Bacterial ecology of coastal and near-shore placer sediments

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By

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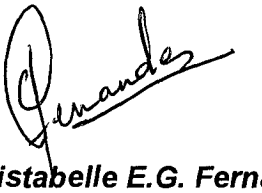
Declaration

As required under the University Ordinance 0.19.8 (vi), I state that the present thesis entitled ***"Bacterial ecology of coastal and near-shore placer sediments"*** is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned. The literature related to the problem investigated has been cited. Due acknowledgements have been made whenever facilities and suggestions have been availed of.



Christabelle E.G. Fernandes

Certificate

This is to certify that the thesis entitled "***Bacterial ecology of coastal and near-shore placer sediments"*** submitted by Ms. Christabelle E. G. Fernandes for the award of the degree of Doctor of Philosophy in Marine Science is based on her original studies carried out by her under my supervision. The thesis or any part thereof has not been previously submitted for any degree or diploma in any University or Institution.

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Certified that all corrections suggested by the examiners have been incorporated in the thesis.

17/12/2010.

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The possession of knowledge does not kill the sense of wonder & mystery. There is always more mystery.

– Anäis Nin, Diary

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"This is the Lord's doing. It is marvellous in our eyes" – Ps 118:23

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Christabelle E.G. Fernandes

Dedicated

to

My Beloved

Mum and Dad

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Abbreviations

AMR	Average metabolic response
ACE	Abundance based coverage estimator
AODC	Acridine orange direct count
ASW	Artificial seawater
ATP	Adenosine tri phosphate
Chl *a*	Chlorophyll *a*
CHO	Sedimentary total carbohydrates
CI	Capacity index
CLPP	Community-level physiological profiling
CMD	Community metabolic diversity
CSUR	Carbon substrate utilization rate
d	days
DD	during disturbance
DW	Distilled water
FC	Final concentration
FDC	frequency of dividing cells
Fe(II)	Ferrous iron
Fe(III)	Ferric iron
FeA	Acid soluble iron
FeM	Microbially reducible iron or hydroxylamine extractable Fe
h	hour
IB	Iron bacteria enumerated using spread plate method
ICoMM	International Census of Marine Microbes
Ilm	Ilmenite
INDEX	Indian Ocean Deep-sea Experiment
IR	Iron bacteria enumerated using agar shake method
LIP	Sedimentary total lipids
LOM	Sedimentary labile organic matter
min	minute
MM	monitoring phase – 2 hours after disturbance
MPN	Most probable number
NB	nutrient broth concentrations
OTU	Operational taxonomic units
PCA	Principal component analysis
PD	pre-disturbance phase – 3 hours before disturbance
PI	pre-disturbance (phase I)
PII	immediately after disturbance (phase II)
PII	24 hours after disturbance (phase III)
PROT	Sedimentary total proteins
RC-0.001	Retrievable counts retrieved on 0.001% nutrient amended media
RC-0.01	Retrievable counts retrieved from 0.01% nutrient amended media
RC-10	Retrievable counts retrieved from 10% nutrient amended media
rpm	revolutions per minute
RT	Room temperature
s	second
SEM	Scanning electron microscopy
Stn	Station
SW	Seawater
TC	Direct total bacterial abundance
Tcarb	Total carbon
TEM	Transmission electro microscopy
TOC	Total organic carbon
TV	Direct total viable counts
TVCa	Direct total viable counts under aerobic conditions
TVCan	Direct total viable counts under anaerobic conditions

Chapter 1

Chapter 1: Introduction

1.1. What are placer deposits?

Placer mineral deposits are segregated group of clastic/unconsolidated sediments, sedimentary rock or its metamorphosed equivalent with economic-grade concentration of one or more valuable dense resistant minerals (Mookherjee, 1999). It is defined as *"a surficial mineral deposit, formed by mechanical concentration of mineral particles from weathering debris. The mechanical agent is usually alluvial but can also be marine, aeolian, lacustrine, or glacial, and the mineral is usually a heavy metal such as gold"* (Gary et al., 1972). Beach sediments containing placer deposits harbor considerable mineral wealth and are evolving as important sites for sand mining. These deposits contain metals such as barium, chromium, gold, iron, rare earth elements, tin, titanium, thorium, tungsten, zirconium as well as gemstones such as diamond. Hence, understanding the ecology of these ecosystems especially at the microbial level is important to appreciate the differences it would make to the systems if the resources were exploited on a continuous or intermittent scale.

The study area, Kalbadevi Bay, Ratnagiri is well known for its fishery resources and is also a potential mining site. The associated beach is nearly 5 km long and ~ 250 m wide with estuaries on either ends. These estuaries are responsible for the deposition of major placer minerals comprising mainly of ilmenite and magnetite, which appear as black particles embedded in beach sand. Such type of placer is known as ***Beach placers* or *On-shore placers* or even as *Black sands.*** A combination of favorable factors like the hinterland, geology, coastal geomorphology, sub-tropical to tropical climate and intricate network of drainage, aided by wind and coastal processes like waves and currents influence the formation of these placers (Loveson and Rajamanickam, 1988; Chandrasekaran et al., 1997; Mohan Das et al., 2004). In tropical environments, minerals are typically released from the parent source rock after subjection to various weathering processes. The humid

