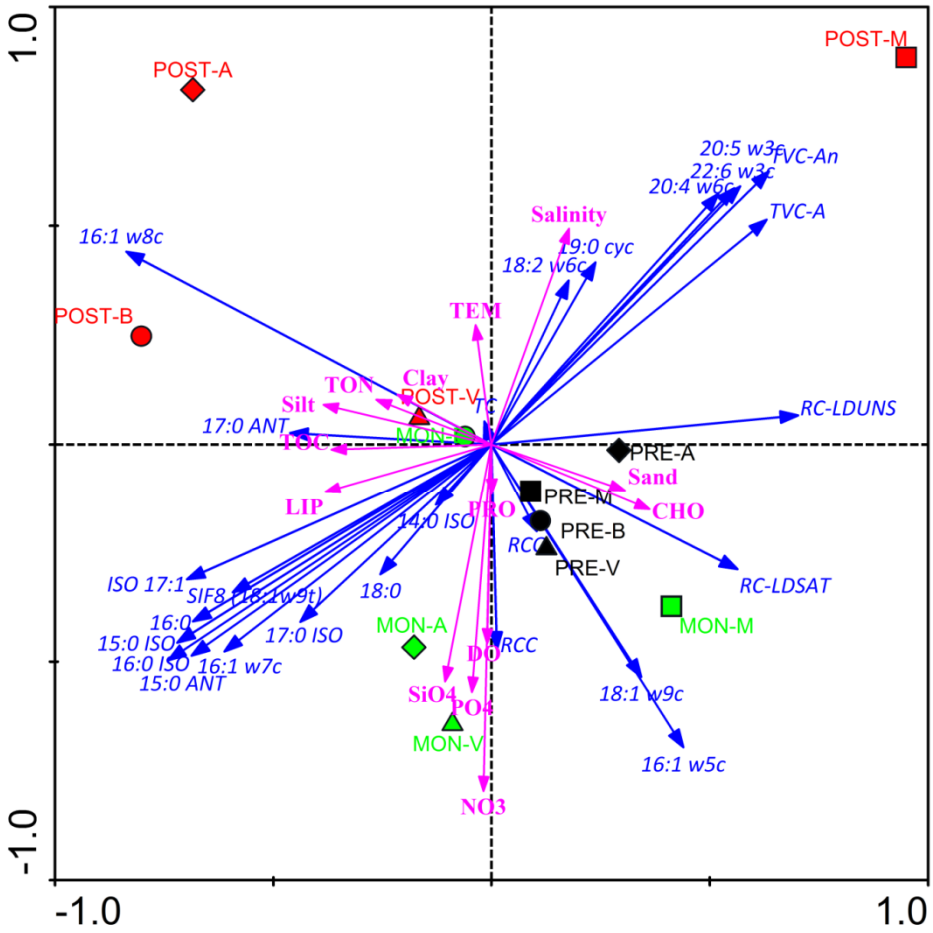

Figure 4.18 Abiotic and biotic factors influencing community at four study locations during three seasons (RDA analysis). B-Barmouth, V-Vaduthala, M – Munambam, A- Arookutty, PRE (Pre-monsoon), MON (Monsoon), POST (Post-monsoon)

4.2.3. Culturable Bacterial Diversity (FAME)

4.2.3.1 Distribution of bacteria

Four hundred and fifty six bacteria of the assemblage RCC, RCO, RC-LD_{SAT} and RC-LD_{UNSAT} were affiliated to different phyla such as Firmicutes, Proteobacteria, Actinobacteria and Bacteriodetes and were further classified

into 6 classes having a total of 41 genera. Firmicutes was the dominant phylum. A total of 84 bacterial species were recorded from the Cochin estuary in the present study. Identities of the 84 species are tabulated in Table 4.12. The chromatogram and composition report of bacteria is given in appendix.

Table 4.12 The list of species recorded in the 4 bacterial assemblages

Phyla	Class	Species	RCC	RCO	RC-LD$_{SAT}$	RC-LD$_{UNSAT}$
Firmicutes	Bacilli	*Bacillus atrophaeus*	+	+	+	+
		Bacillus cereus	+	+	+	+
		Bacillus circulans			+	
		Bacillus coagulans	+			+
		Bacillus filicolonicus	+			
		Bacillus firmus				+
		Bacillus flexus			+	
		Bacillus freudenreichii	+			
		Bacillus halodenitrificans		+		
		Bacillus lentimorbus	+		+	+
		Bacillus macroides	+		+	
		Bacillus marinus	+			
		Bacillus marisflavi	+	+		+
		Bacillus megaterium	+	+	+	+
		Bacillus mycoides				+
		Bacillus oleronius	+	+	+	+
		Bacillus pumilus	+	+	+	+
		Bacillus sphaericus	+		+	+
		Bacillus subtilis	+		+	+
		Brevibacillus agri	+	+	+	
		Brevibacillus brevis			+	+
		Kurthia gibsonii	+	+		
		Paenibacillus apirarius		+		
		Paenibacillus larvae pulvifaciens			+	
		Staphylococcus gallinarum	+			

		Staphylococcus hyicus	+			
		Staphylococcus schleiferi			+	+
		Virgibacillus pantothenticus	+	+	+	+
Proteobacteria	β proteobacteria	*Acidovorax temperans*				+
		Neisseria sicca		+		
		Neisseria flavescens			+	
	γ proteobacteria	*Aeromonas cavae*	+			
		Aeromonas hydrophila				+
		Aeromonas ichthiosmia A/hydrophila	+	+	+	+
		Aeromonas jandaei				+
		Acinetobacter calcoaceticus				+
		Acinetobacter haemolyticus		+	+	
		Acinetobacter junii				+
		Cedecea neteri	+			
		Flavimonas oryzihabitans		+		
		Grimontia hollisae	+		+	
		Klebsiella pneumonea pneumonae			+	
		Listonella anguillarum		+		
		Marinobacter hydrocarbonoclasticus	+		+	+
		Pantoea agglomerans			+	
		Photobacterium damselae		+	+	+
		Pseudomonas aeruginosa		+	+	
		Pseudomonas balearica				+
		Pseudomonas mendocina		+		
		Pseudomonas doudoroffii	+			
		Pseudomonas stutzeri	+			

		Pseudoalteromonas haloplanktis haloplanktis			+	+
		Pseudoxanthomonas sp.			+	
		Pseudoalcaligenes				+
		Psychrobacter immobilis		+	+	+
		Shewanella putrefaciens	+	+	+	+
		Vibrio alginolyticus/harveyi B/photo.leiognathi			+	
		Vibrio carchariae			+	
		Vibrio cincinnatiensis	+			
		Vibrio cholerae Inaba				+
		Vibrio fluvialis/furnissii	+	+	+	+
		Vibrio furnissii				+
		Vibrio harveyi	+			
		Sphingomonas paucimobilis (Pseudomonas paucimobilis)		+		
	α *proteobacteria*	*Brevundimonas vesicularis*	+	+		
		Brevundimonas diminuta		+		
		Methylobacterium organophilum/fujisawaence	+			
		Xanthobacter flavus	+			
		Ochrobactrum anthropi		+		
		Rhodobacter sphaeroides		+		
Actinobacteria	*Actinobacteria*	*Arthrobacter ilicis*	+			
		Cellulomonas fimi	+	+	+	
		Clavibacter michiganase insidiosum			+	
		Curtobacterium flaccumfaciens betae/oortii	+		+	
		Curtobacterium flaccumfaciens flaccumfaciens			+	+

		Kocuria rhizophila				+
		Kytococcus sedentarius				+
		Microbacterium liquifaciens		+		
		Microbacterium laevaniformans	+			
		Nocardia brasiliensis GCB	+			
		Nocardia otitidiscaviarum GCB	+			
		Rhodococcus erythropolis/R.globerulus/N.globerula	+	+		
		Saccharothrix mutabilis GCB		+		
Bacteriodetes	*Bacteriodetes*	*Flavobacterium ferrugineum*	+	+		
		Flavobacterium johnsoniae (Cytophaga johnsoniae)				+

The diversity indices (H') of the phyla in the bacterial assemblage are shown in Table 4.13A. Bacteriodetes was not included in the diversity analysis as only 2 species were isolated. Maximum species diversity was recorded among Proteobacteria phylum (H' 2.22±0.13). There was significant differences in the H' of bacterial assemblage between the phyla ($F=9.5$; df 2,3; $p \leq 0.01$). Season wise variation in the diversity of phyla is shown in Table 4.13B. Maximum species diversity was seen during the monsoon season (H' 2.46±0.08) followed by post- monsoon (H' 2.1±0.26) and pre-monsoon seasons (H' 1.92±0.12). There was significant difference in H' of bacterial assemblage between the seasons, but no significant difference was seen within the seasons ($F=8.52$; df 2, 3; $p=0.02$).

Table 4.13 A &B Shannon Weinner Index - H' of (A) phyla and (B) seasons

A

Bacterial Assemblage	Firmicutes	Proteobacteria	Actinobacteria
RCC	1.96	2.13	1.75
RCO	1.93	2.11	1.08
RC-LD_{SAT}	1.79	2.24	1.46
RC-LD_{UNSAT}	1.61	2.39	0.51

B

Major groups	Pre-monsoon	Monsoon	Post-monsoon
RCC	2.00	2.42	2.21
RCO	1.95	2.57	1.71
RC-LD_{SAT}	1.95	2.47	2.25
RC-LD_{UNSAT}	1.74	2.38	2.24

4.2.3.2. Spatial variation

All the 4 phyla were represented at all the stations, but station wise occurrence in the percentage of phyla was dependant on the type of bacterial assemblage. For instance, RCO bacteria were grouped under 4 phyla and were present at all the 4 stations whereas RCC, RC-LD_{SAT} and RC-LD_{UNSAT} were restricted to 3 phyla and were not present at all the stations. At Barmouth station, the dominant phyla of RCO were Proteobacteria (49%), Firmicutes (32%), Actinobacteria (10%) and Bacteriodetes (9%). At Vaduthala station, the dominant phyla were Firmicutes (53%), Proteobacteria (32%), Bacteriodetes (9%) and Actinobacteria (8%). At Arookutty, the phylum Bacteriodetes was present in the RCC (1%) and RCO (3%) assemblages but was absent in RC-LD_{SAT} and RC-LD_{UNSAT} (Table 4.14). Bacteriodetes were the least at Munambam station and that too only in RCO and RC-LD_{UNSAT} assemblages. Firmicutes was the dominant phylum at all the 4 stations in RCC assemblages and the highest was in Vaduthala station (85%) followed by Arookutty station (83%).

The over dominance of the phylum Firmicutes in all the assemblages was observed at Vaduthala and Arookutty stations compared to Barmouth and Munambam stations (Table 4.14). Phylum Proteobacteria was present in all the 4 assemblages at all the studied stations in varying percentages, but its dominance at Barmouth station (62%) was in RC-LD_{SAT} at Vaduthala (32%) in RCO, at Munambam (55%) both in RCO and RC-LD_{UNSAT} and at Arookutty (45%) in RC-LD_{UNSAT} assemblages. Phylum Actinobacteria was third in the dominance in all the assemblages and in some cases not present. There was significant spatial variation in the percentage of phyla ($F=21.23$ df=3,14, $p>0.001$) but among the assemblage (RCC, RCO, RC-LD_{SAT} and RC-$LD_{UNSAT)}$ there was no significant variation. ANOVA of each of the bacterial assemblage showed that the phylum Firmicutes and Proteobacteria showed significant spatial variation ($F=6.65$;$p=0.01$ and $F=4.74$; $p<0.05$; df 3,3). This variation was also significant for RCO, RC-LD_{SAT} and RC-LD_{UNSAT} ($F=5.02$; $p<0.05$ and $F=5.71$; $p<0.05$; df 3,3). Bacteriodetes showed only significant spatial variation ($F=5.67$; df 2,3; $p<0.05$) whereas Actinobacteria did not show any spatial variation.

Table 4.14 Percentage distribution of different phyla at the 4 stations studied

Types	Phylum	Barmouth	Vaduthala	Munambam	Arookutty
RCC	Firmicutes	73	85	56	83
	Proteobacteria	23	11	35	15
	Actinobacteria	4	4	9	1
	Bacteriodetes	-	-	-	1
RCO	Firmicutes	32	53	36	59
	Proteobacteria	49	32	55	24
	Actinobacteria	10	8	8	14
	Bacteriodetes	9	7	1	3
RC-LD_{SAT}	Firmicutes	37	68	47	54
	Proteobacteria	62	28	53	42
	Actinobacteria	1	4	-	4
RC-LD_{UNSAT}	Firmicutes	64	74	44	45
	Proteobacteria	26	20	55	45
	Actinobacteria	10	6	-	10
	Bacteriodetes	-	-	1	-

Number of bacterial species in assemblage (RCC, RCO, RC-LD_{SAT} and RC-LD_{UNSAT}) in the 4 stations ranged from 15 to 29. There was not much spatial variation in the number of species in different bacterial assemblages in CE (Table 4.15). At Barmouth, Vaduthala and Munambam, the highest number of species were recorded in RCC. At Arookutty, the highest number of species (27) was recorded in RC-LD_{UNSAT} assemblage. The total number of single species recorded in the 4 stations was 27 and the number varied within the phyla and stations.

Table 4.15 Number of species in the bacterial assemblage at the 4 stations studied

Types	Barmouth	Vaduthala	Munambam	Arookutty
RCC	27	29	27	24
RCO	23	26	23	25
RC-LD_{SAT}	24	21	15	24
RC-LD_{UNSAT}	18	17	20	27

Twenty nine bacterial species were found to be common among the 4 stations (Table 4.16). Maximum number of species encountered was among the γ Proteobacteria (12) followed by Firmicutes (11) and Actinobacteria (3) one each was recorded for α and β Proteobacteria and Bacteriodetes.

Table 4.16 Common species present in 4 stations studied

Firmicutes	Proteobacteria			Actinobacteria	Bacteriodetes
	α	β	γ		
B. atrophaeus	*Brevundimonas vesicularis*	*Neisseria sicca*	*Aeromonas hydrophila*	*Cellulomonas fimi*	*Flavobacterium ferrugineum*
B. cereus			*Acinetobacter haemolyticus*	*Microbacterium liquifaciens*	
B. lentimorbus			*Flavimonas oryzihabitans*	*Nocardia brasiliensis*	
B. macroides			*Grimontia hollisae*		
B. marisflavi			*Klebsiella pneumonea pneumonae*		
B. megaterium			*Marinobacter hydrocarbonoclasticus*		
B. oleronius			*Photobacterium damselae*		
B. pumilus			*Pseudomonas aeruginosa*		
B. sphaericus			*Pseudoalteromonas haloplanktis haloplanktis*		
Brevibacillus agri			*Psychrobacter immobilis*		
Virgibacillus pantothenticus			*Shewanella putrefaciens*		
			Vibrio fluvialis/furnissii		
11	**1**	**1**	**12**	**3**	**1**

Out of 28 species recorded, 14 each were present in more than 2 studied stations and their distribution was equal in numbers. However, species distribution varied between phyla and stations (Table 4.17).

Table 4.17 Station wise distribution of number of species present at two or three stations studied

Phylum	Unique species	Barmouth	Vaduthala	Munambam	Arookutty
Firmicutes	*B. freudenreichii*	+	+	+	
	B. marinus		+	+	+
	Brevibacillus brevis		+	+	+
	S. hyicus	+	+		+
	S. schleiferi	+		+	+
	B. coagulans	+	+		
	B. filicolonicus	+	+		
	B. firmus		+		+
	B. subtilis	+		+	
	Kurthia gibsonii			+	+
	P. apirarius			+	+
	P. larvae pulvifaciens	+	+		
	Total 12	**7**	**8**	**7**	**7**
γ Proteobacteria	*A. ichthiosmia A/hydrophila*	+	+		+
	P. doudoroffii	+		+	+
	V. carchariae	+		+	+
	V. cholerae Inaba	+		+	+
	P. stutzeri	+	+		+
	Sphingomonas paucimobilis	+	+		+
	A. jandaei		+		+
	A. junii			+	+
	P. balearica	+	+		
	V. furnissii			+	+
α proteobacteria	*Rhodobacter sphaeroides*	+	+		
	Total 11	**8**	**6**	**5**	**9**
Actinobacteria	*C. flaccumfaciens betae/oortii*	+	+		+
	C. flaccumfaciens flaccumfaciens	+	+		+
	Saccharothrix mutabilis	+	+		+
	Kocuria rhizophila	+			+
	Rhodococcus erythropolis/R.globerulus/N.globerula		+		+
	Total 5	**4**	**4**	**0**	**5**

At Barmouth, Vaduthala, Munambam and Arookutty exclusive species were 8, 4, 8 and 7 respectively. In Barmouth, these species were affiliated to all the 4 phyla (Table 4.18). Unlike Barmouth and Vaduthala, in Arookutty and Munambam exclusive species belonging to Firmicutes were absent.

Table 4. 18 Station specific species in 4 stations studied

	Exclusive species	**Barmouth**	**Vaduthala**	**Munambam**	**Arookutty**
Firmicutes	*B. circulans*	+			
	B. flexus	+			
	B. halodenitrificans		+		
	B. mycoides	+			
	S. gallinarum		+		
	Total	**3**	**2**		
β proteobacteria	*Acidovorax temperans*				+
	Neisseria flavescens	+			
γ proteobacteria	*A. cavae*				+
	A. calcoaceticus			+	
	Cedecea neteri				+
	Listonella anguillarum	+			
	Pantoea agglomerans				+
	P. mendocina			+	
	Pseudoxanthomonas sp.	+			
	Pseudoalcaligenes	+			
	V. alginolyticus/harveyi B/photo.leiognathi	+			
	V. cincinnatiensis			+	
	V. harveyi			+	
α *proteobacteria*	*Brevundimonas diminuta*				+
	Xanthobacter flavus			+	
	Ochrobactrum anthropi				+
	Total	**5**		**5**	**6**
Actinobacteria	*Arthrobacter ilicis*			+	
	Clavibacter michiganase insidiosum		+		
	Kytococcus sedentarius				+
	Microbacterium laevaniformans			+	
	Nocardia otitidiscaviarum		+		
	Total		**2**	**2**	**1**
Bacteriodetes	*Flavobacterium johnsoniae*			+	

4.2.3.3. Temporal variation

Except during pre-monsoon seasons all the 4 phyla were represented during all other two seasons. During pre-monsoon the phylum Bacteriodetes was absent among all the 4 bacterial assemblages. But season wise occurrence in the percentage of phyla was dependent on the type of bacterial assemblage eg. RCO bacteria grouped under 4 phyla and all four were present only during monsoon and post-monsoon seasons whereas RCC bacteria grouped under 4 phyla and all were present only during monsoon. RC-LD_{SAT} and RC-LD_{UNSAT} were restricted to 3 phyla and were not present at all the seasons (Figure 4.19). During pre-monsoon, the dominant phyla of RCO were Firmicutes (45%) followed by Proteobacteria (34%) and Actinobacteria (21%) and the Bacteriodetes were absent in all the stations during this period whereas during monsoon the dominant phyla were Firmicutes (44%) followed by Proteobacteria (39%), Bacteriodetes (9%) and Actinobacteria (8%). During post-monsoon the dominant phyla were Actinobacteria (45%) followed by Firmicutes (42%), Proteobacteria (7%) and Bacteriodetes (6%). At monsoon and post-monsoon seasons the phylum Bacteriodetes were present only in RCC and RCO assemblages. Firmicutes and Proteobacteria were the dominant phyla during all the three seasons and highest percentage recovery was on RCC (91%) and on RC-LD_{SAT} (62%) during pre-monsoon and monsoon respectively. There was not much temporal variation in the percentage of phyla but among the assemblage there was significant variation ($F=10.31$ $df=2, 14$ $p>0.001$).