climate and heavy rainfall further enhances this weathering process. The liberated minerals are transported by streams and rivers and deposited along the shores where sorting and re-distribution of the minerals takes place due to dual action of sea currents/waves and wind. Enrichment of these deposits takes place on shorelines which are stable i.e. shorelines which are neither erosive/abrasive nor accumulative. Many parts of the Indian coastline extending over 7500 km have stable shorelines. These shores along with the exclusive economic zone area of 2.02 million sq. km. harbor some of the largest and the richest placer mineral deposits (Chandra et al., 1996; Joshi, 1996). These deposits may contain ilmenite, rutile, xenotine, leucoxene, zircon, monazite, sillimanite, thorite and garnet. The average heavy mineral grade in these placers varies between 10 to 15%. The typical composition comprises of ilmenite (80%), zircon (10%), leucoxene (5%), rutile (1%), monazite (0.5%) and others (3.5%). Among these deposits, the most important heavy minerals are titanium bearing minerals like ilmenite ($FeTiO_3$) and rutile (TiO_2) (Jade et al., 2004). Ilmenite is the largest constituent of the Indian beach sand deposits, followed by sillimanite and garnet (Rao et al., 2001; Bhattacharyya et al., 2004; Rajamanickham et al., 2004). The major source rocks are the khondalites, charnockites, granites gneisses (Precambrian), Deccan traps (Cretaceous), laterites, sandstones which occur along the Eastern Ghat and the Western Mountain ranges of India. The entire Quaternary deposit of sediments can cumulate as the placer resource of the country. The inferred placer reserves along the Indian coast are 348 Mt ilmenite, 18 Mt rutile, 21 Mt zircon, 107 Mt garnet and 130 Mt sillimanite. The explored Indian resources constitute nearly 35% of world resources of ilmenite, 10% rutile, 14% zircon, 71% monazite (Ali et al., 2001).

1.2. Mining of placer deposits

Ilmenite is an important mineral and a feedstock source for producing titanium metal, titanium dioxide pigment, synthetic rutile, electrodes, ferro-alloys, paints and welding rod coatings (Mohan Das et al., 2004; Gambogi, 2005). Titanium and its compounds is commonly used in desalination plants, electrical components, glass products, cosmetics, artificial jewelry and smoke screens while its alloys are used in high tech airplanes, missiles, space vehicles and

surgical implants (Rajan et al., 2004). The rich availability of these placer minerals coupled with easy separation and mining techniques as well as availability of indigenous technology has made the extraction of placer minerals superior compared to other deposits (Vaikundarajan and Kandaswamy, 2004). However, mining for sand and placers in the near-shore region can lead to creating an imbalance in the material supply and removal ratios. This in turn may promote artificial erosion in one region and accretion in other regions of the coastal tract (Nath et al., 2004). Studies have demonstrated that mineral sand mining involves considerable disturbance and loss of physical, chemical and biological fertility (van Aarde et al., 1998). Mining adversely affects biological communities in and around the mining site. Considerable damage is known to occur on associated coastal habitats such as mangroves, seagrass beds, coral beds, rocky shores, sandy beaches, mud flats, lagoons and algal beds. Hence, to understand and minimize the anticipated impacts on fragile coastal environments such as sandy shores, mining activity needs to be coupled with holistic environmental studies (Nath et al., 2004).

1.3. Beaches as an environmental niche

A typical sandy shore has the following three main components (Fig 1.1).

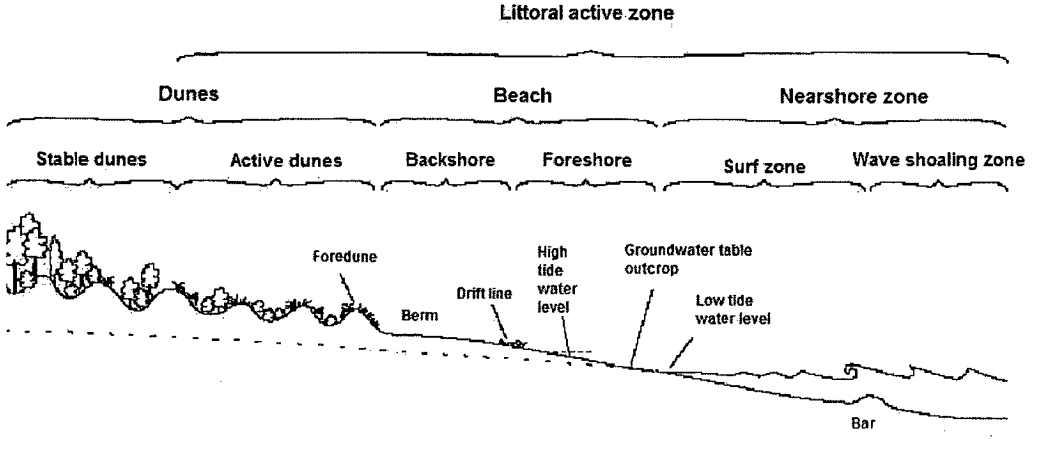


Fig 1.1 Profile of a typical sandy beach environment (*source*: Knox, 2001)

a) Dune zone extending beyond the highest point reached by waves on spring tides.

b) Beach zone extending from the upper limit of the driftline to the extreme low water level and is sub-divided into

(i) Backshore zone which extends above high water, and covered only on exceptional tides

(ii) Foreshore zone which extends from low water up to the limit of the high water wave splash

c) Near-shore zone extending from low water to the deepest limit of wave erosion and is subdivided into

(i) Inner Turbulent zone covering the region of breaking waves

(ii) Outer Turbulent zone

Beaches act as transitional ecosystem as well as functional links between terrestrial and marine environments in the coastal zone (Bird, 1988; Zann, 1997). There exists a continuous exchange of sediments and organic materials between the beach, the dune and the surf zone. Beaches also act as connectors between the terrestrial dune aquifers and coastal seas through the discharge of nutrient-rich groundwater. Further, these shores play a vital role in breakdown of organic materials and pollutants, in water filtration and purification, and in nutrient mineralization and recycling (Davies, 1972; Brown, 2001; Defeo et al., 2009). Economically, beaches act as sites for recreational opportunities (Hosier et al., 1981; Hubbard and Dugan, 2003; Fabiano et al., 2004; Defeo et al., 2009). The physical and chemical processes coupled with biological pressure make beaches highly dynamic systems leading to the characteristic steep environmental gradient and complex biogeochemical processes (Brown and McLachlan, 1990; Sundbäk et al., 1996; Guarini et al., 2000; Brazeiro, 2001; Blanchard et al., 2001). The hydrodynamic forces also play a dominant role in near-shore environments (Eckman, 1985; Berninger and Huettel, 1997; Shimeta et al., 2001). Subsequently, beaches and its associated near-shore environment act as critical habitats for maintenance of biodiversity and genetic resource, nursery areas, nesting grounds, and prey grounds for birds and terrestrial wildlife, thus establishing species diversity, biomass and community structure on the beach ecosystem (Brown and

McLachlan, 1990). These community structures especially the microbial communities are highly specialized in their functioning.

Microbes are important biogeochemical agents and mineral cyclers in any environmental niche since these organisms participate in many chemoautotrophic and mixotrophic reactions in sediments. Microorganisms usually mediate organic matter re-mineralization (Berner, 1980). Resident microbial communities are known to actively break down organic carbon, typically utilizing electron acceptors in the order of decreasing free energy yield (i.e. O_2, NO_3^-, MnO_2, FeOOH, SO_4^{2-}) (Froelich et al., 1979). Under efficient energy metabolism, a major fraction of the metabolized organic matter is transformed to cell material. In aerobic decomposition of organic matter, oxygen-containing radicals such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (.OH) are readily formed and consumed. These radicals are capable of breaking strong chemical bonds and relatively refractory organic compounds rich in aromatic structures like lignin (Canfield, 1993). The anaerobic respiration processes generally occurs in the sequence with sediment depth according to the availability of electron acceptors such as Mn^{4+}, NO_3^-, Fe^{3+}, SO_4^{2-}, and CO_2 (Fenchel et al., 1998). Manganese and iron oxides are electron acceptors for Mn(IV) and Fe(III) reducing bacteria. Furthermore, much of the oxygen uptake is used to re-oxidize the reduced inorganic metabolic products of anaerobic respiration (NH_4^+, Mn^{2+}, Fe^{2+} and H_2S) at the oxic/anoxic interface (Jørgensen, 1983). In addition, because of their high turnover rate and metabolic activity, the structure of microbial assemblage is sensitive to changes in trophic conditions (Hansen and Blackburn, 1992). Microbial assemblages also play an important role in the coastal environment in degrading organic matter.

1.4. Role of heterotrophic bacteria in organic matter degradation

Sedimentary organic material is generally composed of high-molecular-weight and polymeric structures such as cellulose, chitin, lipids, nucleic acids, pectin, phospholipids, proteins and starch (Fabiano and Danovaro, 1998). These substrates are an important source of carbon, nitrogen and energy required for respiration by bacteria. Energy is obtained by bacteria through the

oxidation of these organic compounds. These reactions results in the synthesis of ATP as the chemical energy source and generates simpler organic compounds (precursor molecules) needed by a cell for biosynthetic/assimilatory reactions. However, bacteria are incapable of using the polymeric substrates directly as these cannot permeate the bacterial membranes since their molecular weight is larger than 600Da (Thurman, 1985; Fabiano and Danovaro, 1998). Bacteria are also incapable of phagocytosis. Nevertheless, it is well known that bacteria mineralize and convert nearly 70% of organic matter reaching a marine beach ecosystem into its biomass (Koop et al., 1982; Meyer-Reil, 1991; Azam and Long, 2001). This is usually achieved through highly efficient extracelluar proteinaceous catalysts/exoenzymatic systems. In this mechanism, bacteria secrete the exoenzymes into the environment which catabolize the degradation of large-size fractions of organic matter and the enzyme-catalyzed reaction product is taken up by passive diffusion or active transport. The major extracellular enzymes are proteases, amylases, lipases and phosphatases. These enzymes can decompose high molecular weight biopolymers into simple monomer compounds which diffuse easily into the periplasmic space and interact with permeases. Bacteria are thus competent to decompose a wide spectrum of organic compounds ranging in molecular size from monomers to polymers (Thurman, 1985; Fabiano and Danovaro, 1994; 1998). This activity is usually recognized as the key step in degradation and utilization of organic polymers by bacteria (Gottschalk, 1986; Hoppe, 1991; Meyer-Reil, 1991). Thus in marine sediments, organic matter diagenesis is largely dependent on bacterial activity and contributes significantly to the overall biogeochemical cycles (Azam et al., 1983; Deming and Barross, 1993; Jørgensen, 2000). This activity is further enhanced due to the availability of high concentrations of organic matter in the sediment as well as due to the occurrence of pelagic-benthic coupling (Danovaro et al., 1993; 2000). Organic matter present in the upper layers of sediment is usually degraded by predominant heterotrophic bacteria present in these layers. However, as the sediment depth increases, oxygen is depleted due to bacterial respiration and other functional groups of bacteria continue degradation of organic matter via the use of nitrate, manganese oxide, iron oxide, sulphate as electron acceptors (Fuhrman et al.,