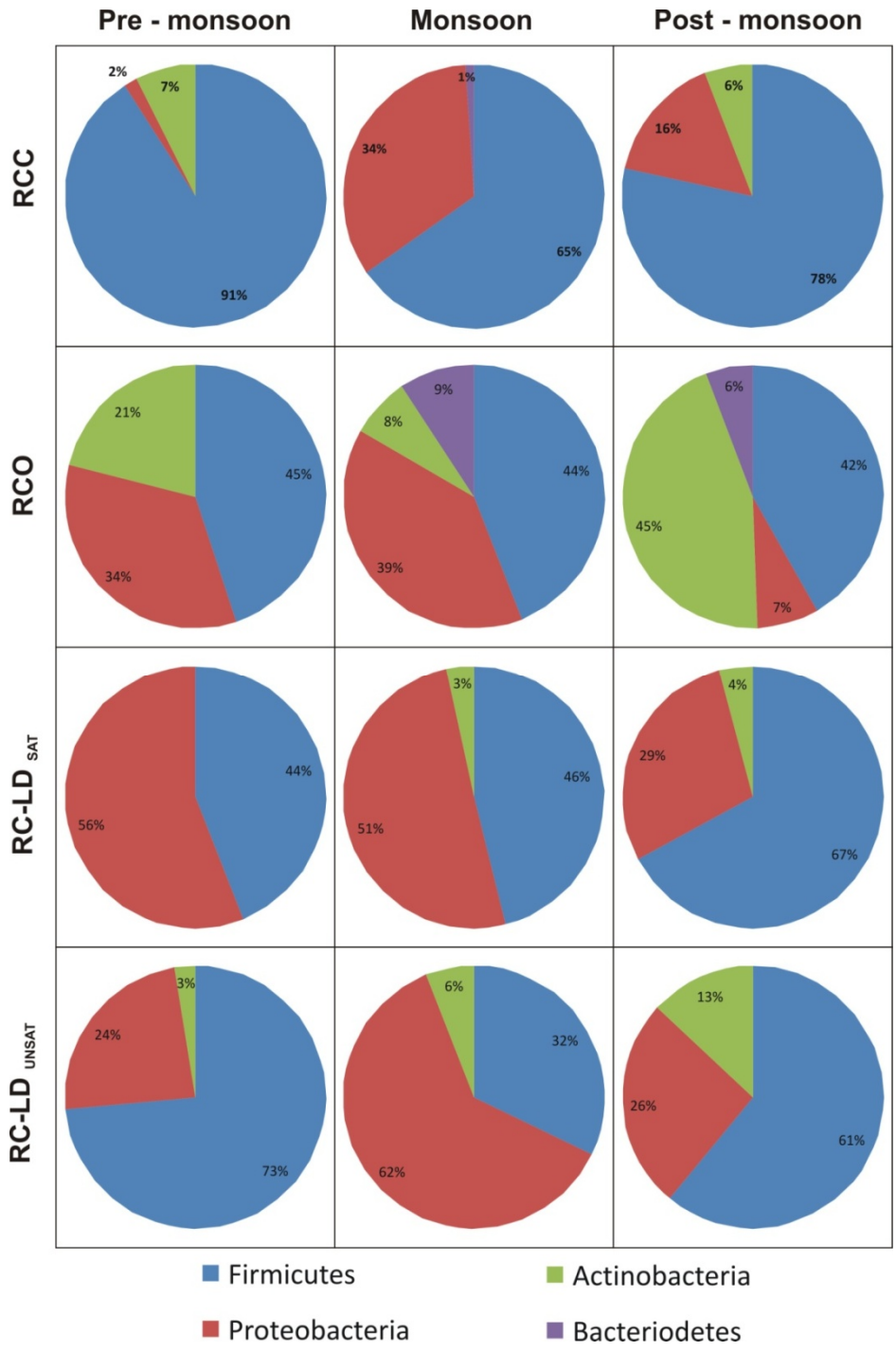

Figure 4.19 Seasonal variation of the bacterial assemblage at 4 stations studied

Number of bacterial species in the assemblage (RCC, RCO, RC-LD$_{SAT}$ and RC-LD$_{UNSAT}$) in the 3 seasons ranged from 9 to 24 (Table 4.19). During pre-

monsoon, the highest number of species (14) was recorded in RCO whereas during monsoon season the highest number of species (20) was in RC-LD_{SAT} and during the post-monsoon season (24) it was in RCC.

Table 4.19 Seasonal variation in number of species in the bacterial assemblage at 4 stations studied

Types	Pre-monsoon	Monsoon	Post-monsoon
RCC	11	19	24
RCO	14	18	9
RC-LD_{SAT}	11	20	19
RC-LD_{UNSAT}	12	17	21

4.2.3.3.1. Station wise temporal variation

Station wise distribution (%) of different phyla in different bacterial assemblages is given in Table 4.20. Temporal variation in different stations was prominent among Firmicutes and Proteobacteria in all the bacterial assemblages. The dominance of Firmicutes depended on the season and station. Interestingly the dominance of these two groups was inverse i.e., when Firmicutes increased Proteobacteria decreased and vice versa. Distribution of Proteobacteria was the highest during monsoon followed by post and pre-monsoon seasons. At Munambam and Arookutty stations, this phylum was absent during pre-monsoon. The phylum Bacteriodetes was observed only during monsoon (1%) at all the stations except Munambam where it was absent during all the seasons. Among Actinobacteria, the highest % distribution was observed during monsoon followed by pre- and post-monsoon seasons. At Barmouth and Arookutty, this group was observed only during post-monsoon. At Vaduthala, it occurred during both post and pre-monsoon seasons.

Among the RCO bacterial assemblage, the seasonal dominance of phylum Firmicutes varied with the stations. Among this assemblage, both Firmicutes and Proteobacteria were the dominant phyla at all the 4 stations. At Vaduthala and Arookutty stations, the highest percentage of Firmicutes was present during post-monsoon followed by pre-monsoon and monsoon seasons whereas at Barmouth, the highest percentage occurred during monsoon followed by post and pre-monsoon. At Munambam, the maximum distribution was observed during pre-monsoon followed by monsoon and post-monsoon. The trend in temporal variation of phylum Proteobacteria was just opposite to Firmicutes at all the 4 stations. The Bacteriodetes were absent during pre-monsoon at all the stations and during post-monsoon at Munambam and Arookutty stations. Among Actinobacteria, the highest percentage occurrence was seen during pre-monsoon followed by monsoon and post-monsoon at Barmouth and Vaduthala stations. At Munambam and Arookutty stations, the percentage was high during pre-monsoon followed by post-monsoon and monsoon seasons.

Among RC-LD_{SAT} bacterial assemblage also, the seasonal dominance of phylum Firmicutes varied based on the stations. Among this assemblage, both Firmicutes and Proteobacteria were the dominant phyla at all the 4 stations. At these stations, the highest percentage distribution of Firmicutes was observed during post-monsoon followed by monsoon and pre-monsoon seasons, except at Munambam station. At Munambam, the highest occurrence was observed during pre-monsoon followed by post-monsoon and monsoon seasons. The trend in temporal variation of phylum Proteobacteria was just reverse of Firmicutes at all the stations. Among

Actinobacteria, low percentage distribution was observed only during monsoon and post-monsoon seasons at all the stations except at Munambam. The Bacteriodetes were absent in this assemblage.

Among RC-LD_{UNSAT} bacterial assemblages also, the seasonal dominance of phylum Firmicutes varied based on the stations. Among this assemblage, both Firmicutes and Proteobacteria were the dominant phyla at all the stations. At these stations, the highest percentage of Firmicutes was observed during pre-monsoon followed by post-monsoon and monsoon seasons except at Barmouth station. Whereas at Barmouth, the highest percentage was observed during post-monsoon followed by pre-monsoon and monsoon. The highest percentage of Proteobacteria was observed during monsoon followed by pre-monsoon and post-monsoon at all the stations except at Munambam station. At Munambam, the highest occurrence was observed during monsoon followed by post-monsoon and pre-monsoon. Among Actinobacteria, the maximum percentage was observed during monsoon followed by pre- and post-monsoon at Barmouth. At Vaduthala and Arookutty, Actinobacteria was observed during post-monsoon. The Bacteriodetes were absent at all the stations except at Munambam during post-monsoon.

Table 4.20 Percentage distribution of phyla in four stations during three seasons

	Types	Phylum	Pre-monsoon	Monsoon	Post-monsoon
Barmouth	RCC	Firmicutes	93	59	76
		Proteobacteria	7	40	17
		Actinobacteria	-	-	7
		Bacteriodetes	-	-	-
	RCO	Firmicutes	9	48	34
		Proteobacteria	77	29	47
		Actinobacteria	14	13	6
		Bacteriodetes	-	10	13
	$RC\text{-}LD_{SAT}$	Firmicutes	22	26	78
		Proteobacteria	78	72	19
		Actinobacteria	-	2	3
		Bacteriodetes	-	-	-
	$RC\text{-}LD_{UNSAT}$	Firmicutes	70	37	75
		Proteobacteria	24	38	21
		Actinobacteria	6	25	4
		Bacteriodetes	-	-	-
Vaduthala	RCC	Firmicutes	97	69	89
		Proteobacteria	1	30	5
		Actinobacteria	2	-	6
		Bacteriodetes	-	1	-
	RCO	Firmicutes	49	41	71
		Proteobacteria	35	40	21
		Actinobacteria	16	5	4
		Bacteriodetes	-	14	4
	$RC\text{-}LD_{SAT}$	Firmicutes	48	74	80
		Proteobacteria	52	26	13
		Actinobacteria	-	-	-
		Bacteriodetes	-	-	-
	$RC\text{-}LD_{UNSAT}$	Firmicutes	90	35	84
		Proteobacteria	10	65	3
		Actinobacteria	-	-	13
		Bacteriodetes	-	-	-
	RCC	Firmicutes	71	50	55
		Proteobacteria	-	49	38
		Actinobacteria	29	1	7
		Bacteriodetes	-	-	-

Munambam	RCO	Firmicutes	69	36	5
		Proteobacteria	12	60	92
		Actinobacteria	19	1	3
		Bacteriodetes	-	3	-
	$RC\text{-}LD_{SAT}$	Firmicutes	74	14	41
		Proteobacteria	26	86	59
		Actinobacteria	-	-	-
		Bacteriodetes	-	-	-
	$RC\text{-}LD_{UNSAT}$	Firmicutes	80	21	22
		Proteobacteria	20	79	75
		Actinobacteria	-	-	-
		Bacteriodetes	-	-	3
Arookutty	RCC	Firmicutes	100	76	85
		Proteobacteria	-	23	13
		Actinobacteria	-	-	2
		Bacteriodetes	-	1	-
	RCO	Firmicutes	54	51	75
		Proteobacteria	17	38	17
		Actinobacteria	29	3	8
		Bacteriodetes	-	8	-
	$RC\text{-}LD_{SAT}$	Firmicutes	36	62	69
		Proteobacteria	64	30	27
		Actinobacteria	-	8	4
		Bacteriodetes	-	-	-
	$RC\text{-}LD_{UNSAT}$	Firmicutes	56	32	49
		Proteobacteria	44	68	26
		Actinobacteria	-	-	25
		Bacteriodetes	-	-	-

4.2.3.4. Diversity index

The species diversity index (H') of major groups in the four stations is given in Table 4.21. The diversity index (H') ranged from 2.01($RC\text{-}LD_{UNSAT}$ at Vaduthala) to 2.84 ($RC\text{-}LD_{UNSAT}$ at Arookutty). However, the indices did not vary significantly between stations and bacterial assemblages, indicating relatively uniform diversity (Table 4.21).

Table 4.21 Spatial variation of species diversity (H') in 4 stations studied

Types	Barmouth	Munambam	Arookutty	Vaduthala
RCC	2.67	2.76	2.27	2.02
RCO	2.66	2.51	2.71	2.83
$RC\text{-}LD_{SAT}$	2.48	2.15	2.62	2.21
$RC\text{-}LD_{UNSAT}$	2.12	2.60	2.84	2.01

The species diversity index (H') of the major groups among three seasons at 4 stations in CE is given in Table 4.22. During pre-monsoon, monsoon and post-monsoon the diversity index (H') ranged from 1.1 – 1.89, 0.65 – 2.41 and 0.8 – 2.38. Analysis of variance of the pooled data showed that the indices were not significantly different during pre-monsoon indicating relatively uniform diversity unlike monsoon and post-monsoon.

Table 4.22 Seasonal variation in diversity index of all bacterial assemblages at the 4 studied stations

Stations	Pre-monsoon				Monsoon				Post-monsoon			
	RCC	RCO	$RC\text{-}LD_{SAT}$	$RC\text{-}LD_{UNSAT}$	RCC	RCO	$RC\text{-}LD_{SAT}$	$RC\text{-}LD_{UNSAT}$	RCC	RCO	$RC\text{-}LD_{SAT}$	$RC\text{-}LD_{UNSAT}$
Barmouth	1.78	1.22	1.45	1.67	2.17	2.41	2.18	1.77	2.25	1.61	1.41	1.56
Vaduthala	1.28	1.89	1.29	1.1	1.94	2.41	1.99	1.73	1.65	1.68	2.01	1.34
Munambam	1.73	1.58	1.56	1.44	2.13	2.19	0.65	1.91	2.11	0.8	1.45	1.68
Arookutty	1.33	1.81	1.62	1.48	2.16	2.29	2.37	2.14	1.74	1.34	1.95	2.38

CHAPTER 5

DISCUSSION

Marine and estuarine habitats exhibit a great deal of temporal and spatial variability due to the higher complexity of physical and chemical components that interact within the biological components to yield a dynamic ecosystem (Clark and Cripe 1993). Tropical coastal ecosystems such as sandy beaches, estuaries, and coastal lagoons, are among the world's most jeopardized environments due to the increased and unscrupulous use of their natural resources. These systems are persistently exposed to anthropogenic activities that cause negative impacts on their biodiversity and habitat suitability. Bacteria in the estuarine sediments influence elemental biogeochemical cycles, benthic ecology and *in situ* bioremediation. The sediment–water interface is a zone of high microbial abundance and the processes/flux occurring are of great biogeochemical importance. Therefore, knowledge of the abundance and composition of microbial assemblages is crucial for the comprehension of the local processes (Zehr and Ward 2002; DeLong and Karl 2005). The concept that bacterial communities are heterogeneous over trophic gradient as regards their abundance (Andrade et al. 2003), physiological activity and diversity (Hewson and Fuhrman 2004) has evolved from the comparative studies on bacterial communities. However, the biogeography of microbial assemblages in tropical estuarine environment has not been well understood (Baldwin et al. 2005) and the taxa that make up these communities are poorly known (Rappé and Giovannoni 2003; Giovannoni and Stingl 2005). Despite its importance, the benthic bacterial studies have eluded researchers and this area has lagged behind its pelagic counterpart due partly to methodological and conceptual

difficulties (Kemp et al. 1990). Very few data on sedimentary bacterial abundance and diversity from tropical estuaries are available in literature.

5.1 Environmental Parameters

Estuarine water column, in general is subjected to wide fluctuations in physical parameters (tides, salinity, temperature, turbidity etc.) (Shiah and Ducklow 1994; Wikner et al. 1999; Cunha et al. 2000) and chemical (substrate availability, substrate complexity) properties (Thingstad and Billen 1994; Amon and Benner 1996; Forman 1998; Covert and Moran 2001). Extensive studies have been carried out, particularly on the physical, chemical and biological aspects of the Cochin estuary and also on the impacts due to dredging by Gopinathan and Qasim (1971), and Rasheed and Balchand (1995). The physio-chemical characteristics of water column of CE in the present study showed spatial variation, with surface variables reflecting significant differences. However, temporal variations in most of the parameters were not so pronounced during the study period. Also the variability seen in the surface parameters was not quite visible in all parameters in the near bottom water. Spatial variation in temperature and pH of surface and near bottom water was significant. The major hydrological variable in the Cochin estuary is the salinity, which is similar to the situation encountered in any other Indian estuary where the salinity gradually declines from ~35 at the mouth of the estuary (Barmouth and Munambam) to ~0.1 at the entry point of rivers (Vaduthala and Arookutty). During the monsoon season, heavy rainfall causes high river discharge and this limits; the salt-water intrusion to a short distance from the mouth into the estuary, resulting

in low salinity even close to the Barmouth. Stratification often develops culminating in a condition with less dense river water at surface and high dense seawater at the bottom layers. Such typical hydrographic features and circulation pattern complicate the sedimentation characteristics of the estuarine channels. Post-monsoon is a transitional period, when the river discharge gradually diminishes, the tidal influx gains momentum and the estuarine conditions turns to a partially mixed type thereby weakening the stratification. Comparatively, high salinity during the pre-monsoon (dry and stable period) at Vaduthala, which is located near river Periyar can be attributed to the low river input at the upstream, resulting in extended seawater intrusion into the estuary. The oxygen concentration at the near bottom water however varied between and within the stations from 1.24 to 4.83 $ml.l^{-1}$ was not significant reflecting uniform and steady minimum state of oxygen in the water at most of the stations. Overall, the hydrography of the Cochin estuary is influenced by two main factors viz. the short term changes induced by the tides and the longer term seasonal changes brought about by the monsoon system. The tides at the Cochin estuary are mixed and semidiurnal nature. Two high (flood) and two low (ebb) watermarks occur every day and differ in amplitude (height). All water quality parameters (temperatures, salinity, DO, pH, nutrients etc), are primarily influenced (distribution in the estuary and concentration) by the tides. The changes in the hydrology are also controlled by the prevailing seasons. Nitrate, nitrite and ammonia of the water column showed spatial variation. As compared to nitrate (average 15.99 ± 9.17$\mu mol.l^{-1}$), the concentration of nitrite in the estuary was low (average 0.84 ± 1.23 $\mu mol.l^{-1}$) and the concentration of both

these constituents were high at the near bottom layers probably formed as a result of decomposition of organic nitrogen within the sediments (Sankaranarayanan and Qasim 1969; Qasim 2003). Among other nutrients, silicate showed significant spatial variation both in the surface and near bottom waters but the temporal variation was seen only in the near bottom waters. Silicate concentration in the Cochin estuary has been reported to be always excessive (Jyothibabu et al. 2006) and this may be associated with the freshwater influx and land runoff (Qasim 2003) especially during southwest monsoon season. Another important source of silicate input into the estuary could be the diatom as the silicate content in marine sediments has a close link with biosiliceous (diatom) productivity in the overlying surface waters (Conley 1998). Temporal variation in the silicate content at the bottom indicates either the difference in the rate of utilization or sedimentation of particulate organic matter. In the estuary coupling has been shown to exist between biogeochemical processes in the surface sediment and the water column in shallow marine systems (Nixon 1981; Graf 1992). Nutrients recycled in the sediment diffuse or are transported back to the over-lying water which supports water column production (Nixon and Pilson 1983). In addition to autochthonous input, nutrients and pollutants are also introduced into the estuary as a result of domestic and industrial waste discharge which influences the chemical characteristics of the estuary. Cochin estuary receives contaminated freshwater, industrial effluents and partially treated sewage at many points throughout its tidally mixed zone. Large variations in the water quality parameters observed at Munamabam and Vaduthala stations located in the northern part of the estuary may be

due to the stratification and large scale inputs from industrial units, sewage works and agricultural runoffs (Anirudhan et al. 1987; Menon et al. 2000).