1993; Martinez et al., 1996). These processes are also operational in anaerobic pockets in the aerobic surface realms such as beach sediments which allow the release of electron donors/acceptors through redox reactions, e.g. release of soluble iron where a great fraction probably comes from ilmenite in the present study site directly or indirectly by the fall in pH due to the degradation of organic matter

1.5. Interaction between the element iron and bacteria

Depending on the environmental conditions, iron forms stable compounds in the divalent as well as the trivalent state. In the presence of oxygen, ferrous [Fe(II)], the geochemically mobile form is stable only under acidic conditions and gets autoxidized to ferric [Fe(III)], even in the presence of minute quantities of molecular oxygen and at pH values greater than 6.0. The ferric iron spontaneously precipitates as a constituent of one of a variety of oxides, hydrated oxides, or hydroxides (Nealson, 1982; Schwertmann and Fitzpatrick, 1992; Lovley, 2000). Average Fe(III)/ Fe(II) ratio in the environment is usually 1.35 (Murad and Fischer, 1988). Iron has gained importance as a key metal in environmental microbe-metal interactions due to its ability to readily switch between the Fe(III) and Fe(II) states (Lovley, 2000). Microbes may determine the speciation of iron which is found abundantly in the Earth's crust and at the same time derive energy from both Fe(III) reduction and Fe(II) oxidation. Virtually, all organisms with the exception of *Lactobacilli* require iron for growth and various other metabolic processes (Archibald, 1983). Iron plays a key role in the detoxification of reactive oxygen species (O_2^- and H_2O_2) and is present in enzymes such as catalase, peroxidase and superoxide dismutases (Sunda, 2001). In living cells, protein bound iron complexes such as cytochromes and FeS redox proteins act as vital electron mediators in the metabolic processes such as photosynthetic and respiratory electron transport, nitrate and nitrite reduction, nitrogen fixation and sulfate reduction. Iron complexes are involved in intracellular respiration, oxygenic and non-oxygenic photosynthesis and are utilized by respiring higher organisms for oxygen transport (Falkowski and Raven, 1997; Sunda, 2001). Within aquatic photosynthetic organisms, iron is relevant to marine phototrophs since it plays a central role in photosynthesis and nitrogen assimilation (Sunda, 2001). Iron

is an essential component in photosynthetic apparatus such as photosystems - PSI, PSII and cytochromes and ATP synthase (Jacobs and Worwood, 1974). Iron plays a relatively more significant role in controlling rates of metabolism and growth as compared to cell yields. This is because it mainly functions in electron transport and redox catalysis rather than in structural components of cells (Sunda and Huntsman, 1997). Due to this metabolic function, cellular Fe:C ratios normally increase with increasing iron availability, in contrast to the nearly fixed N:C and P:C ratios observed in marine plankton (Sunda, 1997). However, iron presents a dual paradox especially for aerobic iron-requiring organisms. The dilemma lies in it being useful and hazardous, as well as abundant but poorly available at the same time. Though iron is found abundantly in the crust, most iron is unavailable to the biota as it is present as an insoluble precipitate. However, bacteria (and other cellular organisms) utilize a variety of biochemical mechanisms to counter the problems presented by their dependence on iron. Thus, this fraction of microbial community plays a central role in making iron available to the higher trophic levels (Neilands, 1973; Archibald, 1983; Raymond and Dertz, 2004). On the other hand, iron toxicity is alleviated through the production of anti-oxidants (e.g. glutathione) and enzymes (e.g. superoxide dismutases, catalases, peroxidases) that degrade the reactive oxygen species and by repair systems (e.g. endonucleases) that repair the damage inflicted during redox stress (Andrews, 1998).

1.6. Understanding bacterial ecology in placer-rich beach sediments

Thus understanding ecological phenomena from all angles form an important basis for managing and harnessing our ecosystems better. This is especially pertinent at coastal and near-shore systems, as these regions form a direct interphase between land and the sea. These are also active sites for human habitation and activity. While ecological studies of beach systems at higher level of trophic echelons have been generally attempted, those at the microbial level have been restricted to very few studies. However, the growing realisation that the microbes especially bacteria are the "unseen majority" and are the main mediators of all biogeochemical processes, they are beginning to occupy the centre stage of all ecological studies. Further, understanding

bacterial interaction in sandy beach sediments rich in placer deposits had not been attempted. Comprehending bacterial distributory patterns, their diversity and their metabolic profiles would help develop early warning systems about the state of ecosystems as these organisms have a short doubling time and therefore high turnover. This type of study would also be pertinent given that beach ecosystems with placer deposits form the focal zones of sand mining along the coasts for the heavy minerals. Also, measuring the environmental impact of anthropogenic activities on sediment microbial processes and diversity is gaining importance for broadening and strengthening our knowledge of ecosystem functioning. In future, these studies would help assessing human-induced changes on beach and associated bay systems. The present study therefore focuses on bacterial interactions in this placer rich sediments with special emphasis on their responses to simulated mining on sandy shores.

Yet another important reason would be to understand the interactions of microbes in these systems with the heavy minerals under varying environmental conditions. Bacterial interaction with heavy minerals like ilmenite would pave way for biotechnological application like exploiting bacterial ability in leaching titanium from these minerals. More interestingly, it would be an opportunity to understand how ecosystems respond to the increased availability of iron due to bacterial interactions. This study on the ecology of coastal and near-shore placer rich sediments is dedicated to understand such responses. It is hypothesised that the steady release of bio-available iron from heavy minerals would have profound effect on bacterial abundance, diversity and activity. Further, it could also impact the primary production in near-shore systems positively, as iron is paradoxically abundant in coastal systems but yet biologically unavailable to that extent, due to complexation with organic molecules in the environment. Though the Ratnagiri beach, the site of study is rich in both ilmenite and magnetite, the laboratory studies revolve mainly around the more abundant ilmenite in these placer rich sediments. Field observations over a year covering all seasons as well as stations along a beach transect which includes Berm, Dune, High, Mid and Low tidal marks give baseline information about the bacterial ecology of

these sediment systems. Simulated mining throws light on the short time *insitu* effect on the bacterial abundance and activity both on the beach and in the water column. *Exsitu* laboratory experiments complement field observation to answer pertinent questions related to the influence of iron released from ilmenite on bacterial growth and activity. This is one of the first few studies attempting to understand bacterial ecology in beach ecosystems influenced by placer minerals.

The present study is an attempt to understand the bacterial interaction in placer sediment with 3 main objectives

1.7. Objectives

1) To study the spatial and temporal variation of bacterial population of the coastal and near-shore placer-rich sediments and their response to the biochemical properties of these sediments
2) To identify the lineages of dominant bacteria and their contribution to the iron oxidation/reduction processes mediated through ilmenite in placers
3) To delineate the response of bacteria to sand mining

Chapter 2

Chapter 2: Review of literature

This chapter covers existing review of literature on placer research.

2.1. Beach placer deposits: Their occurrence in India

The geological aspect as well as the economic potential of the heavy mineral placer deposits has been extensively studied (Mohan and Rajamanickam, 2001). Beach placer deposits such as ilmenite, monazite, rutile etc., have been reported from Tropical regions such as Australia, Egypt, Sri Lanka as well as Temperate zones such as Canada, New Zealand, USA (Jade et al., 2004). In India, these deposits were first discovered in 1909 by a German scientist Schorrberg in Quilon beach sands and the world's first heavy mineral sand production plant was established in India in 1911 at Manavalkurichi (Tamil Nadu). Occurrences of placer deposits from different locations of India's 7000 km coastline have been reported in parts of Gujarat, Maharashtra, Goa, Karnataka, Kerala, Tamil Nadu, Andhra Pradesh, Orissa and West Bengal (Patel, 1936; Siddiquie and Rajamanickam, 1979; Gujar et al., 1989; Ramana et al., 1990; Dutta, 1991; Rao and Wagle, 1997; Ali et al., 2001).

The 720 km coastline of Maharashtra is well known for its placer deposits (Patel, 1936; Krishnan and Roy, 1945; Roy, 1958; Mane and Gowade, 1974; Siddiquie and Rajamanickam, 1979; Siddiquie et al., 1979; Siddiquie et al., 1982; Velayudhan, 1982; Rajamanickam and Gujar, 1984; Siddiquie et al., 1984; Gujar et al., 1986; 1988; 1989; Ramana et al., 1990; Dutta, 1991; Rao and Wagle, 1997). Krishnan and Roy (1945) reported the occurrence of ilmenite placers on the beach, estuaries and offshore region. Siddiquie et al., (1979) suggested that the placer deposits extended about 2 to 5 km offshore and around 9-12 m below sea floor. Occurrences of heavy minerals have been identified around Purangad, Gaonkhede, Randapur, Bhatya, Ratnagiri, Kalbadevi, Newra and Malgund in Ratnagiri district of Maharashtra. On the basis of drainage, hinterland geology, coastal geomorphologic features and

heavy mineral concentrations, Ratnagiri region is divided into three zones, viz, Northern (Arnala to Jaigarh), Central (Jaigarh to Vijaydurg) and Southern (Vijaydurg to Redi point) (Wagle et al., 1989; Gujar, 1995). The Central zone which is a 130 km strip is marked by 13 arcuate bays and tidal inlets. This zone harbors rich deposits of onshore and offshore placers containing appreciable quantities of ilmenite and magnetite (Siddiquie et al., 1982; Rajamanickam, 1983; Gujar, 1995; 1996). Ilmenite concentrations are the highest in Kalbadevi bay followed by Mirya and Ratnagiri bay (Rajamanickam and Gujar, 1984). Studies have shown that Ratnagiri ilmenite does not reveal pronounced alteration to leucoxene. The average concentration of TiO_2 in ilmenite is nearly 52.8% which is close to the theoretical limit of 52.75% TiO_2 in ilmenite (Deer et al., 1966; Ali et al., 1989; Sukumaran and Nambiar, 1994). The general chemical composition of ilmenite is given in Table 2.1.