The sediment characteristics in relation to changing hydrography of the Cochin estuary have been investigated (Murty et al. 1985; Nair et al. 1993). Jos Anto (1971) and Murty and Veeraiah (1972) reported the presence of silty clays with phi–mean values up to 9.2 in this region. The study area experiences heavy rains during most of the year caused by monsoonal winds. Run-offs from the local streams and minor rivers connected to the estuarine region are the main sources of sediments in the region of the study area. These studies have revealed the seasonal spatial grading of particles as sand, silt and clay. The present study also corroborates this finding. The sediments in the northern stations of the upper estuary, viz. Vaduthala and Munambam were mainly composed of sand particles while in the lower estuarine regions, the abundance of sand was seen during the monsoon season which points at the downward movements of the estuarine bed. Otherwise, large areas of the estuarine bed were predominantly covered by sand and silt fractions throughout the year. Finer sediments were restricted to the Barmouth. The sediment distribution pattern at Arookutty station indicated the dominance of finer terrigenous sediments such as clays and clayey-silts. Sand undergoes rapid pore water exchange resulting in physically unstable environments that tend to diminish geochemical gradients. Comprehensive reports on interstitial adsorbed forms of phosphates in sediments of this estuary are available (Anirudhan 1988; Nair et al. 1993) and enhanced amount of bioavailable P are reported in high saline waters (Balchand and Nair 1994).

Sediments are pivotal components of aquatic ecosystems where important transformations and exchange processes take place (Levin et al. 2001). The organic matter content plays an important role in every ecosystem and it is likely to influence the microbial composition in the sediments. Estuarine sediments are the sink for organic carbon produced by plankton at different trophic levels (bacterioplankton, phytoplankton and zooplankton) and also from terrestrial plants and by aeolian and river transport. Sediments of all the stations received the above inputs at varied quantity on a temporal and spatial scale. Besides the biological and geographical variation, the stations organic inputs were dependent on the socio- economic environment like urbanisation, industrialisation and regional development (Galois et al. 2000). Zimmerman and Canuel, (2000) found that anthropogenic activities within estuarine watersheds can exert a substantial influence on carbon cycling in estuaries. The Cochin estuary is a highly productive ecosystem with a seasonal shift in net pelagic production from autotrophy to heterotrophy, due to allochthonous organic matter inputs (Thottathil et al. 2008a). As a result, the sedimentary concentration of TOC of CE is influenced by a variety of environmental conditions and deposit processes, notably primary productivity, water column remineralization, sedimentation rate, bottom water oxygen concentration and exposure time (Hodell and Schelske 1998). Abundance and variability of sedimentary OM is a reflection of the changes in any of these factors (Meyers 1997; Zimmerman and Canuel 2000; Pinturier-Geiss et al. 2002; Zimmerman and Canuel 2002). The TOC concentration in CE varied from 0.79 to 3.52% whereas TON concentration ranged from 0.05 to 0.42%. The TOC

concentration in the estuary was 3 orders higher than the typical range (0.42–2.46 mg g^{-1}) of organic matter in open-ocean surface sediments (Santos et al. 1994; Gogou and Stephanou 2004; Schefuß et al. 2004). The relative significance of these sources is determined by local environmental factors such as climate, hydrodynamic conditions and nutrient supply. Absence of spatial variability in CE suggests that the sources of input in the 4 study locations was either the same or was uniformly distributed due to tidal mixing. However, there was significant temporal variation in TOC and TON concentrations both apparently influenced by seasonality. Such seasonal variations in sediment organic carbon and nitrogen in this estuary have been earlier reported (Balachandran et al. 2005). The amount of TOC was comparatively higher towards the estuarine mouth. Munambam and Vaduthala stations, on the northern part of the estuary exhibited low values, signifying low retention of organic carbon in the sandy sediments. Rapid pore water advection is likely to enhance the degradation leading to low organic content in marine sands. North limb of CE did not consistently have high organic carbon in the sediment. The distribution of organic matters in the surface sediment suggests that the organic matter of the four studied sites was a mixture from marine authogenic and terrestrial inputs and corresponded to their location and condition. The terrestrial input was the predominant source of organic matter for the sites closest to the river mouth. Although there were only small variations in TOC and TON contents between the locations, remarkable seasonal decreases in TOC and TON were observed. An obvious cause for the variation in TOC is likely to be related with the shift in sediment type as discussed earlier, with a seasonal

increase in silt/clay that gradually decreased towards the mouth where the sediments became sandier (high at Barmouth). It is well known that the organic carbon and nitrogen contents of muddy sediments are generally higher than those in sandy sediments (Mayer 1994; Keil and Cowie 1999).

C/N ratios provide information on the origin of the preserved organic matter. The ratios for the autochthonous organic water generally range from 6 to 9 because of high protein content in organisms such as algae and zooplankton (Uzaki and Ishiwatari 1986), whereas those of higher plant-derived organic matter give larger values because of a high content of non-protein material (Ishiwatari et al. 1977). However, the presence of clay-bound nitrogen components, including inorganic ammonium, may decrease the C/N ratios (Müller 1977). In estuaries, C:N ratios (proxies for the sources of organic carbon) are useful in differentiating between OM sources since marine organisms are usually enriched in nitrogen (C:N=6-8) compared to terrestrial plants (C:N >12) (Prahl et al. 1980; Meyers et al. 1984). The C:N ratios of sediments in the CE estuary varied between stations with generally higher values at the northern stations indicating a shift of organic matter input from different sources (Table 4.5). The C:N weight ratios of sediments ≥ 12, recorded at some stations are on the higher range reported in literature from productive sites (Uchida et al. 2005; Niggemann and Schubert 2006). The C:N ratios in the Harney River decrease steadily from the upper to the lower estuary. Values typical for higher plants were observed at the upper estuary, while typical marine end-member values were obtained for the Florida Shelf site (Jaffé et al. 2001). The C:N distribution in the Harney River is indicative of estuarine mixing of marine-derived and terrestrial OM. Similar

trends of decreasing C:N have been observed in other, larger estuaries (Kennicutt II et al. 1987). In contrast, the C:N ratios for the Taylor River estuary did not change significantly between sites, although a small decrease in the ratio was observed at a particular site due to the increased marine influence and regions with slightly higher C:N ratios attributed to higher terrestrial OM inputs, most likely derived from mangroves (Jaffé et al. 2001).

The high C:N values close to the upstream sites suggests the important contribution of terrigeneous matter (Stein et al. 1994), decreasing towards the sea. Oxygen demand and the quantities of nutrients regenerated are not a simple function of the quantity but also quality of organic matter present (i.e. origin, carbon and nitrogen ratio (C:N ratio)), and the latter is affected by numerous biological and physical factors (Cowie and Hedges 1992; Fiordelmondo and Pusceddu 2004). It remains uncertain whether increased water-column productivity or decreased mineralization or both are the cause in the shift of autochthonous OM in the surface sediment of CE. At the northern upstream stations (Vaduthala and Munambam), the C:N ratios of sediments were close to that of terrestrial plants while at the Barmouth site and southern station (Arookutty), the C:N ratios approached that of marine phytoplankton, indicating the relative importance of terrestrial versus marine organic inputs between the upper and lower parts of the estuary. Further, the decrease in the ratio could be also due to monsoon – induced changes in the size fractionated phytoplankton biomass and production rate in CE (Madhu et al. 2010). C:N values between 8 and 9 is typical of algal derived OM sources (Meyers 1994). The range of typical C:N

ratios of marine material rich in amino acids is 5.0–7.7 (Jasper and Gagosian 1989; Westerhausen et al. 1993). Decrease of C:N could be either due to preferential re-mineralization of N relative to TOC overtime or to increase in deposition of algal versus terrestrial OM. In CE there was a partial shift in organic matter input from terrestrial to marine source. Such variation based on d13C distribution patterns have been observed in many estuarine systems (Cai et al. 1988; Fogel et al. 1992). The seasonal shift from high terrestrial input at stations close to river to station near the mouth was not dependent on the distance from the origin. That is steady decrease in trend from upper to the mouth. The absence of such trend in C:N ratio is a reflection of localized episodic autochthous or/and allochthonous organic matter inputs in a particular sampling site and sampling season. Such inputs induce hypoxic or anoxic conditions in bottom waters during the degradation of organic matter an event frequently seen at cultural eutrophic sites (anthropogenically induced increase in primary production) (Rabouille et al. 1993). And the location of eutrophic site sediments in the estuary favors the sedimentation of fresh marine material. Low level of oxygen at near bottom water compared to the surface and high nitrate at the surface sediments signals de-nitrification. So the high C:N ratio observed need not always indicative of higher-plant derived material but also an outcome of de-nitrification (Meyer et al. 2005).

The gross measure of total organic matter content in sediment furnishes only scant information on its actual availability to consumers (Newell and Field 1983; Bianchi and Levinton 1984).Therefore it is necessary to assess the nutritional value of sediment organic matter, from its

biochemical composition (Fabiano et al. 1995). Specific labile components (carbohydrate, lipid and protein) of the organic matter fraction give an estimate of the nutritional value of the sediment, which are potentially available for the organisms (Fichez 1991). Though this approach is not free of interpretation problems, it has however been widely used (Fabiano and Danovaro 1994; Fabiano et al. 1995; Danovaro 1996). The LOM concentration in the sediment ranged from 0.78 to 2.22 mg g^{-1} which was ~.001% of the TOC of the sediment. Most of the deposited OM has not been accounted for by labile organic compounds in CE. Such observation has been made from non estuarine areas (Danovaro et al. 1993). Further, the morphodynamic and physicochemical characteristics of the station may also be a determining factor for the presence of organic matter. Total LOM showed temporal variation whereas CHO and PRO showed both temporal and spatial variation and this variation was seen within the stations. The variations may be due to degradation of these labile components of organic matter at the sediment surface by the microbial activity, resulting in remineralization and regeneration of inorganic carbon and nutrients (Rowe et al. 1975; Klump and Martens 1981; Azam 1998). The dominance of proteins over carbohydrates observed in CE is characteristic of highly eutrophic environments (Danovaro et al. 1993) and such high protein concentration is reported in the coastal Baltic Sea (Meyer-Reil 1983). The protein to carbohydrate ratio (PRO:CHO >7) was >7 in CE. This ratio was comparatively higher than the 0.01 ratio reported from oligotrophic deep-sea sediments in the Eastern Mediterranean Sea (Danovaro et al. 1993) and less than 10 seen in coastal Antarctic sediments (Pusceddu 1997). This

intermediate ratio suggests the presence of fresh organic matter (Danovaro et al. 1993) in the shallow CE. The high protein concentration in the sediment indicates that the protein content is more related to phytoplankton distribution in the water column which is seen throughout the year in CE (Madhu et al. 2010). Study by Danovaro et al. (1999) has shown that the sedimentary protein is dependent upon the downward flux of phytoplankton pigments. Habitat variation is assessed through geochemical characteristics. According to the accepted concept that environmental parameters are expected to vary with space and season in the estuary, in the CE the environmental data partially sustain this assumption as the Cluster analysis grouped the sampling site and sampling stations into 3 clusters based on the morphometric and chemical characteristics of the sediment. Cluster I consist of sampling season pre-, post- and monsoon of Munambam station and post and monsoon seasons of Vaduthala station. Cluster II consisted of the three seasons of Barmouth station and monsoon sampling season of Vaduthala station. Vaduthala station is close to the Barmouth. Arookutty station, was an exclusive cluster with all 3 sampling seasons and was distantly placed. This station, is at the southern region with input from Muvatupuzha river with different watersheds. Clusters 1and 2 are fed by the same river with Munambam situated at the upstream point. Though, Barmouth also receive fresh water influx from the Arookutty region, it is comparatively less than the northern river. All the three clusters are subjected to varying degrees of riverine influence depending on the season, tide and geographic locations. In conclusion based on the environmental parameters, it can be deduced that the overall hydrochemistry of the Cochin estuary is influenced by

monsoon and associated run off results in changes in the geochemistry of the surficial sediment. The OM of CE was generally high and is comparable to other productive ecosystem. The nutritional quality is also relatively high.

5.2. Bacterial Abundance

The vertical transport of biogenic particles out of the water column (sinking) to the sediment and their biotransformation are important processes in the estuarine surficial sediments. The importance of benthic bacterial abundance both as a food source and a major contributor to biogeochemical processes in the ecosystems has been widely recognized (Montagna 1984; Alongi 1988; Kuwae et al. 1998). Despite the significant ecological role of benthic bacteria, information on their abundance, metabolic and physiological state in marine sediments in general, is extremely scarce (Douglas et al. 1987; Karl and Novitsky 1988; Van Duyl and Kop 1990; Proctor and Souza 2001) and practically no information is available on the fraction of viable versus dormant benthic bacterial cells. The average TC, TVC-A and TVC-An bacterial densities in CE were 3.99 $x10^9$, 4.5 $x10^5$ and 4.57 $x10^5$ g^{-1}, respectively and there was no significant variation both spatially and temporally. The uniformity in the distribution of bacteria was in agreement with most of the earlier studies where quite conservative bacterial densities have been reported (Danovaro et al. 1993; Danovaro et al. 1995). The TC of CE was comparable to TC of the central Indian deep sea sediments (Raghukumar et al. 2001; Das, 2011) and mangrove swamps of Goa (Krishnan 2009). It was also one order less to benthic biomass of 6.3 $x10^8$ g^{-1} for estuarine sediments from Apalachee Bay, Florida based on the

concentration of PLFA (Baird and White 1985). In CE TVC-A and TVC-An (10^5 g^{-1}) were almost equal distribution and TVC–A brought about 80% of the variation in the anaerobic abundance and 64% variation in TC. Higher aerobic viability encountered may be due to predominance of oxic condition in CE with occasional hypoxic condition. The level of oxygen concentration in the near bottom water ranged from 2 to 5ml.l^{-1}. Nitrate (inorganic ions as a source of energy for growth showed negative relationship with TVC-AN. The tendency to flourish under anoxic conditions could be a measure of TVC's facultative nature and/or the frequency of encountering anaerobic regimes in space or in time. Strong relationship between TVC-A and TVC-AN suggests that their abundance were governed by the same variables or source of input had a constant ratio.

The quantification of the bacterial fraction actually responsible for the activity is of primary relevance in addressing the important ecological questions involving organic matter degradation rates and nutrient cycling, as well as the factors that are controlling these processes (Joux and Lebaron 1997).The abundance of retrieval counts represents only a small fraction (0.1% ± 10%) of the active microbial community (White et al. 1998), and in CE it was 2-3 order less than TC, commonly observed in all aquatic systems.

Among the different RC bacterial assemblages RCC, RC-LD_{SAT} and LD_{UNSAT} showed positive inter relationship. The low nutrient i.e., RCO population was in the same order as the copiotrophic population and this implies that a considerable fraction of sedimentary bacteria of the estuary are adapted to the trophic gradients seen in estuary ie varying concentration of organic matter and its associated variable (discussed above under

environment parameters). These observations also suggest that the bacteria are highly resilient or the ecosystem is heterogeneous in nature in its nutrient requirement. In most estuaries, the variation in hydrodynamic conditions and salinity brings about rapid changes bacterial abundance of the water column (Painchaud et al. 1995) but such an influence was not seen on the benthic bacterial parameters of CE. The autochthonous and allochthonous input (TOC, TON and LOM) showed seasonal variation in CE and the scenario was different in abundance for each group of influenced mostly by the sediment characteristics. Though grain size showed significant variation both in temporal and spatial scales, it did not show any direct correlation with bacterial abundance despite lower bacterial density in sand to clay. Lower abundance of bacteria in coarse grained sediments has been documented (Llobet-Brossa et al. 1998) and was attributed to large grain size with relatively low specific surface area results in rather low adsorption capacity leading to low organic matter content (Jickells and Rae 1997; Llobet-Brossa et al. 1998) and subsequently low microbial activities (Keil et al. 1994; Jickells and Rae 1997).The organic carbon (TOC) was positively correlated with clay which could be explained by the larger specific surface area (Meyer-Reil et al. 1978; Mazure and Branch 1979; Luna et al. 2002) higher pore water content and organic matter. The high bacterial biomass was associated with finer sediment in CE. Even though there was a gradient in the sediment organic content none of the culturable bacterial assemblages showed correlation with TOC except RC-$LD_{UNSAT.}$ Being a eutrophic estuary with high productivity and excess nutrient, it's intuitive that bacterial abundance was not limited by organic matter (Balachandran et al. 2003;

Qasim 2003; Madhu et al. 2010). It was just not the autochthonous organic content that lead to high organic matter but was also fuelled by allochthonous sources like other estuaries (Peduzzi and Herndl 1992; Mann and Wetzel 1995; Conan et al. 1999). Though TOC and LOM were positively correlated, the lipid content of the LOM was negatively correlated with TOC. Saturated degraders did not show any relationship suggesting that lipids associated with TOC may be the less labile unsaturated form. TC was correlated with sediment CHO and lipid and these variables brought about 21% and 31% variation in TC. In the case of RCC, CHO was responsible for 25% of the variation. Though protein was higher than CHO and lipid, it did not influence the abundance of bacteria as it was non limiting and there was no constraint for heterotrophic metabolism in the sediments (Fabiano et al. 1995). It is well known that heterotrophic bacteria require lipids as a source of carbon and energy (Arts et al. 1992). Lipids are actively assimilated by bacteria and used in respiratory processes or in biosynthesis of cellular structures (Meyer-Reil 1983). Considerable amounts (i.e. 2 to 45%) of lipids are being accumulated by ciliates, zooplankton, phytoplankton, benthos and detritus. Hence the sources of lipids could include dead phytoplankton, zooplankton, meiobenthos, macrobenthos and also detritus (Siuda et al. 1991; Albers et al. 1996; Harvey et al. 1997). TON had a negative influence on the lipid content, but however, during pre-monsoon, there was a marginal influence on this parameter from Barmouth and Arookutty sediments. Total viable bacteria (aerobic and anaerobic) were not influenced by any of the geochemical parameters except distantly by TON. On the contrary, TVC were negatively influenced by PRO and CHO. This was more

pronounced during the pre- and post-monsoon seasons at Munambam station. All the three sampling stations and sampling seasons showed negative correlation with PRO, TOC and CHO. RCO counts were abundant in samples taken from fresh water stations like Vaduthala during monsoon and post-monsoon seasons and from Arookutty during monsoon and pre-monsoon seasons and were more influenced by geochemical parameters like TOC, PRO and CHO and to a lesser extent by TON whereas post-monsoon samples from Arookutty were influenced more by TON than TOC, PRO and CHO. TVC-A and TVC- An were abundant and correlated significantly ($r=.943$ $p<.001$) during all the seasons at Munambam whereas the RCO counts were depleted. Study by Deming and Yager (1992) has demonstrated that bacterial abundance and distribution is dependent on the availability of labile organic compounds. In CE, the viable biomass of the bacterial community were site and time specific and were influenced by the geochemical parameters. Lipid was the key player influencing the oligotrophic counts, while TOC and protein negatively influenced the oligotrophic bacteria. Further, the morphodynamic and physicochemical characteristics of the sampling sites determine the factors for the presence of organic matter which in turn could translate into higher or lower bacterial load. A phenomenon commonly associated with sediments of aquatic system, viz. estuaries, lakes and bays (Luna et al. 2002; Adriano et al. 2005; Grandlic et al. 2006; Ziervogel and Arnosti 2009; Rodrigues et al. 2011a). In CE the enrichment of different types of bacterial biomass at different locations and season were brought about by the studied geochemical variables.