Table 2.1: Chemical composition of ilmenite (Sukumaran and Nambiar, 1994).

Sample	TiO2 (%)	Total Fe as FeO (%)	Co (ppm)	Cr (ppm)	V (ppm)	Mn (ppm)	Ni (ppm)	Zn (ppm)
Kalbadevi bay	51.25	43.79	194	621	872	2107	84	232
Kalbadevi bay	56.25	37.5	178	773	791	2057	93	197
Kalbadevi bay	50.00	45.08	93	513	661	2210	78	179
Kalbadevi bay	53.13	42.55	153	583	811	2218	94	208
Ratnagiri bay	51.25	40.64	153	724	875	2189	82	223
Ratnagiri bay	53.13	41.47	123	740	849	2344	87	235
Kalbadevi beach	54.38	40.95	114	508	738	2228	86	207
Honnavar beach	50.63	42.76	29	519	100	8891	-	316
Honnawar beach	49.34	43.48	34	1731	120	11769	19	242
Chavara beach	57.50	37.43	9	1106	537	2491	13	211
'Q" grade ilmenite of Quilon sector	60.60	30.01	-	821	840	-	-	-

2.2. Geology of the study area - Kalbadevi

The Kalbadevi Bay is an arcuate bay having creeks towards the Northern and Southern ends. This region is rich in heavy mineral deposits. According to Krishnan and Roy (1945), Mane and Gowade (1974) and Siddique and Rajamanickam (1979), the heavy minerals contains magnetite and non-magnetite fractions and the main source for these minerals are the Deccan traps. The shape of the bay possibly generates converging or circulatory currents during the monsoons, favoring the deposition of sandy material along the shore and silts rich in heavy minerals at the central region of the beach or the bay. Thus the source, geometry of the bay and processes operating in the bay apparently control the distribution of the heavy minerals (Siddiquie et al., 1979). The quantity of sediment input from a mixed origin or change in depositional environment may contribute to the variation in magnetite and ilmenite distribution (Rajamanickam and Gujar, 1984). Bhattacharyya et al., (2007) while studying the size and modal distribution of minerals of offshore and onshore Kalbadevi samples found that the onshore placer sand is rich in ilmenite. However, there was no direct relationship between the heavy mineral variation and grain size (Rajamanickam and Gujar, 1984). Occurrence of seasonal variation in heavy mineral deposition on the beach was more prominent in the berm region (Valsangkar, 2005). Sand formed the dominant component of the sediments (>95%) and was comparatively higher in the pre-monsoon season of May and the January transitional period than during post-monsoon of October (Valsangkar, 2007).

Heavy mineral reserve was estimated to be nearly 25% of the total raw sand reserve of 0.60Mt. Ilmenite constituted nearly 99% of this heavy mineral reserve (Siddiquie et al., 1979). The grade of total heavy minerals (THM) in Kalbadevi is 30-35% and ilmenite constituted nearly 83-92% of THM (i.e. 15-30% of raw sand). Presence of small quantities of limonite, hematite and magnetite was also recorded (Ali et al., 1989; Bhattacharyya et al., (2007). Other heavy minerals in the placer are magnetite and pyroxenes (Sukumaran and Nambiar, 1994).

2.3. Effects of mining on the environment

Ilmenite has been mined from the sandy beaches for its economic importance. Activities like mining and dredging create new environmental conditions. The most obvious effect is often the disturbance or displacement of large quantities of sediment. Monitoring studies prior to dredging, during dredging and post dredging have clearly shown this effect (Nayar et al., 2007). Studies based on the statistics of the fisheries products has shown large negative differences of fisheries products between the period of pre- and post-mining and sand mining has been attributed as the causative factor (Son and Han, 2007). Environmental monitoring studies have reported adverse effect on the resident community structure due to anthropogenic activities (Gray et al., 1988; Heip et al., 1988; Wilson and Elkaim, 1992). Study by Hilton and Hesp (1996) has shown that the risk of sand mining affecting the coastal processes and landforms is high. It adversely affects the marine habitat by causing erosion and plume formation. Sand mining along streambeds is known to alter the hydrology of the stream, thereby affecting flora and fauna due to the change in water quality and salinity. Thus, removal of sand from the seabed not only destroys the habitat and nursery environment but also affects sand dune vegetation and fisheries (Masalu, 2002).

2.4. Studies on the beach environment

Sandy marine sediments cover nearly 70% of coastal zones. It is well known that sandy beaches are subjected to a variety of physical and biological disturbances, which make them one of the most dynamic and challenging natural environments. Several studies have focused on issues pertaining to geomorphology and management of sandy beaches (Hanson et al., 2002; Thomalla and Vincent, 2003), ecological studies relating to faunal community structure and development (Dexter, 1992), macrofauna, meiofauna and phytobenthos (Jaramillo et al., 1996; Lercari et al., 2002; Wilber et al., 2003). In addition, considerable information is available on macro- and meiofauna and their functioning in beach benthic ecosystems (Novitsky and Macsween, 1989; Sundbäk et al., 1996; Albertelli et al., 1999; Fabiano et al., 2002; Moreno et al., 2006; Papageorgiou et al., 2007). The role of sedimentary organic matter in controlling the spatial distribution of the benthic organisms

as well as their metabolism and dynamics in this environment has also been carefully reviewed (Graf, et al., 1983; Bretschko and Leichtfried, 1987; Grant and Hargrave, 1987; Duineveld et al., 1997). In India, the ecology of meiobenthos, macrobenthos (Ansari and Gauns, 1996; Ingole et al., 2006) has also been studied.

However, ecology and composition of microbial communities and their structure in the sandy sediments has received little attention (Nair and Loka Bharathi, 1980; Huettel and Rusch, 2000; Rusch and Huettel, 2000; Wieringa et al., 2000; Rusch et al., 2001; D'Andrea et al., 2002; Rusch et al., 2003; Ishii et al., 2004; de Beer et al., 2005; Bühring et al., 2005). Also, scarce information is available on the horizontal distribution of microorganisms on sandy intertidal beaches especially between high and low tide (Pearse et al., 1942; Meadows and Anderson, 1966; Steele and Baird, 1968; Anderson and Meadows, 1969; Pugh et al., 1974; Meyer-Reil et al., 1978). To date, bacteriological studies of marine beaches are mainly concerned with sanitary pollution and bacterial numbers (Papadakis et al., 1997; Mudryk et al., 2001).

Bacteria inhabiting sandy beaches are well adapted to the extreme conditions of coastal ecosystems and are predominant in these sediments (Novitsky and MacSween, 1989). Bacterial abundance ranges from 10^{8-10} cells g^{-1} dry sand and are generally found attached to the sand grains (Meadows and Anderson, 1966). This abundance increases in finer sediments as well as when the surface area increases (Dale, 1974; Meyer-Reil et al., 1978; Mazure and Branch, 1979). The 10^{8} cells g^{-1} dry sand abundance is not only recorded in the surface sediments but also has been recorded in the sub-surface sediments (McLachlan, 1979). Bacterial abundance varied with the increasing distance from the low tide mark. Bally (1983) while studying the distribution of bacteria in the sediments of a South African sandy beach found that the bacterial abundance was highest at the low water mark and decreased towards the high tide level. However, other studies estimated higher numbers at the high tide mark (Pugh et al., 1974; Anderson, et al., 1981). The microbial activity in the sandy sediments is generally lower than finer sediments and was attributed to lower organic matter content and less microbial cell numbers

(Jickells and Rae, 1997; Llobet-Brossa et al., 1998). The large grain size with relatively low specific surface area results in rather low adsorption capacity and subsequently low microbial activities (Keil et al., 1994; Jickells and Rae, 1997). However, despite their low abundance, these assemblages have high organic matter turnover rates (Llobet-Brossa et al., 1998; Huettel and Rusch, 2000; Rusch et al., 2001; Rusch et al., 2003). In turn, sandy beach sediments harbor microbial communities that are important and metabolically active (Meyer-Reil et al., 1978).

Microorganisms scavenge nutrients required for their growth from the environment, either, directly or indirectly (McLean et al., 1996). These interactions may in turn influence the local environmental chemistry. In indirect reactions, physicochemical sorption of ions onto charged microbial surfaces takes place passively. Subsequently microbial influence on the local solution chemistry may alter the environmental pH and Eh. In direct reactions, processes are metabolically mediated and involve the assimilation of required nutrients and inorganic ions as a source of energy for growth. One such nutrient is iron.

2.5. Interaction of the element iron and its form with living cell

Iron (Fe) is the fourth most abundant element in the Earth's crust comprising nearly 4.3% by mass (Wedepohl, 1995; Canfield et al., 2005). It exists in the trivalent, ferric [Fe(III)] and divalent, ferrous [Fe(II)] oxidation states and can undergo both oxidation and reduction reactions (Murad and Fischer, 1988; Wackett et al., 1989; Turner et al., 2001). Fe is insoluble in aerobic environments at neutral and alkaline pH (Crowley et al., 1987).

Iron is well known for its redox chemistry, photochemistry, biogeochemistry, organic complexation, adsorption/desorption on particles, biological interactions, uptake and cycling by organisms (Haese, 1999; Wessling-Resnick, 1999; Turner and Hunter, 2001; Crosa et al., 2004; Ussher et al., 2004; Butler, 2005; Rickard and Luther, 2007). The abundance of Fe imparts a significant redox buffering capacity. Studies by Stumm and Morgan (1981), Davison (1993), de Vitre et al., (1994), have clearly shown that both Fe(II) and

Fe(III) are important constituents especially in the aquatic environments. They exist as a plethora of solid and dissolved forms. In natural environments, iron present on sediment surface acts as a redox buffer. Under oxic conditions, it builds up as a stock of Fe(III) which is utilized by benthic bacteria during stagnation. In addition, iron binds hydrogen sulfide produced by sulfate reduction to form insoluble and nontoxic sulfides (Propp and Propp, 2001).