5.3. Microbial Community Structure

Microbial communities in the estuaries actively involve and play important roles in a number of rate processes that include particulate carbon export, nutrient regeneration and biogeochemical cycling (Binnerup et al. 1992; Sinsabaugh and Findlay 1995; Pinckney et al. 2001; Yokokawa and Nagata 2010).At the same time, these are highly diverse communities which respond rapidly to changing environmental conditions, including changes in the nutrient concentrations in sediments and sediment-water interface (Van Duyl et al. 1993).The importance of studying microbial ecology at the community level for marine environments has been appreciated for a long time but the progress in this direction was hindered due to limited availability/access to methodology. With the advent of methods that circumvent culture bias (culture independent approaches), researchers were allowed to acquire detailed information on the phylogeny and spatial pattern of non-cultivable microorganisms in aquatic and sediment environments. Over the past 10 years, the approach of analyzing the microbial communities has changed dramatically. Many new and novel methods and approaches are now available, allowing soil microbiologists to gain access to more diverse groups of microorganisms residing in sediment, for better assessment and understanding of the microbial diversity (Hewson et al. 2006; Gontang et al. 2007; De Corte et al. 2008).This has also resulted in evolving the taxonomic composition of the complex microbial communities in coastal oceans (Crump et al. 1999; Scala and Kerkhof 2000; Pernthaler et al. 2002; Koizumi et al. 2003) and the elucidation of crucial factors in structuring the sediment communities of estuaries. Signature fatty acids (FA)

are being used for the past three decades (Bobbie and White 1980) and are still considered as reliable tools in the ecological investigations of sediments (Crump et al. 1999; Scala and Kerkhof 2000; Pernthaler et al. 2002; Koizumi et al. 2003; Freitag et al. 2006) for the quantitative estimates of microbial biomass and community structure and for the spatial pattern of non-cultivable microorganisms in aquatic and sediment environments (Hewson and Fuhrman 2004; Gontang et al. 2007; De Corte et al. 2008).

5.3.1. Community analysis using PLFA Profiling

In CE, a total of 33 individual fatty acids belonging to seven groups were identified which differed between the stations and seasons. The PLFA based community profile identified in CE was close to the number of major groups reported from elsewhere. From surficial sediment of a subtropical Pearl River 33 different PLFA were identified (Hu et al. 2006a). These, however, were less than the number of PLFA (38) identified from the sediment near the mouth of a tropical estuary along the east coast of India (Harji et al. 2010). Variations were also reported from other regions. For instance, in the sub temperate sediments of Japan, the number of PLFA identified varied between 31 and 65 (Rajendran et al. 1992a), 31 in Hiroshima Bay (Rajendran et al. 1993a), 65 in Osaka Bay (Rajendran et al. 1994), 60 in Saroma Ko lagoon (Rajendran et al. 1995b), 30 in Kojima Lake (Rajendran et al. 1995a) and in the intertidal region of Florida it was 25 (Findlay et al.1990). A total of 34 different PLFA were detected in soil, south of Sweden (Frostegård and Bååth 1996). In CE, the relative proportion in the PLFA profiles also exhibited variation. The straight chain saturated PLFA

(<19) (40.76 – 46.07%), were predominant followed by iso and anteiso methyl branched saturated (22.45 – 29.2%), monounsaturated (MUFA) (9.67 – 11.71%), branched monounsaturated (3.31 – 7.92%) and saturated (>19 9.37-1.06%) fatty acids in all the samples. In addition 4.34% PUFA and 1.73% cyclopropanes PLFA were also recorded. Wide occurrence and distribution of these fatty acids in CE was contributed by the different groups of microorganisms present at these stations. The most abundant fatty acids, C 16:1 ω7, C 16:0 and C 18:1 ω7, are generally common in marine sediments (Boschker et al. 2001; Rütters et al. 2002b; Rütters et al. 2002a). C 16:0 is ubiquitous in marine life forms and is extensively biosynthesized *de novo,* and therefore not suitable for detailed biomarker studies (Sargent and Whittle 1981). In contrast, monounsaturation at the ω7 position is generally considered as typical for bacteria (Sargent and Whittle 1981; Lechevalier and Lechevalier 1988). C 16:1 ω 7 and C 18:1 ω 7 are constituents of marine phytoplankton (Birgel et al. 2004), but are also commonly associated with bacteria (Abraham et al. 1998; Boschker and Middelburg 2002). MUFA 18:1 ω7 is a well-known bacterial marker found in both aerobic and anaerobic bacteria, and it is related to bacteria in the surface sediments more widely in the studied area (Hu et al. 2006b). PLFA i15:0, a15:0, 15:0, i16:0, 16:1 ω9, 16:1 ω7t, i17:0, a17:0, 17:0, cyl7:0, 18:1 ω7, and cy l9:0 were identified as representative of bacteria (Federle 1986; Tunlid et al. 1989; Frostegård et al. 1993a; Frostegård and Bååth 1996). Variation in their proportions indicated differences in the lipid contributing microbial communities. Similar difference in lipid contributing microorganisms have been observed in bays of Japan (Rajendran et al. 1992a; Rajendran et al. 1993a; Rajendran et al. 1993b;

Rajendran et al. 1997) and near the mouth of an east coast Indian estuary (Harji et al. 2010). As a whole, PLFA were couple of times more abundant in the upper 2 cm than the deeper layers of the core (Rajendran and Nagatomo 1999), indicating the importance of surficial sediments in the biogeochemical cycles. Moreover, these constituents being lighter than the other two constituents of LOM, are more abundant at the surface.

5.3.2. Major microbial groups

A total of 17 PLFA biomarkers including SIF 8 (18:1 ω9t) were identified in CE, 9 of which were bacteria, 3 of fungi, 2 of diatoms and diniflagellates, 2 of Type I and II methanotrophs and 1 of protozoa. The most dominant groups observed at all the four stations throughout the study period were (I) Gram-positive Firmicutes and gram-negative anaerobic bacteria; (II) Gram negative Proteobacteria; (III) sulphate reducers such as *Desulfovibrio desufuricans*; (IV) Arbuscular mycorrhyzal fungi (AMF); (V) Ectomycorrhizal Fungi (EMF); and (VI) Type II methanotrophs. The biomarkers specific for Type I methanotrophs, acidophilic bacteria, microeukaryotes such as diatoms, dinoflagellates and protozoa were observed at some locations. The high proportions of fatty acids ranging from C_{10} to C_{20}, indicated the predominance of prokaryotes in CE. Such dominance of prokaryotes has been reported from other regions also (Guckert et al. 1985; Rajendran et al. 1995a; Polymenakou et al. 2005).

In the marine environment, 90% of bacteria are Gram-negative with different characteristics and the Gram-negative cell wall is better adapted for survival in the marine environment. In the surficial sediment of CE, the

most predominant microbial group was anaerobic gram-negative bacteria and gram-positive bacteria, especially Firmicutes, represented by the biomarker i and ai15:0. Their dominance in deeper layer of saline Qinghai lake as well as anoxic sediments have been reported (Dong et al. 2006). Rajendran et al. (1994) also reported the predominance of anaerobic bacteria and gram-positive prokaryotes, characterized by the high proportions of branched PLFA in the eastern and northeastern sides of Osaka Bay, Japan. Gram-positive and anaerobic Gram negative in Damariscotta river estuary ranged from 0.4 to 1.2% and 1.2 to 3.0% of total fatty acids, respectively (Findlay and Watling 1998).

The second dominant group throughout the CE was gram negative Proteobacteria. Aerobic gram-negative bacteria are characterized by larger amounts of monounsaturated fatty acids (16:1 ω7c) (Ratledge and Wilkinson 1988). Among the detected PLFAs, monounsaturated fatty acids made up a dominant pool in the core samples of East Mediterranean Sea and Barbados Trench, indicating the predominance of aerobic Gram-negative bacteria in the analyzed surface sediments (Guezennec et al. 1996; Polymenakou et al. 2005). Similar dominance have been reported in sub tropical regions (Rajendran et al. 1992a; Rajendran et al. 1993a; Rajendran et al. 1993b; Rajendran et al. 1997) and this was consistent with earlier reports (Zelles et al. 1992; Zelles et al. 1995; Bossio and Scow 1998; Bossio et al. 1998).

The biomarkers specific for SRB group along with genus (*Desulfovibrio*) and species (*Desulfovibrio desulfuricans*) were detected from the sediments in CE during the entire study period. Although the branched

PLFAs i-15:0, a-15:0, i-16:0 and i-17:0 are common to many bacteria including Gram-positive bacteria (Lechevalier and Lechevalier 1988), Gram-negative anaerobic bacteria and sulfate reducing bacteria (Boon et al. 1977; Boon et al. 1978; Edlund et al. 1985; O'leary and Wilkinson 1988; Findlay and Dobbs 1993; Wakeham 1995; Rütters et al. 2002a; Harvey et al. 2006; Widenfalk et al. 2008), the predominance of iso over anteiso appears to be characteristic of the SRB (Dowling et al. 1986). The branched C15 and C17 fatty acids can be attributed to *Desulfococcus multivorans*, whereas 10Me16:0 is characteristic of *Desulfobacter* (Londry et al. 2004). In this study also, the branched iso fatty acids were dominant over anteiso. This indicates that the dominant anaerobic bacteria in CE belonged to sulphate reducers. Harji et al. (2010) has also reported the dominance of SRB group in harbor sediments at Visakhapatnam (east coast of India). The phospholipid fatty acid (PLFA) and lipopolysaccharide hydroxyl fatty acid profiles are consistent with the presence of large numbers of sulfate-reducing bacteria (SRB) in black, sulfide muds surrounding the seeps (Fang et al. 2006). SRB occurrence have been reported from the anoxic environments of the Black Sea Barbados Trench, the Arabian Sea (Wakeham 1995; Guezennec et al. 1996; Wakeham et al. 2002) and also from bays. Rajendran et al. (1992a), suggest the presence of anaerobic bacteria, particularly SRB in marine sediments.

The polyunsaturated fatty acids (represented primarily by 18:2 ω6,9 and 18:1 ω9) are associated primarily with fungi (Federle 1986; Frostegård and Bååth 1996). Marine fungi occupy an important place in ubiquitous organisms, helping in the decomposition and cycling of nutrients. Biomarker

16:1ω5c representing arbuscular mycorrhizae were present at all the four stations in CE, but were less in quantity. This group is mostly reported from soil and metal polluted region (Frostegård and Bååth 1996).

Type I methanotrophs (monoenoic PLFA (16:1 ω8c) was present at 2 stations, indicating their localized distribution in CE. On the other hand, Type II methanotrophs (18:1 ω9t as SIF 8) was present in high abundance (mol %) at all four stations in CE during the entire study period. The cyclopropane fatty acid (19:0 cyclo c11-12) biomarker, specific for acidophilic bacteria was also encountered. In CE, 5 biomarkers (PUFA) of microeukaryotes (Volkman et al. 1980) were recorded, representing ectomycorrhyzal fungi, diatoms, dinoflagellates and protozoa. These group has been reported from bays (Findlay et al. 1990; Rajendran et al. 1992a; Rajendran et al. 1995a). The community was always dominated by aerobic species, but there were also significant occurrence of both fungi and anaerobic bacteria in salt marsh throughout the year (Keith-Roach et al. 2002). In the sediments of CE, there was the predominance of branched and monounsaturated fatty acids during the study period indicating that aerobic gram positive and anaerobic prokaryotes were the dominant groups in the benthic microbial community, a pattern similar to the sub tropical Bay (Rajendran et al. 1994).

5.3.3. Spatial and temporal variation in microbial community

In CE, PCA of PLFA profiles indicated that the microbial community structure varied primarily by location and season. Such spatial and seasonal variations of a microbial community in different ecosystem like estuary, bay,

lake and salt marsh have been reported by very few researchers (Findlay and Watling 1998; Rajendran and Nagatomo 1999; Smoot and Findlay 2001; Keith-Roach et al. 2002). The scores for individual PLFAs showed three clusters and cluster I (wet and transitional periods) expressed maximum diversity (6 groups). Cluster II (dry period) had a great influence on sulphate reducers especially *Desulfovibrio desulfuricans,* and *Arbuscular mycorrhizae.* Aerobic bacteria start multiplying earlier in the year than do anaerobes, as indicated by an increase in aerobic PLFA from spring onwards while the anaerobic biomarkers start increase significantly only from summer to late summer. Also, the amount of aerobic biomarker PLFA increased 7-folds between mid-winter and late summer, as opposed to 4.5 folds for the anaerobes over the same period (Keith-Roach et al. 2002). In CE, SRB were consistently predominant throughout the year and this differed from the earlier reports of seasonality in their abundance (Rajendran et al. 1995a).

Maximum number of microbial groups was observed during post-monsoon season at Munambam station (cluster III). This cluster was influenced by microeukaryotes like diatoms, dinoflagellates and protozoa, ectomycorrhyzal fungi and acidophilic bacteria. One of the reasons for the absence or low occurrence of these communities has been attributed to spatial patterns of reduction-oxidation conditions of the sediment (Rajendran and Nagatomo 1999). Munambam station receives terrigenous inputs from two rivers during the monsoon period and is carried further to the post-monsoon period. Seasonality has been reported for fungal biomass, with a maximum in the late summer months, perhaps due to increased input of

plant detrital material towards the end of the growing season. This finding is consistent with studies of soil microbial communities which often reveal late summer peaks in fungal growth (Doetsch and Cook 1973). Fungi were detectable throughout the year and the amounts and proportions of this group varied between seasons in salt marsh ecosystem (Keith-Roach et al. 2002). The community was influenced by different types of microbial groups in different seasons. Rajendran and Nagatomo (1999) reported that microbial community structure in Hiroshima Bay was dominated by aerobic prokaryotes and eukaryotes in spring, and Gram- positive bacteria in autumn. There was a variation in both size and structure of the microbial community in salt marsh ecosystem and both aerobic and anaerobic bacteria were detected throughout the year and the amounts and proportions of these groups varied between seasons (Keith-Roach et al. 2002). Findlay et al (1998) have studied the patterns of seasonal variation in the structure of a marine benthic microbial community and the annual variation in microbial community structure appeared to be driven, at least in part, by changes in biomass of phototrophic eukaryotes. This was in consistent with the reports from Acton Lake sediments, where increased biomass from algal bloom contributed to total microbial biomass during early spring season (Smoot and Findlay 2001). In Cochin estuary, as the biomass of phototrophic eukaryotes was generally high throughout the season (Madhu et al. 2010) it would not be a major factor to bring about the seasonality in the microbial community.

Spatial variation in the community was less pronounced than the seasonal variation. The aerobic prokaryotes and Gram-negative anaerobes were predominant in pre-monsoon and monsoon seasons whereas in post-

monsoon season, Type I methanotrophs was predominant. However, since there was no great interchange between dominant groups of microbes and no appearance of previously absent groups, the microbial community structure in CE was apparently steady throughout the year.

5.4. Culturable Bacterial Diversity

Heterotrophic bacteria are one of the most important groups of microorganisms active in the decomposition of organic matter. Bottom sediments are the zone where these organisms occur in abundant in particular. To address the processes limiting organic matter cycling in shallow water marine ecosystems, the diversity of the culturable microorganism responsible for carrying out these various processes must be identified and characterized. Studies in the early seventies have concentrated on the distribution, taxonomy and cytology of these culturable marine bacteria using traditional method. The first report of Oliver and Colwell (1973) contained the phospholipids content of 20 marine and estuarine bacteria. Since then, difference in composition of FA in various genera is being used to differentiate most of the marine bacteria. FAME analysis became a valuable standard and routine phenotypic marker in taxonomic studies for characterizing pure cultures (Guckert et al. 1991; Vainshtein et al. 1992; Sasser 1997; Yamamoto et al. 1998). Descriptive patterns of prominent ester–linked PLFA recovered from isolated microbes grown on standardized media have been developed for over 4500 species (Microbial Identification System, MIDI, Newark, DE) and are regularly updated in MIDI Library. The culturable bacterial diversity of CE was

identified based on this library. In this study, bacteria belonging to copiotrophs, oligotrophs, RC-LD_{SAT} and RC-LD_{UNSAT}, were retrieved to partly overcome, the inadequacy of selective growth of marine bacteria. And identification based on FAME has been shown to provide a reliable estimate of the microbial composition.

The microbial ecology of shallow water sediments is not well understood and the culturable microbial diversity is also poorly characterized. A few studies from India, have attempted to characterize microorganisms to the generic species level and these studies were from Mandovi-Zuari estuary (Divya et al. 2009; Khandeparker et al. 2011) Cochin estuary (Jose et al. 2011) Palk bay (Nithya and Pandian 2010) Visakhapatnam (Clark et al. 2003). A few other studies were on either certain groups or functionally associated groups in mangrove and intertidal beach (Krishnan 2009; Fernandes 2010; Fernandes 2011; Rodrigues et al. 2011b). In CE, the culturable bacteria were affiliated to 4 phyla. The dominant phylum was Gram-positive Firmicutes followed by Proteobacteria. PLFA approach also showed the dominance of Firmicutes community in CE. The occurrence and dominance depended on the habitat and region. For instance, Firmicutes and Proteobacteria are ubiquitous in sediments and reported from Palk Bay (Nithya and Pandian 2010) and intertidal sand of Goa and Ratnagiri coasts (Fernandes 2011) as well from Tyrrhenain sea sediment (Ettoumi et al. 2010), Sub- arctic river sediment in Northeastern Ice (Markúsdóttir et al. 2012), Dapeng Bay of South China (Jiang et al. 2010) etc.. Similarly, the dominance of Firmicutes in the coastal and deep sediment of the Arabian Sea and shallow lakes have also been reported (Dang et al.

2009; Divya et al. 2010). In other regions like Pacific, the dominant phyla was Proteobacteria (DeLong et al. 1997; Musat et al. 2006; Kobayashi et al. 2008). This dominance and occurrence were seen also at the class level. Among the classes, Bacilli were predominant followed by γ-Protoeobacteriae. Limited number of studies focusing on sediment microorganisms have detected a mixture of both aerobic and anaerobic microbial groups, many of which fall within the Proteobacteria (Llobet-Brossa et al. 1998; Hunter et al. 2006). Functionally versatile and physiologically adaptable genera like *Vibrio*, *Pseudomonas, Acinetobacter* under γ-Proteobacteria and *Flavobacteria* of phylum Bacteroidetes were commonly found in CE. Among the γ-proteobacteria the dominant genera were *Aeromonas* and *Shewanella* in CE. Among the β- proteobacteria, the genera *Neisseria* and *Acidovorax* were dominant In CE whereas in sandy beaches *Alcaligenes* was dominant (Fernandes 2011). Such variations have been reported earlier from various habitats of different regions (Krishnan 2009; Fernandes 2010; Jiang et al. 2010; Nithya and Pandian 2010; Das 2011; Fernandes 2011; Khandeparker et al. 2011; Markúsdóttir et al. 2012). The Flavobacterium group was also detected in the present study. These gram-negative bacteria which are taxonomically reclassified into the subgroup Bacteroidetes, are specialized in the degradation of complex macromolecules (Reichenbach and Dworkin 1992) and are also adapted to low nutrient levels (Höfle 1983; Stoeck et al. 2002). In CE, there were seasonal and spatial variations not only in number and abundance of major groups, for example at Barmouth the abundance of Firmicutes and Proteobacteria were 73% and 23%, whereas at Vaduthala these were 85%

and 11%, respectively. Temporally variation of Firmicutes at Barmouth was 93%, 59% and 76% during pre-, monsoon and post-monsoon periods, respectively. In the case of species also, such spatial and temporal variations were seen (Tables 4.14 - 4.20). Variations in the dominance of the groups was due to the local difference in topography (influenced by tidal and terrestrial input), hydrobiology (high nutrients and DO) and geochemistry of sediment (repository of organic matter) of the stations, which is a common feature of tropical estuaries (Qasim 2003). These variables dictated the presence of heterotrophic bacteria of α, β, and γ-Proteobacteria and the Flavobacterium/ Bacteroides throughout the study period. Heterotrophic bacteria plays an important role in the decomposition of organic matter at the surficial sediment in CE as protein decomposition is done by proteolytic *Pseudomonas* and other eubacteria, chitin by chitinolytic or chitinoclastic *Bacillus*, *Pseudomonas* and *Vibrio* etc. Further, being a eutrophic shallow estuary, aerobic metabolic processes (microbial activity) dominate the community metabolism (Guezennec and Fiala-Medioni 1996; Polymenakou et al. 2005), due perennial sedimentation of labile organic matter in water-sediment interface.

5.5. Factors affecting the community structure

Microbial communities are highly diverse in coastal oceans and respond rapidly with changing environments, which are often involved with variations of nutrient contents in sediments and near bottom waters (Van Duyl et al. 1993). CE is a monsoonal driven eutrophic tidal estuary into which

drains two large rivers with varying input of fresh water (Qasim 2003). The environment change in the estuary is often cyclic in nature and is caused by seasonal changes in the geochemistry of the surficial sediment. Changes in the microbial community structure are brought about by physical, chemical and biological factors (photrophic biomass and grazers) (Rajendran and Nagatomo 1999).

PCA and Redundancy analysis showed that the wet and transition seasons had influenced community diversity at all the stations except Munambam. Among the communities, bacteria were influenced the most. The positive loading along PCA factor 1 was driven by Gram positive Firmicutes and Gram negative anaerobes especially SRB. The microeukaryotes had negative loading on PCA factor. The variations in loading are the consequence of the direct influence of variable on the community change or the cascading effect of the other environment variables.

The composition and abundance of the microbial diversity is expected to vary depending on the quantity and quality of organic matter manufactured by the primary producers (Zelles et al. 1992; Borga et al. 1994; Bååth et al. 1995; Bossio et al. 1998). Among the different components of organic matter, lipid content of sediment had influenced the community structure at Barmouth and Vaduthala stations during the three seasons. Whereas carbohydrate and protein contents in sediments were found to influence the community at Barmouth, Vaduthala and Arookutty stations during monsoon and pre-monsoon seasons. At Arookutty station, TOC and TON was influenced during pre- and post-monsoon seasons.

These observations clearly demonstrated that in CE, the influence of organic matter on the community depends not only on the quality and quantity of the matter but also on the location and the season.

Studies have revealed that a higher relative abundance of aerobic gram-negative bacteria occurred with higher inputs or availability of organic substrates and decreased with flooding and other anaerobic conditions (Zelles et al. 1992; Zelles et al. 1995; Bossio and Scow 1998; Bossio et al. 1998). Bacteroidetes were found during monsoon period when there was high algal input to the sediment. High algae-based aggregates association was seen with Bacteroidetes group (Tang et al. 2009). High abundance of Type I Methanotrophs associated with anaerobic bacteria may be because of the availability of nutrients as carbon source. Another factor that would have influenced its occurrence may be pollutants. Type II Methanotrophs were present at all stations and all these stations have pollution impact. In contaminant plumes, the reduced conditions near the pollutant source are often associated with PLFAs indicative of sulfate-reducing bacteria (Albrechtsen et al. 1995; Pfiffner et al. 1997; Sundh et al. 1997; Ludvigsen et al. 1999). The co-existence of Methanotrophs and sulfate reducers in the boreal peat land soil in Sweden has been reported near the contaminant source due to depletion of other electron acceptors (Madsen et al. 1991). Such association may be the fact that obligate anaerobes are able to survive in presence of oxygen (Fukui and Takii 1990; DeLaune et al. 1995) and the ability of Methonotrophs to tolerate anaerobic conditions for extended periods (Roslev and Iversen 1999). The pollutant effect (Rajendran et al. 1992a) and reduced availability of oxygen in the overlying waters of

sediments and existence of anoxic conditions in the sampling area may have caused decrease in the microeukaryotes and aerobic bacteria and the increased in SRB and other anaerobes. Cis isomers of monounsaturated PLFA are commonly present in microbial cellular components (Guckert et al. 1987; Findlay et al. 1990; Kieft et al. 1994; Fang et al. 2004) but presence of trans isomers a marker associated with stress reflects survival strategies of the organisms during physiological stress (Guckert et al. 1985; Guckert et al. 1986; Nichols et al. 1986; Guckert et al. 1987). In CE, the community seems to be partly under stress as this marker was detected at all the four stations. Harji et al. (2010) have reported that tran-monounsaturated PLFA was one of the major PLFAs in the sediments of the Visakhapatnam harbour. It may be because the harbours are subjected to pollution due to municipal sewage, factory discharge, sulfur and petroleum products (Sarma et al. 1982; Kadam and Bhangale 1993).

The dominant anaerobic bacteria were sulphate reducers. It can be postulated that anaerobes and SRB occurrence may be due to the prevailing hyoxic condition associate with water–sediment interface (the redox boundary) brought about by the occasional low flushing during dry season (Srinivas et al. 2003) and the prevailing stratification that prevents the renewal of the bottom waters with oxygen. Harji et al. (2010) has also reported the dominance of SRB group in harbor sediments at Visakhapatnam (east coast of India). According to them the predominance of these PLFAs may be due to the anoxic conditions that were prevailing at most of the stations due to constant stagnation of the water (Sarma et al. 1982; Kadam and Bhangale 1993). Another factor that influenced the SRB

may be the high input of 0.26 × 10^3 $m^3d^{-}1$ of domestic waste (CPCB 1996). These effluents (anthropogenic inputs) with abnormal polluting properties such as high temperature, high chlorinity, alkalinity, suspended matter, COD, BOD and high ammonia have contributed to phenomenal regional diversity in CE (Jayaraj et al. 2008).

In CE, the occurrence of *Arbuscular mycorrhizae* was influenced by pH as the bottom water pH varied between 6.2 and 8.12. The relatively drop in pH from neutral value and the presence of lime (calcium carbonate) in the sediments (Balachandran 2004) had facilitated the occurrence of this fungus. Yeast and moulds tend to predominate at lower pH and in limed (high pH) soil (Frostegård et al. 1993a). Baath et al. (1995) reported a decrease in fungal PLFA with increasing in pH. Temperature influenced Type I methanotrophs and SRBs along with TOC, TON and nature of the sediment. This influence was more prominent during monsoon and post-monsoon period when maximum temperature variability was observed. In sub tropical sediments, temperature varied the abundance of anaerobic bacteria and fungi during the warm-water months (Rajendran and Nagatomo 1999). Temperature had less influence on the community unlike the subtropical and temperate regions (Findlay et al. 1990). Most of the anaerobes were influenced by organic content and the water variables esp DO, NO_3 and PO_4. The presence of SRB would have made the environment conducive for the flourishing of other anaerobic bacteria as sulfide would have partly come from organic matter (rich in organic matter) but also from bacterial reduction of sulphate. Rajendran et al. (1994) also reported the predominance of anaerobic bacteria and gram-positive prokaryotes, where high

concentrations of pollutants have been reported. The presence of anaerobic bacteria in oxic areas may be due to microniches of anaerobic conditions formed by the microbial activity of bacteria (White et al. 1979). The microeukaryotes had a more irregular distribution in the sediments. Similarly, the irregular distribution was seen in organically contaminated and oxygen-depleted environment (Smith et al. 1986; Findlay et al. 1990; Rajendran et al. 1992b) and in mud flat estuary, Apalachee Bay, Florida (White et al. 1979). In CE, the microeukaryotes was also influenced to a lesser (low correlation) by TC.

Overall, the quantity of particulate and organic matter inputs and the distribution of these inputs were the crucial factors which controlled the structuring of the sediment communities of Cochin estuary. A combination of lipid and percentage of sand in the sediment regulated the microbial community which was influenced by the hydrodynamics of the estuary. Anthropogenic inputs also exerted a substantial influence on the community.

CHAPTER 6

SUMMARY AND CONCLUSION

In the estuarine and coastal systems, organic matter (OM) is derived not only from autochthonous primary production, but also from allochthonous (terrestrial) organic matter (OM) delivered by river discharge and runoff. A significant portion of the OM sinks through the water column and is ultimately stored in carbon pool in the sediments. The combined flux of this OM to estuarine and coastal sediments forms the base of a diverse food web and is an important factor in determining the benthic biomass. Microorganisms play a vital role in the spatial variability of the deposited OM, transformation and degradation. Microbial activity within the estuary has distinct affect both in the chemical conditions of the estuary and the behavior of the elements within the estuary. Hence changes in the microbial community structure can result in geochemical changes. Therefore, the central paradigm for understanding the microbial processes in the estuarine ecosystem is the study on microbial community. This area has been rarely explored, in spite of having a direct implication in sustainable management of the estuary as it can provide useful and important information in addressing environmental issues. The thesis on "**Spatial and Temporal Variation of Microbial Community Structure in Surficial Sediments of Cochin Estuary**" is an attempt in understanding the structure of the benthic microbial community and its interaction with environmental parameters with special reference to bacterial community. In this study, PLFA profiling of sediment samples give the structural composition attributes of microbial communities along with the spatial and seasonal dimensions.

As the main **objective of** this study was to establish the spatial and temporal variation in benthic microbial community structure of Cochin estuary, it was necessary to utilize an analysis which looks at the communities "*in situ*" rather than at only cultured fraction that represent only a small percentage of the extant micro- biota. The use of this biochemical method helps to eliminate the bias from incomplete release of organisms from the sediments, the necessity for growth, and the variance created by subsampling the homogenized sediment samples for enumeration. These methods also can be adopted with a high level of reproducibility and generate a fingerprint of signatures that can be used to define the microbial community. PLFA provides quantitative measures of the abundance and distribution of viable microbes, composition and also insight into the nutritional and physiological status of the community. Although no single approach could provide a complete depiction of microbial characteristics, each one of the approaches provides a slightly different perspective, and with the use of multiple approaches, a more complete representation of microbial characteristics can be achieved. This information, combined with data on the population of microorganism from dilution platings, can enable a more comprehensive assessment of microbial community. In this study, culture dependant FAME identification of the bacteria was mainly carried out to understand the finer composition of the community. The environmental parameters of the surficial sediment and overlying water column, from 4 locations in Cochin estuary covering three seasons, were investigated to delineate the governing factors of the community. Munambam and Vaduthala stations are situated in the northern part and Barmouth and

Arookutty stations are more towards the southern part of the estuary. Vaduthala and Arookutty are located in the upstream region whereas Munambam and Barmouth are in downstream region and opening into the sea. The depths of the stations were less than 5 m, except the Barmouth which has a depth of 10 m.

To delineate the governing factors on the community, physio-chemical parameters of the water column (surface and near bottom) as well as geochemical characteristics of the surficial sediment were estimated. Ambient physico-chemical parameters like temperature, pH, salinity, dissolved oxygen and nutrients (ammonium, nitrite, nitrate, phosphate and silicate) of the water column were measured. Bulk sediment properties like texture (sand, slit and clay), organic matter (TOC and TON), and labile organic matter ie the total of carbohydrate (CHO), protein (PRO) and lipid (LIP) were measured using standard techniques.

The bacterial abundance in the sediment is affected by the changes in the hydrodynamic conditions and the geochemistry of the sediment. Since these changes are linked to the community structure and their metabolic potential, direct total (TC), viable (aerobic (TVC-A) and anaerobic (TVC-An) microbial counts, heterotrophs [copiotrophs (RCC), and oligotrophs (RCO)] and lipid degraders [saturated ($RC\text{-}LD_{SAT}$) and unsaturated ($RC\text{-}LD_{UNSAT}$)] were enumerated in the sediment. TC and TVC were estimated using AODC method. RCC and RCO were estimated by culturing in 100% nutrient medium (NA) and 10%NA. ($RC\text{-}LD_{SAT}$) and ($RC\text{-}LD_{UNSAT}$) were enumerated in NA medium amended with 1% of tween 40 and 80 respectively.

Determination of the composition of the complex surficial microbial community which largely drives the biogeochemical cycles and fertility of the sediment still remains the most daunting task to accomplish. Signature fatty acids (PLFA) is a reliable tools in the ecological investigations of sediments as it overcomes the problem of selective culturing while providing a relatively unbiased view of the community structure. PLFA method was used for quantitative estimation of microbial community structure and for the spatial and temporal pattern of their distribution. The diversity of the culturable microorganism responsible for carrying out these various processes was identified and characterized. FAME profiling of the bacteria was also used to understand the finer composition of the culture dependant bacterial diversity.

In order to understand the interrelationships among the variables, the data sets were also subjected to various statistical analyses using the software XLSTAT 2012, Primer 6 and CANOCO 4.5

The highlights of the results are listed below**:**

Environmental Study

- The salinity in CE ranged from 0.11 to 34.55 psu and there was no significant difference in salinity between surface and near bottom water except at Barmouth. During the study period, a tropical monsoonal pattern was observed in salinity distribution. Salinity dropped substantially during the monsoon especially in the upper region of the estuary.
- Near bottom water was oxic in nature during most of the time with occasional hypoxic condition at Barmouth and Arookutty during monsoon

and post-monsoon seasons. This occasional hypoxic condition seen in CE would have been caused by both physical stratification due to increased fresh water influx and biological parameters enhanced by OM production triggered by high nutrient input.

- Nutrients were generally high at all stations throughout the study period classifying it as a eutrophic estuary
- The sediment texture comprised mainly of sand, silt and clay. The percentage of sand was high at all the stations except Arookutty and seasonal and spatial grading of particles was observed. The variation in texture may be attributable to hydrological variables such as riverine input and tidal influx.
- The TOC concentration ranged between 0.79 and 3.52% and was comparatively high towards the mouth of the estuary. TON concentration was low and was one order less than TOC. Low C:N ratio (6.5 – 16.2) indicates the presence of freshly deposited organic matter and reduced vascular inputs in the estuary. High C: N at Munambam and Vaduthala in the northern estuary, may be due to either a shift in organic matter input from different sources (fresh autochthonous and alllochthonous (labile and refractory) or due to low retention of organic carbon in the sandy sediments. In CE most of the organic matter was within the utilizable range for bacteria.
- Nutritional value of the labile components-LOM (carbohydrate- CHO, lipid –LIP, and protein-PRO) of the organic matter fraction reflects the potentially available substrate for the organisms. The LOM concentration in the sediment ranged from 0.78 to 2.22 mg g^{-1} which was ~0.001% of

the TOC of the sediment. Total LOM showed temporal variation, which may be attributed to seasonal changes in phytoplankton productivity and community structure. The proportion of CHO: PRO: LIP was 5:1:1. The dominance of PRO to CHO in CE is a typical characteristic of highly eutrophic environments. CHO and PRO showed temporal and spatial variation and were positively correlated. The variability in LOM concentration was due to the variability in these two components. The variations may be due to the variation in input or due to microbial degradation of these labile components of organic matter at the sediment surface (re-mineralization and regeneration of inorganic carbon and nutrients), accounting for the high nutrient present in CE.

- Cluster analysis based on environmental variables of the near bottom water and sediment grouped spatially distant stations during different seasons in the same clusters suggesting that in CE at a particular season distantly located stations had the same environment characteristics. Thus clustering was season driven rather than station. Geochemical parameters had a greater influence than near bottom water characteristics on the grouping of stations as stations remained within the same cluster when water parameters were excluded from the analysis.

Microbial Abundance study

- The average TC, TVC-A and TVC-An bacterial densities in CE were 3.99 $x10^9$, 4.5 $x10^5$ and 4.57 $x10^5$ cells g^{-1}, respectively. The abundance was consistent throughout the estuary as no significant spatial and temporal variation were observed. This may be because TC is a reflection of the

balance between the longer processes of growth (uptake though abundance) and mortality (grazers and cell lysis). This is because there is net balance between production and loss and this value is quite conservative over several ecosystem. TC was limited by CHO and LIP. This limitation shifts when all the variables are taken into consideration (Redundancy analysis).The influence of CHO and LIP was reduced. TC count was more influenced by protein and to a lesser extent by TOC which was very distinct at Arookutty station and the order of influence was monsoon>pre-monsoon>post-monsoon.

- TVC-A and TVC-AN were positively correlated suggesting either interdependence (syntrophic relation) among these fractions or the same variables were controlling their abundance or there could be overlap due to facultative microbes. Since these two fractions were not limited by any geochemical variables (correlation analysis), the interplay between the two groups determine the abundance. Community analysis based on PLFA also supported the occurrence of these two communities in the estuary. TVC was positively correlated with near bottom NO_3. This relationship may be due to the co variable effect of nitrate on the productivity of water column. Maximum TC and TVC were observed during the post-monsoon season (transitional period).
- The abundance of retrieval copiotrophic counts(RCC) represents only a small fraction (0.1% ± 10%) of the active microbial community and it was 2-3 order less than TC commonly observed in all the stations. RCO was in the same order as that of RCC and this implies that a considerable fraction of sedimentary bacteria of the estuary is adapted to the trophic

gradients seen in the estuary. The distribution of RCO was monsoon driven and high numbers were recorded in pre-monsoon and monsoon seasons.

- The presence of high biomass of saturated and unsaturated fatty acids degrading bacteria at all the stations (equal to RCC 10^5 CFU g^{-1}) suggests a constant inputs of lipids (combined) from terrestrial and marine algae. Being a shallow eutrophic estuary, labile form of lipid is deposited in the surficial sediment. The presence of low and almost uniform distribution of lipid in the sediment points to 1) uniform distribution brought about by tidal flux, 2) dominance of sand fraction as evidenced by its positive relation with lipid and 3) active microbial metabolism of lipids as suggested by RDA analysis which showed the positive influence on lipid degraders (Figure 4.13).
- The high abundance of all fractions of bacteria showed relationship with each other and was influenced by the geochemistry of the sediments (RDA) reiterating that sediment-water interface of CE is an active diagenetic zone associated with active microbial community.
- RDA analysis of geochemical factors with the abundance, improved the factors affecting the abundance. The total variation in abundance and geochemical variables were explained with cumulative 64.8%.The first canonical axis accounted for 29.4% of the variance and second axis accounted for 26.9% of variance. RDA clearly showed that variability of TVC was independent of geochemical characteristics' of the sediment. The RCO counts were highly influenced by silt and clay texture and distantly influenced by TON and TOC. Based on RDA analysis the four

stations were grouped into two. The first axis brought about variation in Barmouth and Munambam stations. Barmouth and Munambam stations were very similar and were influenced only by sand and LIP. Arookutty and Vaduthala were mainly influenced by TC, RCC and RCO and each of these was differently influenced by TOC, TON, CHO, PRO, silt and clay.

- Microbial degradation influenced the geochemistry of the sediment water interface, which in turn was influenced by the physical, chemical and biological processes of the estuary. High abundance of heterotrophic bacteria in CE would have contribute to the degradation process. Tidal mixing, high precipitation and riverine drain would have re-distributed the organic matter and enhanced the rate of microbial degradation.

Microbial Community study

- A total of 33 fatty acids were identified from the surficial sediment samples, including PUFA, monounsaturated (MUFA), and saturated and branched fatty acids.
- The community comprised of 12 groups. Regional variation in the community was less pronounced than the seasonal variation.
- The PLFA profiles in the sediments of Cochin estuary showed dominance of prokaryotes over microeukaryotes. Among the prokaryotes, Gram positive aerobes especially Firmicutes and gram negative anaerobes especially sulphate reducers (SRB) were dominant. Microeukaryotes were minor components of the community, represented by Ectomycorrhyzal fungi, diatoms, dinoflagellates and protozoa.

- The dominant anaerobic bacteria were SRB. SRB group along with genus (*Desulfovibrio*) and species (*Desulfovibrio desulfuricans*) were present during the entire study period. There occurrence may be due to the anthropogenic effect being experienced in the estuary (cultural eutrophication) or due to microniches of anaerobic conditions formed by the microbial activity.
- Type II Methanotrophs were present in high abundance (mol%) at all the four stations during the entire study period.
- Type I Methanotrophs was present only at Barmouth and Arookutty stations whereas acidophilic bacteria were recorded only at Munambam station.
- The variability in the distribution of microbial communities differed from station to station depending on the seasons. There were stations which had the same number of communities and species within the community. Maximum number of groups was isolated during pre- and post-monsoon period. Dry period had a great influence on sulphate reducers especially *Desulfovibrio desulfuricans*, and Arbuscular mycorrhizae in CE.
- The diversity of the culturable fraction of the community comprise of 4 phyla, 6 classes, 41 genera and 84 species. The species diversity was comparatively high at all stations during the entire study (H' >2.0). Maximum number of species (19) was found in Bacilli. About 27 species were present at a particular station and in a particular period. The predominance of Pseudomonas and Bacillus shows that these groups play an important role in the degradation of organic matter

- Significant relationship between sedimentary parameters and community revealed that these parameters play an important role in the distribution of the microbial community structure. Hydrodynamic conditions intimately affect the structure of microbial communities. The main abiotic players were salinity, DO, nutrients (NO_3, PO_4 and SiO_4), sediment texture and organic carbon (TOC and LOM). The low levels of polyunsaturated fatty acids (PUFA) in CE suggest that fatty acids derived from algae were effectively recycled. Therefore, it can be speculated that bacteria within the sediments mainly utilize a labile pool of organic matter derived from algae for their growth.
- Among the different fractions of bacteria, TC was centrally placed on the canonical axis in the RDA triplot and substantially influenced all the communities. Intermediate influence on the community was brought about by the heterotrophic bacteria. The occurrence of micro eukaryotes depended on the viable bacteria.
- The community was also influenced by anthropogenic factors as community stressed biomarkers (18:1 ω9t and 19:0 cyclo) were detected in the sediment.

GENERAL CONCLUSION

The following conclusions can be drawn regarding the distribution of microbial communities in Cochin estuary sediments.

1. Analysis of spatial and temporal variation in benthic microbial community of a tropical estuary was conducted for the first time using non selective measures that affirms that PLFA approach is a sensitive and reliable

method in determining microbial community structures of surficial sediments of estuary.

2. The close relationship between the concentrations of the microbial fatty acids and total biomass indicates that bacteria could account for the largest proportion of the biomass in the sediments.

3. This is first study that has documented the changes in microbial community composition linkage to biotic and abiotic variables in benthic estuarine ecosystem. This contemporaneous community will be the backdrop for understanding the response of autochthonous community to increasing anthropogenic stress.

FUTURE PLAN

- It is conceivable that this microbial community composition might be a useful indicator of the changes occurring in the vulnerable Cochin estuary. However, being a very complex ecosystem, it is expected that this or similar combinations of techniques will provide only limited information. The application of 454 pyrosequencing technique could provide yet another aspect of understanding of bacterial diversity of Cochin estuary. Further additional information on the functional relationships among the members of this community would elucidate the biogeochemical processes in the estuary.

- Natural populations exist in localized patches and rarely exhibit a random or uniform distribution in the environment. To characterize the microbiota over the extent of a habitat, the sample must be large enough to

eliminate variations caused by this patchiness, but still small enough to be manageable. The size of the samples and the pattern in which they are taken, are of significance in understanding the community. This work has to be further extended over a smaller scale both spatially and temporally. Test systems for extrapolating the fate and effects of anthropogenic inputs from the field to the laboratory should be included for a more holistic appreciation of the Cochin Estuary.

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Appendix

APPENDIX

BACTERIAL PARAMETERS

MEDIA Readymade Hi-media (Mumbai) was used.

- **Nutrient Agar (100%)**

Peptone	-	5.0 g
Beef extract	-	3.0 g
Sodium chloride	-	5.0 g
Agar	-	15.0 g
Aged seawater	-	500ml
Distilled water	-	500ml
Final pH	-	7.3 ± 0.2

- **Nutrient Agar (10%)**

Peptone	-	0.5 g
Beef extract	-	0.3 g
Sodium chloride	-	0.5 g
Agar	-	15.0 g
Aged seawater	-	500ml
Distilled water	-	500ml

- **Nutrient Agar amended with Tween 40**

Peptone	-	5.0 g
Beef extract	-	3.0 g
Sodium chloride	-	5.0 g
Calcium chloride (fused)	-	0.1g
Tween 40	-	10ml
Agar	-	15.0 g
Aged seawater	-	500ml
Distilled water	-	500ml
Final pH	-	7.0 – 7.4

- **Nutrient Agar amended with Tween 80**

Peptone	-	5.0 g
Beef extract	-	3.0 g
Sodium chloride	-	5.0 g
Calcium chloride (fused)	-	0.1g
Tween 80	-	10ml
Agar	-	15.0 g
Aged seawater	-	500ml
Distilled water	-	500ml

- **Soyabean Casein Digest Agar (SCDA)**

Casein enzyme hydrolysate	-	15.0 g
Peptic digest of Soyabean meal	-	5.0g
Sodium chloride	-	5.0 g
Agar	-	15.0 g
Distilled water	-	1L
Final pH	-	7.3 ± 0.2

STAINS/SOLUTIONS

- **Acridine Orange**

Acridine orange	-	0.1g
Formalin (5%)	-	100ml

- **Yeast Extract (1%)**

Yeast extract	-	0.1g
Distilled water	-	10ml

Dissolve and dispense in different vials, sterilize by autoclaving at 121^0C (15lbs) for 15 minutes and store at 4^0 C in a refrigerator

- **Antibiotic Cocktail**

Nalidixic acid	-	8mg
Piromedic acid	-	4mg
Pipemedic acid	-	4mg
Saturated NaOH solution	-	50µl
Distilled water	-	10ml

Dissolve the antibiotics separately in saturated sodium hydroxide solution and mix with water. Sterilize by filtration through 0.22µm pore size filter and store in autoclaved vials at 4^0 C in a refrigerator.

- **Sodium sulphide (Na_2S) (0.5%)**

  Sodium sulphide - 0.05g

  Distilled water - 10ml

  Dissolve and dispense in different vials, sterilize by autoclaving at 121^0C (15lbs) for 15 minutes and store at 4^0 C in a refrigerator.

- **Buffered formalin**

  38% Formalin - 100 ml

  Hexamin - to saturation

  Filter through 0.22µm pore size filter and store at room temperature.

REAGENTS

Protein estimation

- **Reagent A**

  Sodium carbonate (Na_2CO_3) (2%) - 2g

  NaOH (0.1N) - 0.4g

  Distilled water - 100ml

- **Reagent B**

  Copper sulphate ($CuSO_4$) - 0.5g

  Sodium potassium tartarate - 1g

  Distilled water - 100ml

- **Reagent C**

  Reagent A - 50ml

  Reagent B - 1ml

- **Folin's Reagent (1N)**

  Folin's reagent (2N) - 5ml

  Distilled water - 5ml

Lipid estimation

- Potassium dichromate ($K_2Cr_2O_7$) - 0.75 g

  Distilled water - 10ml

  Conc. Sulphuric acid (H_2SO_4) - 500ml

- **Organic solvent**

  Chloroform **-** 100ml

  Methanol - 200ml

  Distilled water - 80ml

Carbohydrate estimation

- **5% phenol reagent**

  Phenol (powder) - 5 g

  Distilled water - 100ml

- **5% TCA**

  Trichloroacetic acid (TCA) powder - 5 g

  Distilled water - 100ml

Reagents for MIS

- **Reagent 1 - Saponification Reagent**

  Sodium hydroxide (certified ACS) - 45 g

  Methanol (HPLC grade) - 150 ml

  Deionized distilled water - 150 ml

  Combine water and methanol. Add NaOH pellets to the solution while stirring. Stir until the pellets dissolve.

- **Reagent 2 - Methylation Reagent**

  6.00N Hydrochloric Acid - 325 ml

  Methanol (HPLC grade) - 275 ml

  Add acid to methanol while stirring.

- **Reagent 3 - Extraction Solvent**

  Hexane (HPLC Grade) - 200 ml

  Methyl tert-butyl ether (HPLC Grade) - 200 ml

  Add the MTBE to the hexane and stir well.

- **Reagent 4 - Base Wash**

  Sodium hydroxide (certified ACS) - 10.8 g

  Deionized distilled water - 900 ml

  Add NaOH pellets to the water while stirring. Stir until the pellets are dissolved.

- **Additional Reagents**

  Saturated NaCl: Dissolve 40g ACS NaCl in 100 ml distilled water.

Reagents for PLFA analysis

- **50mM phosphate buffer**

  Dipotassium phosphate (K_2HPO_4) - 8.7g

  Distilled water - 1L

  pH - 7.4

- **0.2 M Methanolic KOH**

  Potassium hydroxide (KOH) - 4.5 g

  Methanol (HPLC grade) - 400ml

- **1M Acetic acid**

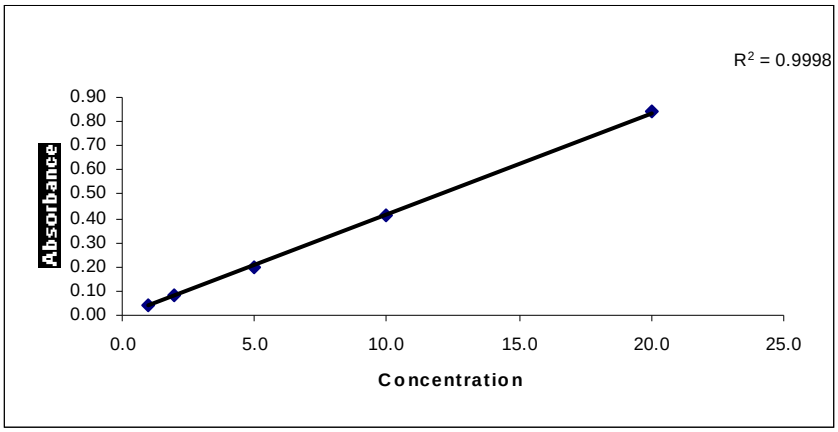
  Acetic acid (99.5% glacial) - 5.7ml

  Distilled water - 100ml

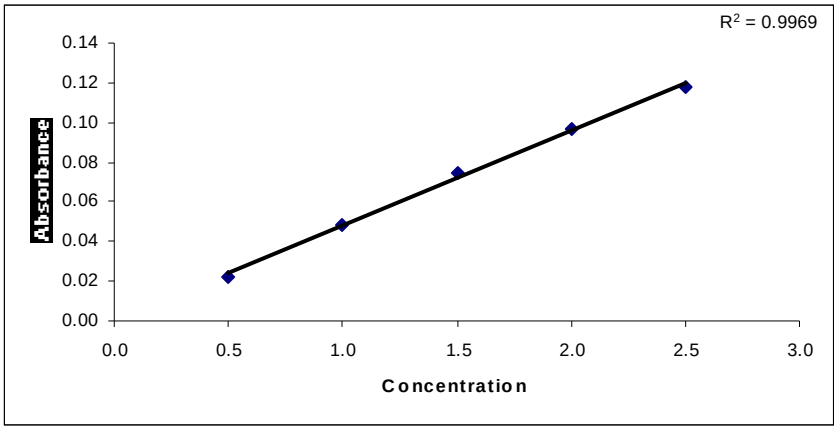
STANDARD GRAPHS

Nutrients

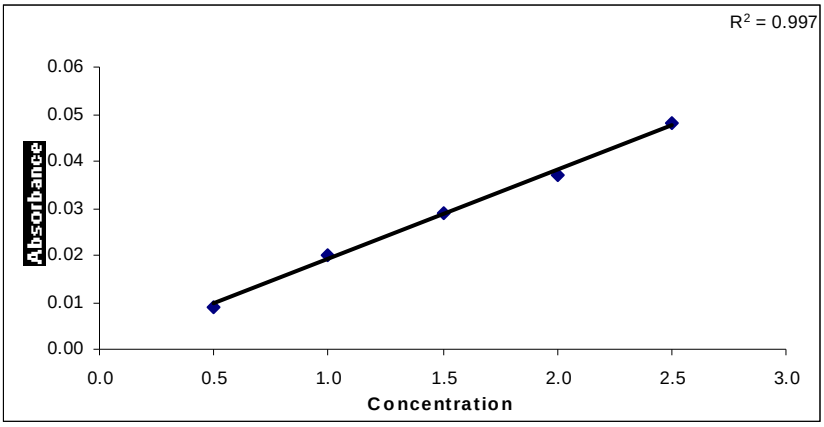
1. Nitrate



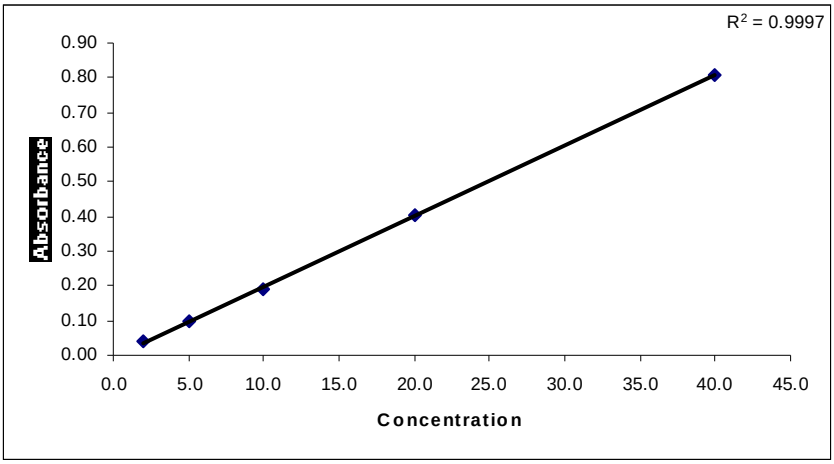
2. Nitrite



3. Phosphate



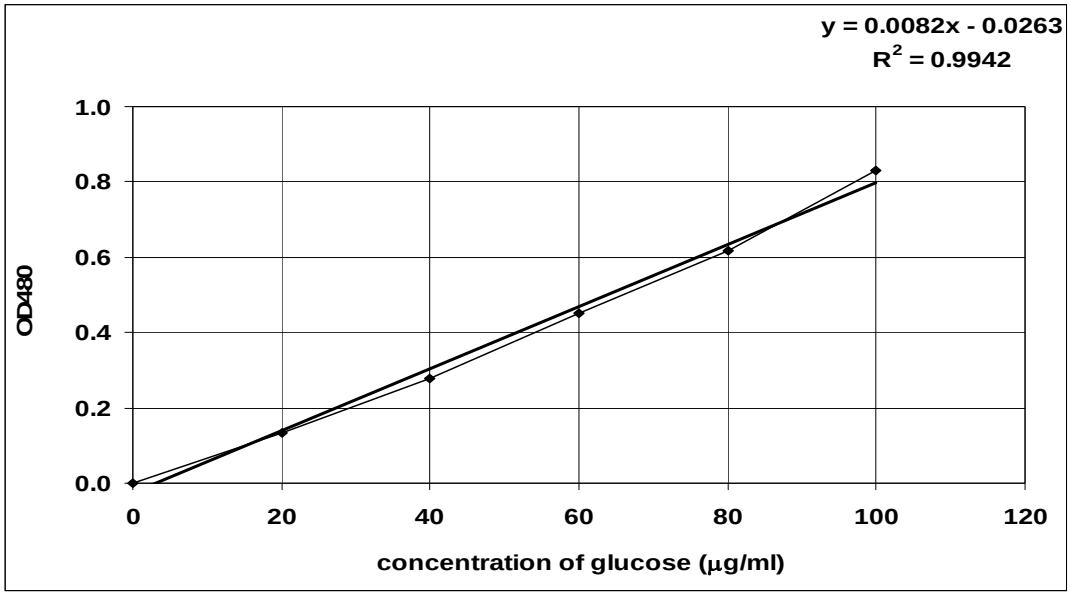
4. Silicate



Labile Organic Matter (LOM)

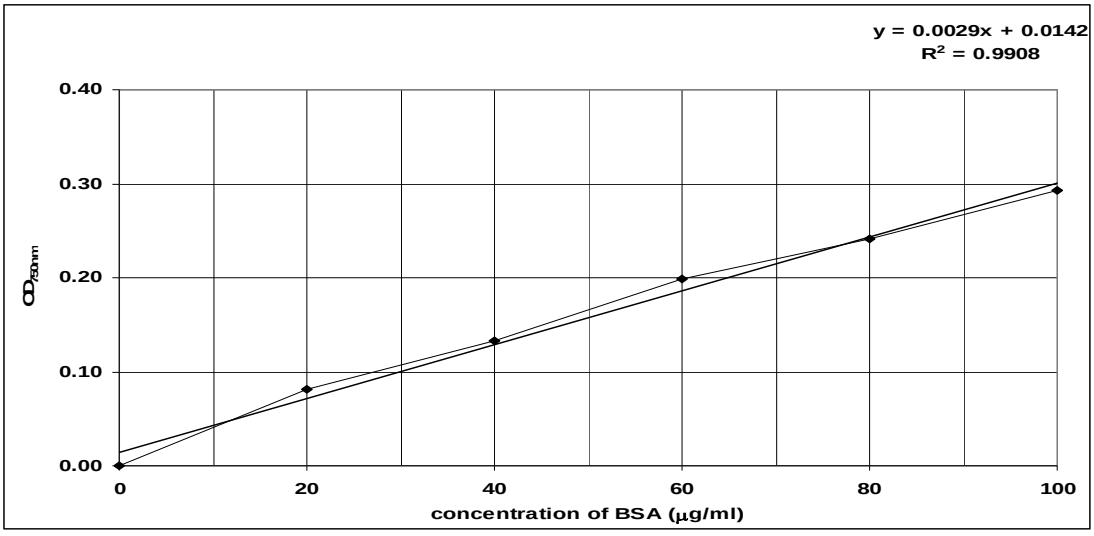
Carbohydrates

Standard Curve for carbohydrate estimation using glucose as standard



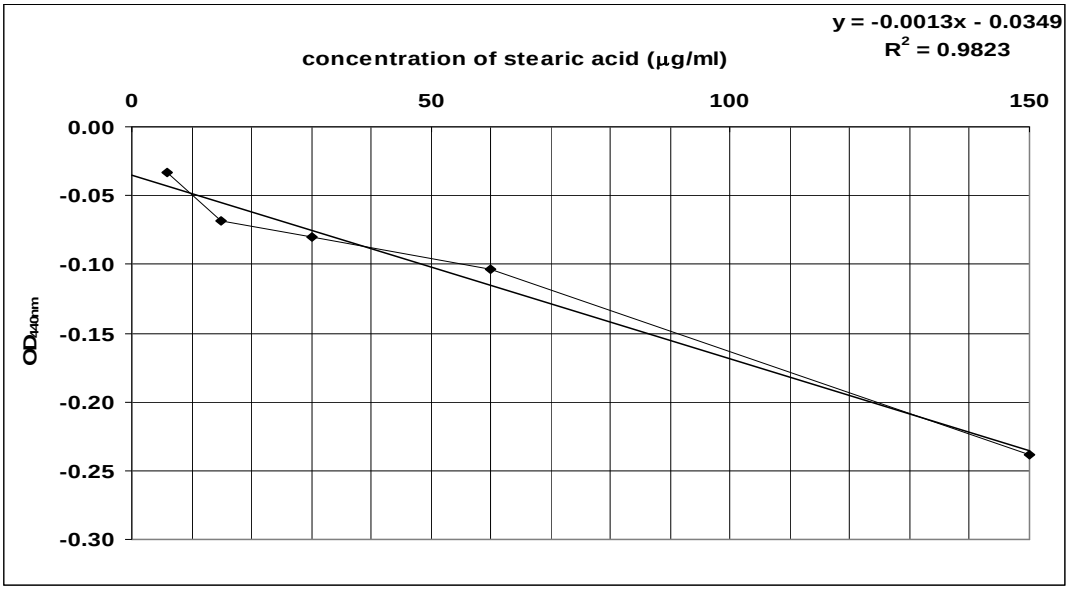
Proteins

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



Lipids

Standard Curve for lipid estimation using stearic acid as standard



THE CHROMATOGRAM AND COMPOSITION REPORT OF SEDIMENT

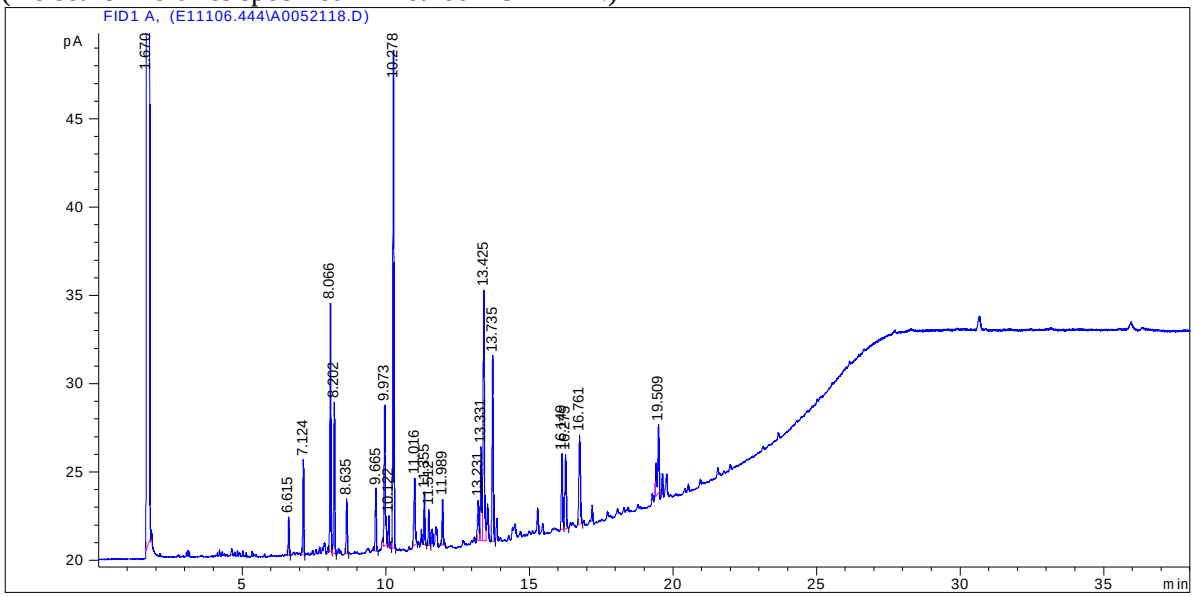
Sherlock Sample Report E11106444A Page 9

Volume: DATA File: E111064.44A Samp Ctr: 5 ID Number: 2118
Type: Samp Bottle: 4 Method: EUKARY
Created: 1/6/2011 2:53:54 PM Created By: administrator
Sample ID: C4S3S2-3a

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.670	5.485E+8	0.029	----	6.985	SOLVENT PEAK	----	< min rt	
6.615	9910	0.036	1.063	13.619	14:0 ISO	1.39	ECL deviates 0.001	Reference 0.009
7.124	25567	0.037	1.050	13.998	14:0	3.54	ECL deviates -0.002	Reference 0.006
8.066	67224	0.038	1.030	14.622	15:0 ISO	9.12	ECL deviates 0.001	Reference 0.009
8.202	43675	0.040	1.027	14.712	15:0 ANTEISO	5.91	ECL deviates 0.001	Reference 0.009
8.635	15418	0.039	1.018	14.998	15:0	2.07	ECL deviates -0.002	Reference 0.006
9.665	18084	0.041	1.001	15.626	16:0 ISO	2.39	ECL deviates 0.000	Reference 0.008
9.973	48229	0.047	0.996	15.814	16:1 w7c	6.33	ECL deviates -0.003	
10.122	8108	0.040	0.994	15.904	16:1 w5c	1.06	ECL deviates -0.005	
10.278	150886	0.042	0.992	15.999	16:0	19.72	ECL deviates -0.001	Reference 0.007
11.016	24636	0.049	0.981	16.431	ISO 17:1 G	3.19	ECL deviates -0.003	
11.355	15409	0.041	0.977	16.629	17:0 ISO	1.98	ECL deviates 0.000	Reference 0.007
11.512	10371	0.040	0.975	16.722	17:0 ANTEISO	1.33	ECL deviates 0.000	Reference 0.006
11.989	14388	0.043	0.969	17.000	17:0	1.84	ECL deviates 0.000	Reference 0.006
13.231	15635	0.053	0.956	17.711	18:2 w6c	1.97	ECL deviates -0.008	
13.331	32873	0.047	0.955	17.768	18:1 w9c	4.14	ECL deviates -0.002	
13.425	91975	0.049	0.954	17.822	Sum In Feature 8	11.57	ECL deviates 0.000	18:1 w9t.
13.735	60251	0.046	0.952	18.000	18:0	7.55	ECL deviates 0.000	Reference 0.003
16.149	27109	0.051	0.933	19.390	20:4 w6c	3.33	ECL deviates -0.002	
16.275	28023	0.052	0.933	19.463	20:5 w3c	3.44	ECL deviates 0.010	
16.761	37676	0.057	0.930	19.744	Sum In Feature 12	4.62	ECL deviates 0.000	20:1 w12c
19.509	29043	0.057	0.919	21.381	22:4 w6c	3.52	ECL deviates -0.003	
----	91975	---	----	----	Summed Feature 8	11.57	18:1 w9t.	18:1 w9t
----	37676	---	----	----	Summed Feature 12	4.62	20:1 w12c	20:1 w11c

ECL Deviation: 0.003 Reference ECL Shift: 0.007 Number Reference Peaks: 11
Total Response: 774490 Total Named: 774490
Percent Named: 100.00% Total Amount: 758884

(No search libraries specified in method EUKARY.)



Sherlock Version 6.0B [S/N 160284] Created on 06-Jan-2011

THE CHROMATOGRAM AND COMPOSITION REPORT OF BACTERIA

Sherlock Sample Report E10129442A Page 10

Volume: DATA File: E101294.42A Samp Ctr: 11 ID Number: 1592
Type: Samp Bottle: 20 Method: TSBA6
Created: 1/29/2010 2:41:08 PM Created By: administrator
Sample ID: 15a-oct

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
0.824	471	0.045	----	5.561		----	< min rt	
1.386	405	0.032	----	6.750		----	< min rt	
1.524	4.255E+8	0.026	----	7.044	SOLVENT PEAK	----	< min rt	
1.653	6249	0.009	----	7.316		----	< min rt	
1.736	16793	0.019	----	7.492		----	< min rt	
1.765	14839	0.025	----	7.554		----	< min rt	
1.836	6906	0.019	----	7.704		----	< min rt	
1.869	1506	0.036	----	7.774		----	< min rt	
1.978	628	0.043	----	8.004		----	< min rt	
2.152	1034	0.065	----	8.373		----	< min rt	
6.383	3396	0.033	0.991	13.61	14:0 iso	5.02	ECL deviates 0.000	Reference -0.008
6.891	1312	0.032	0.984	14.00	14:0	1.93	ECL deviates 0.000	Reference -0.008
7.834	19418	0.036	0.974	14.62	15:0 iso	28.20	ECL deviates 0.000	Reference -0.008
7.970	30191	0.036	0.973	14.71	15:0 anteiso	43.79	ECL deviates 0.000	Reference -0.008
8.410	697	0.038	0.969	15.00	15:0	----	ECL deviates 0.003	
9.038	890	0.065	0.964	15.38	16:1 w7c alcohol	1.28	ECL deviates -0.003	
9.440	1646	0.037	0.962	15.62	16:0 iso	2.36	ECL deviates 0.001	Reference -0.007
9.649	1854	0.041	0.960	15.75	16:1 w11c	2.66	ECL deviates -0.002	
10.053	4378	0.040	0.958	15.99	16:0	6.26	ECL deviates -0.001	Reference -0.009
10.865	365	0.038	0.954	16.47	Sum In Feature 4	0.52	ECL deviates -0.004	17:1 iso I/anteiso B
11.137	2140	0.041	0.953	16.63	17:0 iso	3.04	ECL deviates 0.000	Reference -0.010
11.295	3484	0.039	0.952	16.72	17:0 anteiso	4.95	ECL deviates 0.000	Reference -0.010
----	365	---	----	----	Summed Feature 4	0.52	17:1 iso I/anteiso B	17:1 anteiso B/iso I

ECL Deviation: 0.002 Reference ECL Shift: 0.009 Number Reference Peaks: 8
Total Response: 69074 Total Named: 69074
Percent Named: 100.00% Total Amount: 67723

Matches:

Library	Sim Index	Entry Name
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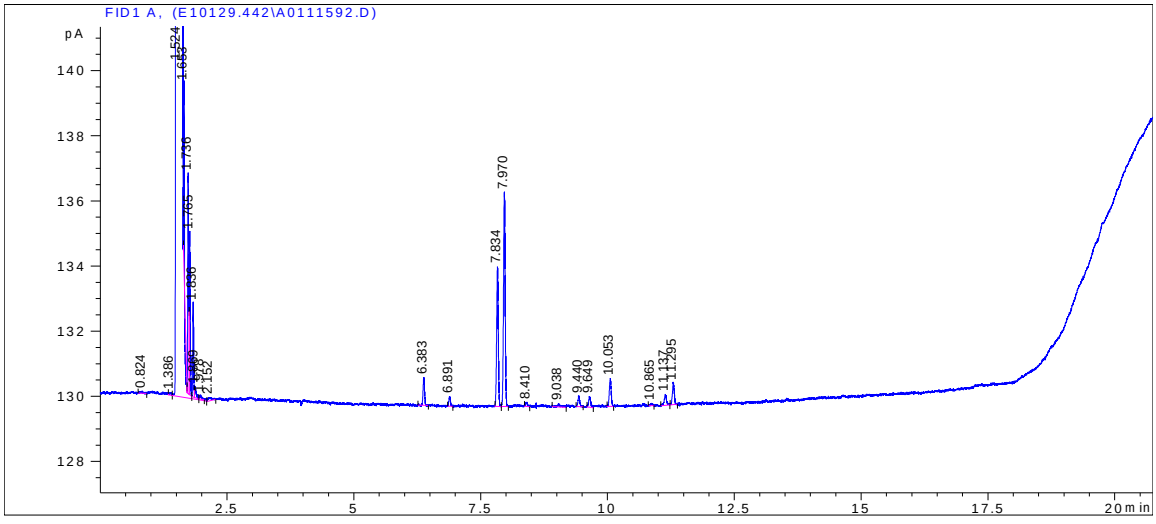

Sherlock Version 6.0B [S/N 160284] Created on 29-Jan-2010

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BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF *BACILLUS PUMILUS* ISOLATED FROM COASTAL ENVIRONMENT IN COCHIN, INDIA

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ABSTRACT

Bacillus species constitute a diverse group of bacteria widely distributed in soil and the aquatic environment. In this study, *Bacillus* strains isolated from the coastal environment of Cochin, India were identified by detailed conventional biochemical methods, fatty acid methyl ester (FAME) analysis and partial 16S rDNA sequencing. Analysis of the data revealed that *Bacillus pumilus* was the most predominant species in the region under study followed by *B. cereus* and *B. sphaericus*. The *B. pumilus* isolates were further characterized by arbitrarily primed PCR (AP-PCR), antibiotic sensitivity profiling and PCR screening for known toxin genes associated with *Bacillus* spp. All *B. pumilus* isolates were biochemically identical, exhibited high protease and lipase activity and uniformly sensitive to antibiotics tested in this study. One strain of *B. pumilus* harboured cereulide synthetase gene *cesB* of *B. cereus* which was indistinguishable from rest of the isolates biochemically and by AP-PCR. This study reports, for the first time, the presence of the emetic toxin gene *cesB* in *B. pumilus*.

Key words: *Bacillus pumilus*; FAME; 16S rDNA; *cesB*; AP-PCR.

INTRODUCTION

The Gram-positive, aerobic, rod-shaped endospore-forming bacteria of the Genus *Bacillus* are the most widely represented organisms in the soil. Due to their ability to form spores and withstand a range of variable environmental conditions, *Bacillus* spp. adapt easily to diverse habitats (25). The diverse physiology of *Bacillus* spp. requires elaborate biochemical tests for their identification (30). Advances in chromatographic analysis of whole cell fatty acid methyl ester (FAME) profiles have made this technique sufficiently sensitive and reliable for grouping of *Bacillus* at species level (31). Further, nucleic acid based techniques such as 16S rDNA (3,34) and gyrase B (*gyrB*) sequence analysis have proved to be of immense value for phylogenetic analysis of bacteria (35). Based on the 16S rDNA sequence analysis, 5 groups have been identified within the genus *Bacillus*, of which the group 1 (*B. subitilis* group) comprises of *B. amyloliquefaciens*, *B. subtilis* and *B. pumilus* (3,12).

Several species of *Bacillus* inhabit coastal and marine environments, though it is hard to strictly classify them as indigenous to these habitats. Together with *B. cereus* and *B. subtilis*, *B. pumilus* is considered as a major component of marine bacterial communities (8,15,22,27). Recently, *B. pumilus* has also been reported to be the second most predominant *Bacillus* species in spacecrafts (17). This bacterium is highly resistant to extreme environmental conditions such as low or no nutrient availability, desiccation, irradiation, H_2O_2 and chemical disinfections (19). The ecological role of *B. pumilus* is emphasized by the fact that they do produce compounds antagonist to fungal and bacterial pathogens (4,6). Thus, *B. pumilus* is of considerable research interest to understand its physiological diversity, genetic relatedness with other *Bacillus* spp. and the possible presence of toxigenic factors. In the study reported here, we describe i) isolation and identification of *Bacillus* spp. from environmental samples by conventional methods, FAME and 16S rDNA sequencing and ii) further

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phenotypic and genetic characterization of *B. pumilus*, the predominant *Bacillus* group of bacteria in the coastal region under study.

MATERIALS AND METHODS

Isolation and biochemical characterization of *Bacillus* spp.

Sea water, sediment, fish and shellfish were collected off Cochin, West coast of India and processed for the isolation of *Bacillus* spp. Fish, shellfish or sediment samples were homogenized in phosphate buffered saline (PBS 0.05 M, pH 7.2), serially diluted in the same medium and spread plated on nutrient agar prepared in 50% seawater. One hundred micro liters of seawater samples were directly spread plated on the same medium and incubated at 30ºC for 24-48 h. The colonies that came up on agar plates were purified and stored at -80ºC in nutrient broth containing 30% glycerol. For taxonomic identification, the isolates were subjected to a series of biochemical tests (11), which included nitrate reduction, anaerobic growth, gas production from glucose, Voges-Proskauer (VP), growth at different NaCl concentrations, temperature and pH ranges, degradation of starch, casein, urea, tween 20, gelatin, chitin, acid production from arabinose, mannitol, xylose, glucose, lactose, citrate utilization and production of DNAse. The production of extracellular enzymes namely caseinase, chitinase, protease, alkaline phosphatase, gelatinase and lipase was studied following the protocol described by Smibert and Krieg (29)

Fatty acid methyl ester (FAME) analysis

Gas chromatographic analysis of whole cell fatty acid methyl ester (FAME) was performed for further identification and grouping of isolates. Fatty acid methyl ester extraction was performed using standard procedures (28). The fatty acid profiles generated were compared against an inbuilt Sherlock TSBA Library version 3.9 (MIDI Inc., DE, USA). A similarity index of >60% was used for clustering of isolates at species level.

Antimicrobial susceptibility assay

The inhibition of *B. pumilus* strains by various antibiotics was tested by standard disc diffusion technique (7). The cultures were grown in nutrient broth overnight and plated on Muller Hinton agar (Hi-Media, Mumbai). The following antibiotic discs with their concentrations indicated in parenthesis were used; amoxicillin (25 mcg), penicillin (10 mcg), ciprofloxacin (5 mcg), gentamycin (10 mcg), cotrimaxazole (25 mcg), chloramphenicol (30 mcg), bacitracin (8 mcg), tetracycline (30 mcg), kanamycin (30 mcg), erythromycin (15 mcg), vancomycin (30 mcg).

DNA isolation and purification

Pure genomic DNA was isolated following the method of Ausubel *et al*. (5). Briefly, the cultures were grown overnight in 3 ml nutrient broth with shaking at 30ºC. A 1.5 ml of the culture was centrifuged at 12 000 *g* for 10 min and the resultant pellet was resuspended in 567 µl 1× TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). Proteinase K and SDS were added to final concentrations of 100 µg/ml and 0.5% respectively, and incubated at 37ºC for 1 h. After incubation, NaCl (5 M) and CTAB/NaCl (10% w/v cetyl trimethyl ammonium bromide in 0.7 M NaCl) were added and incubated at 65ºC for 10 min. The mixture was extracted once each with an equal volume of chloroform-isoamyl alcohol (24:1) and phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated from the aqueous phase using 0.6 volumes of isopropanol and washed once with 70% ethanol. The DNA pellet obtained after final centrifugation was vacuum dried and dissolved in 50 µl 1× TE buffer. DNA quantification was done using a UV-1601 spectrophotometer (Shimadzu Corporation, Japan).

16S rDNA sequencing and AP-PCR

The 16S rDNA of 4 strains (NIOB 005, NIOB 133, NIOB 485 and NIOB 525) were PCR amplified using universal primers and PCR conditions described by Iwamoto *et al*. (16) (Table 1). The resultant 454 bp products were purified using a PCR purification kit (Qiagen, Germany) and sequenced. The sequences were subjected to homology search using BLAST programme (2) of the National Center for Biotechnology Information (NCBI).

AP-PCR was performed using primer CRA22 described by Neilan (18) (Table 1). All the reactions were carried out in 30 µl volumes consisting of a 10×buffer (100 mM Tris-HCl, 500 mM KCl and 20 mM $MgCl_2$), 200 µM concentrations of each of the four dNTPs, 30 picomoles of primer, 3 U of Taq polymerase (MBI Fermentas). All PCR amplifications were carried out in an eppendorf mastercycler (Eppendorf, Germany). In all the reactions, 300 ng of the pure genomic DNA was used. The amplification products were separated on a 2% agarose gel, stained with ethidium bromide and photographed. Amplification profiles obtained were analyzed and a dendrogram was generated using BioNumerics version 4.6 software (Applied Maths, Belgium)

PCR detection of cereulide synthetase genes

B. pumilus were screened for the presence of cereulide synthetase genes *cesA* and *cesB* using primers previously described (Table 1). The expected amplicons with *cesA* and *cesB* gene-specific primers were 188 bp and 635 bp respectively.

Nucleotide sequence accession numbers

The partial 16S rDNA and *cesB* sequences derived in this study have been deposited in GenBank under the accession numbers EU283326, EU283325, EU283323, EU283322, EU283320, EU283318, EU167933, EU283321, EU167932, EU167924, EU289221

Table 1. PCR primers used in this study.

Primer	Sequence (5'-3')	Target gene	Reference
EUB-F	gcacaagcggtggagcatgtgg	16S rDNA	16
EUB-R	gcccgggaacgtattcaccg		
CER1	atcataaaggtgcgaacaaga	Cereulide synthetase (*cesA*)	14
EMT1	aagatcaaccgaatgcaactg		
EM1-F	gacaagagaaatttctacgagcaagtacaat	Cereulide synthetase (*cesB*)	10
EM1-r	gcagccttccaattactccttctgccacagt		
CRA22	ccgcagccaa	Random primer	18

RESULTS

Taxonomic identification of *Bacillus* spp. isolated from coastal environment of Cochin

Eighty-two *Bacillus* spp. were isolated and identified by biochemical tests and fatty acid methyl ester analysis (FAME). These included *B. pumilus* (16), *B. cereus* (15), *B. sphaericus* (11), *B. subtilis* (10), *B. amyloliquefaciens* (8), *B. megaterium* (6), *B. lentimorbus* (5), *B. coagulans* (4), *B. licheniformis* (4), *B. circulans* (1), *B. flexus* (1) and *Bacillus* GC group 22 (1). The *Bacillus* GC group 22 corresponds to the gas chromatographic profile of a *Bacillus* species in the Sherlock TSBA Library version 3.9 (Microbial ID, MIDI Inc.), the 16S rDNA sequence of which does not match any known species of the genus *Bacillus*. In our study, *B. pumilus* was the most predominant species followed by *B. cereus* and *B. sphaericus*. Ivanova *et al*. (15) in their study found that *B. pumilus* and *B. subtilis* were the most abundant *Bacillus* spp. associated with marine sponges, ascidians, soft corals, and seawater.

Characterization of *B. pumilus*

Sixteen *B. pumilus* strains isolated from different sources (Table 2) exhibited uniform phenotypic properties (Table 3). Physiological tests revealed the production of detectable protease and lipase but not amylase, phosphatase, DNase, gelatinase and chitinase. The partial 16S rDNA sequences (≈ 500 bp) of *B. pumilus* determined in this study revealed 99-100% homology with *B. pumilus* 16S rDNA sequences in the GenBank. The antibiotic susceptibility profiles of *B. pumilus* were identical. All 16 isolates were uniformly inhibited by amoxicillin, ciprofloxacin, gentamycin, cotrimaxazole, chloramphenicol, bacitracin, tetracycline, kanamycin, erythromycin, vancomycin. All isolates exhibited resistance to penicillin.

AP-PCR typing of isolates

The random primer CRA 22, which consistently yielded 4-10 bands with *B. pumilus*, was chosen for typing of isolates by AP-PCR. The analysis of AP-PCR fingerprints revealed heterogeneity among *B. pumilus* isolates with 10 distinct patterns (Fig. 1). Despite this overall genetic diversity, near identical profiles were obtained between strains NIOB 485 and NIOB 525 (from crab), strains NIOB111 (fish) and NIOB 426 (from sediment), strains NIOB096 (from crab) and NIOB169 (from fish), strains NIOB163 (from fish) and NIOB431 (from starfish).

Table 2. Sources of *B. pumilus* strains isolated in this study.

Strain	Source
NIOB005	Sediment
NIOB014	Sediment
NIOB018	Sediment
NIOB026	Oyster
NIOB096	Crab
NIOB111	Fish
NIOB113	Fish
NIOB133	Fish
NIOB137	Fish
NIOB163	Fish
NIOB169	Fish
NIOB190	Fish
NIOB426	Sediment
NIOB431	Starfish
NIOB485	Crab
NIOB525	Crab

Detection of cereulide synthetase gene in *B. pumilus* by PCR

One isolate *B. pumilus* NIOB 133 isolated from an estuarine fish yielded 635 bp amplicon with *cesB*-targeted primers EM1-f and EM1-r (Fig. 2). The product of *cesB* PCR was sequenced and nucleotide sequence analysis of the PCR product revealed

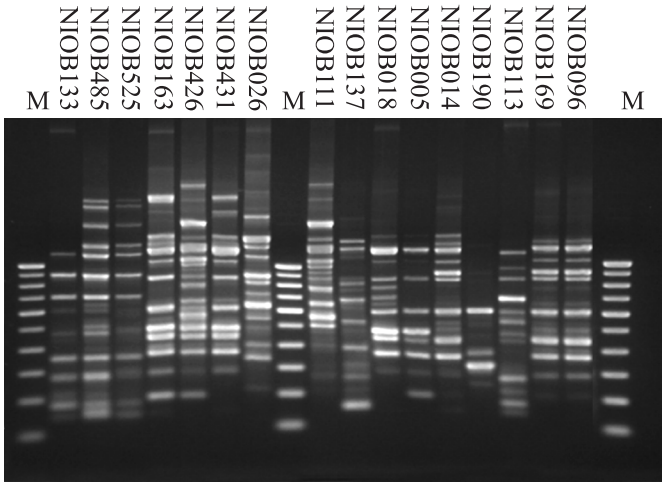

Figure 1. AP-PCR patterns of *B. pumilus* obtained with primer CRA22. M= 100 bp DNA ladder (GeneRuler, Fermentas).

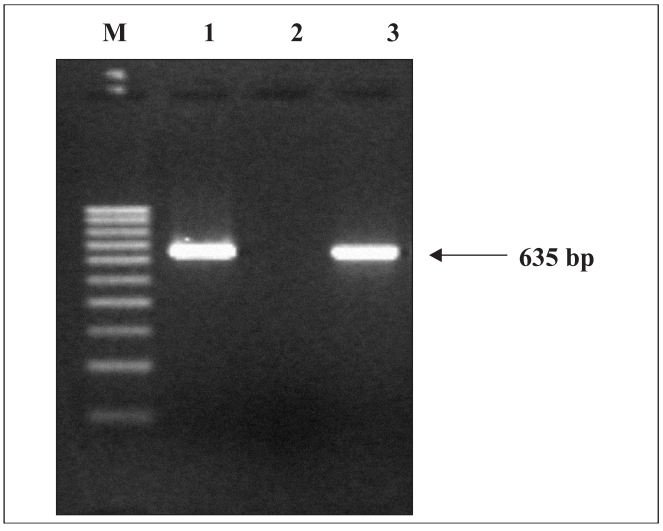

Figure 2. Detection of cereulide synthetase gene *cesB* in *B. pumilus* by PCR using primers EM1-F and EM1-r.
M= 100 bp DNA ladder (GeneRuler, Fermentas). 1: *B. pumilus* NIOB133 *cesB*$^+$; 2: *B. pumilus* NIOB 137 *cesB*$^-$; 3: *B. cereus* NIOB 020 (*cesB*$^+$ reference strain, environmental isolate).

Table 3. Growth and substrate utilization characteristics of *B. pumilus* observed in this study.

Characteristic	*B. pumilus* phenotype	Characte-ristic	*B. pumilus* phenotype
Amylase	+	Substrate utilization	
Protease	+	D-Glucose	+
Lipase	+	L-Arabinose	+
Phosphatase	-	D-Xylose	+
DNase	-	D-Mannitol	+
Gelatinase	-	Galactose	+
Chitinase	-	Fructose	+
Growth temperature	5-50ºC	Mannose	+
Growth ph	5-11	Nitrate	-
Nacl tolerance	10%	Adonitol	-
Oxidase	+	Dulcitol	-
Catalase	+	Sorbitol	-
Indole production	-	Inositol	-
Voges-Proskauer	+	Urea	-
Citrate utilization	-		

96% similarity with corresponding sequence of *cesB* of *B. cereus* (GenBank accession no. DQ889676) (26), while the deduced amino acid sequence showed 92% homology with a few amino acid mismatches (Fig. 3). This strain was negative by *cesA* PCR using primers CER1 and EMT1.

DISCUSSION

This study on the diversity of *Bacillus* spp. isolated from a coastal environment by biochemical assays, FAME analysis and 16S rDNA sequencing revealed that *B. pumilus* was the predominant species followed by *B. cereus*. *B. pumilus* belongs to *B. subtilis* group of aerobic spore-forming organisms, which has lately evoked considerable research interest due its involvement in cases of food-poisoning. Recently, a pumilacidin-producing *B. pumilus* has been implicated in a case of food poisoning (13). A study by Brophy and Knoop (9) reported experimental induction of enterocolitis in guinea pigs, while some compounds produced by *B. pumilus* were reportedly toxic to mice, eukaryotic cells and humans (20,21,24,32).

Sixteen environmental *B. pumilus* strains isolated from different sources (Table 2) exhibited uniform phenotypic properties (Table 3). The identity of these isolates was further confirmed by fatty acid methyl ester analysis with a similarity index of >60% (data not shown). The partial 16S rDNA sequences (500 bp) of these strains revealed 99-100% similarity with *B. pumilus* 16S rDNA sequences in the GenBank. Thus, a combination of conventional physiological tests and genetic analysis enabled unambiguous identification of *B. pumilus* from coastal environments of Cochin. None of the strains exhibited resistance to the antibiotics tested in this study except to penicillin. This observation is interesting, since penicillin

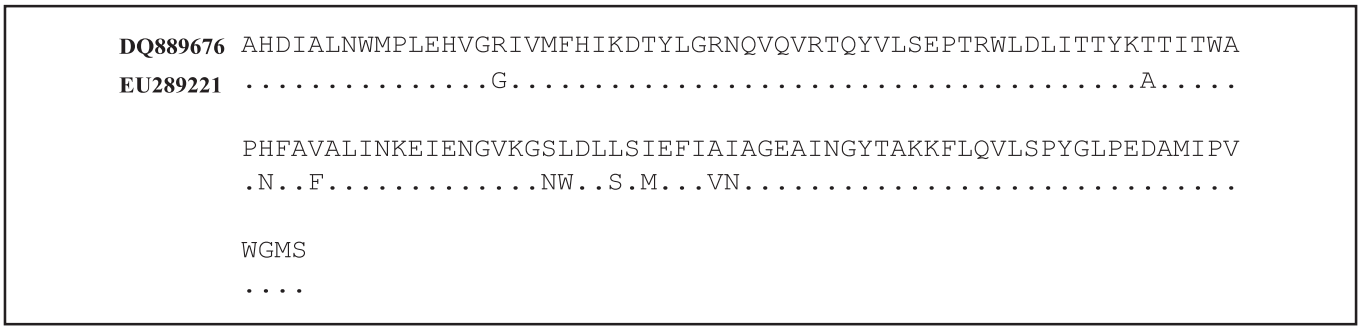

Figure 3. Alignment of deduced partial amino acid sequence of *cesB* derived in this study from *B. pumilus* strain NIOB 133 (EU289221) with the corresponding GenBank sequence of plasmid pCER270 (DQ889676). Dots indicate identical amino acids.

resistance has not been reported in *B. pumilus*. Studies on antibiotic resistance of *B. pumilus* are limited, since the organism is not considered infectious to humans and animals. However, some recent studies have revealed that several *Bacillus* species including *B. pumilus* can cause infections, ranging from skin infection to life threatening bacteremia in immunocompromised individuals (23,33). Thus, more studies need to be performed to understand the human health significance of *B. pumilus*, genetic basis of infections and resistance to antimicrobials.

The whole genome comparison of *B. pumilus* strains by AP-PCR demonstrated that *B. pumilus* were genetically diverse. Though the isolates shared several common amplification bands, overall heterogeneity among *B. pumilus* studied was apparent (Fig. 1). Despite this, some strains isolated from similar sources during different points time exhibited identical or near identical profiles. However, it was not possible to attribute isolates to a particular source solely based on the AP-PCR profiles, since some strains isolated from different sample types such as fish and sediment also exhibited identical AP-PCR profiles. The random primer CRA 22 used in this study has sufficient discriminating power and will be useful for studying genetic diversity among the *Bacillus* group of bacteria.

In order to understand the toxigenic potential of environmental strains of *B. pumilus*, the isolates obtained in this study were screened for the presence of cereulide synthetase genes *cesA* and *cesB* by PCR. Surprisingly, one strain (NIOB 133) was PCR positive for *cesB*-targeted gene (Fig. 2). This strain however, did not harbour *cesA* gene as revealed by *cesA*-specific PCR using primers CER1 and EMT1. Cereulide is a small heat stable cyclic dodecadepsipeptide produced by some strains of *B. cereus* which has high toxicity to humans (1,36). Cereulide is synthesized by a non-ribosomal peptide synthetase, encoded by the *ces* genes located on a 270-kb pXOI-like virulence plasmid named pCER270 (10,26), more often found associated with clinical isolates of *B. cereus*. A recent study has reported the presence of *cesA*, but not *cesB*, in *B. pumilus* and *B. licheniformis* isolated from bovine mastitis (20). However, the *cesB*-positive strain in our study lacked *cesA*. Therefore, it appears that cereulide-encoding genes may be distributed over a wide species range within the *Bacillus* group of bacteria in the environment. The *cesB*-positive *B. pumilus* in the present study was indistinguishable from the rest of the strains by biochemical assays or by its fatty acid profile. Further, the AP-PCR profile of this strain was similar to other non-toxigenic strains (Fig. 1). The production of toxin and the consequent ability of this strain to initiate emetic symptoms need to be established by suitable animal feeding experiments. Our study constitutes the first report on the presence of *cesB* in *B. pumilus*.

In conclusion, *Bacillus* spp. constitute key components of coastal-marine heterotrophic bacterial communities owing to their diverse and flexible physiological properties. Isolation and characterization of *Bacillus* spp. from these environments will help in identifying novel mechanisms of environmental survival, diverse metabolic activities, production of biotechnologically valuable compounds such as enzymes and antimicrobial substances and the presence of putative toxigenic factors. In our study, a combination of methods involving FAME, 16S rDNA sequencing and biochemical assays enabled complete identification of *B. pumilus*. Though, the biochemical and enzymatic properties of *B. pumilus* isolated in this study were uniform, intraspecific genetic diversity was evident from AP-PCR analysis. The detection of cereulide synthetase gene *cesB* in *B. pumilus* is significant, since *ces* genes were previously thought to be restricted to emetic strains of *B. cereus*. The organization of *ces* operon in *B. pumilus* is a subject for further study.

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RESUMO

Caracterização bioquímica e molecular de *Bacillus pumilus* isolado do ambiente costeiro de Cochin, Índia

As espécies de *Bacillus* constituem um grupo diversificado de bactérias amplamente distribuídas no solo e no ambiente aquático. Neste estudo, cepas de *Bacillus* isoladas do ambiente costeiro de Cochin, Índia, foram identificadas através de métodos bioquímicos convencionais, análise de ésteres metílicos de ácidos graxos (FAME) e sequenciamento de 16S rDNA. A análise dos dados revelou que *Bacillus pumilus* foi a espécie predominante na região estudada, seguido de *B. cereus* e *B. sphaericus*. Os isolados de *B. pumilus* foram caracterizados através da reação em cadeia da polimerase com primers arbitrários (AP-PCR), perfil de sensibilidade a antibióticos e triagem por PCR de genes de toxinas associadas com *Bacillus* spp. Todos os isolados de *B. pumilus* foram bioquimicamente idênticos, apresentaram elevada atividade de protease e lipase e foram uniformemente sensíveis aos antibióticos estudados. Um dos isolados de *B. pumilus* apresentou o gene *cesB* de *B. cereus*, que não foi não distinguível dos demais isolados por testes bioquímicos nem por AP-PCR. Este é o primeiro relato da presença do gene *cesB* da toxina emética em *B. pumilus.*

Palavras-chave: *Aspergillus flavus*, pimenta em pó, alterações nutricionais, aflatoxina.

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