Iron is an essential element and is indispensable for growth and proliferation of most of the microorganisms except *Lactobacilli* that lack heme (Archibald, 1983; Briat, 1992). However, Fe has both beneficial as well as detrimental effects. Studies have shown that at lower concentrations, it is essential, and at high levels, it becomes toxic to the cells (Wessling-Resnick, 1999; van Ho et al., 2002; Beutler, 2004; Hentze et al., 2004). Extensive studies on the importance of iron at the cellular level have revealed its importance in biological processes. Importance of iron is derived from its ability to cycle between the two redox states, oxidized Fe(III) and reduced Fe(II). This redox property of iron makes it a suitable catalytic factor for several enzymes that carryout critical biological processes. This includes DNA biosynthesis/repair, respiration, photosynthesis, nitrogen fixation, methanogenesis, H_2 production, the trichloroacetic acid (TCA) cycle, oxygen transport, gene regulation and detoxification of free radicals (Crichton and Ward, 1992, 1998; Sambrook et al., 2001; Andrews et al., 2003; Massé et al., 2003; Andrews, 2005; Ollinger et al., 2006). Thus, it is present in all heme enzymes (proteins having an Fe cofactor), including cytochromes and hydroperoxidases, and is a constituent of ribonucleotide reductase. It is crucial for the activity of nitrogenases and plays a major role in the reduction of oxygen for synthesis of adenosine triphosphate (ATP), reduction of ribonucleotides precursors of DNA and formation of heme (Neilands, 1995). In excess, iron can have a deleterious effect. It reacts with hydrogen peroxide or dioxygen to produce hydroxyl radical, which is a free radical species that is responsible for cellular damage. Damages include lipid peroxidation, oxidation of proteins, and cleavage of DNA or RNA molecules (Halliwell and Gutteridge, 1984). Thus living cells possess mechanism that maintains and ensures that required non-toxic levels of iron are present in the cell (Hentze et al., 2004).

Fe is known to play a critical role in biogeochemical cycling. The production of energy, particularly when Fe is used as an electron acceptor, is important for its impact on the geologic milieu. For example, some bacteria contain a chain of magnetite crystals that allows them to use the Earth's magnetic field to assist in their search for food (Mann and Frankel, 1989). However, these bacteria have in turn produced localized magnetic strips. It is well known that microorganisms can gain energy from both Fe(III) reduction and Fe(II) oxidation. Consequently, these changes in the redox state of iron have a major impact on the biogeochemical cycling of carbon, sulfur, nitrogen, phosphorus and (trace) metals (Haese, 1999). Fe can mediate electron transfer between these two photosynthetic energy reservoirs because of the position of Fe(II)/Fe(III) redox couple at potentials between that of organic matter and molecular oxygen (O_2) (Canfield et al., 2005). Bacteria is known to participate directly in the reduction of Fe by using membrane-bound electron transfer proteins, wherein, intracellular redox transfer processes is coupled to the extracellular reduction of Fe(III) (LaKind and Stone, 1989). Several studies have revealed that increased iron availability has important consequences on the carbon and nitrogen cycling in marine ecosystems. This includes improvement of cyanobacterial nitrogen fixation (Michaels et al., 1996; Falkowski and Raven, 1997), increase in C:Si and N:Si uptake ratios in diatoms (Hutchins and Bruland, 1998; Hutchins et al., 1998; Firme et al., 2003), as well as a reduction in the amount CO_2 respired by heterotrophic bacteria per a given quantity of organic carbon consumed (Tortell et al., 1996). The importance of iron is also characterized by its involvement with regulating coastal and open ocean microbial diversity, controlling primary productivity levels in large areas of the open ocean, and controlling phytoplankton populations in upwelling regions (Ryther and Kramer, 1961; Barber and Ryther, 1969; Kirchman et al., 2000). Over the past three decades iron has become established as an essential micronutrient that can limit primary production in large portions of the ocean (Martin and Fitzwater, 1988; Martin et al., 1989; Martin, 1990; Kolber et al., 1994; Martin et al., 1994; Behrenfeld et al., 1996; Behrenfeld and Kolber, 1999; Boyd et al., 2000).

Although Fe is a vital cell component, the amount required as a nutrient is relatively low. However, it is difficult to meet the requirement of Fe because of its low solubility at physiological conditions. Most microorganisms require micromolar (μM) concentrations of Fe to support growth (Neilands and Nakamura, 1991). Under aerobic conditions, the microorganisms have to deal with ~<10 orders of magnitude between available Fe (~10^{-17} M) and their metabolic requirement for Fe (~10^{-7} M). Thus, In order to obtain it, microbes such as bacteria and fungi use various strategies depending on the environment in which they exist. Hence, microorganisms living in aerobic environments at near-neutral pH acquire Fe under low oxic conditions (Schwertmann, 1991). The most common strategy is the synthesis of small molecules, siderophores, having an affinity for Fe(III) which bind the metal by chelation (Neilands, 1981; Ratledge and Dover, 2000). Microbial siderophores are typically classified as catecholates, hydroxamates, and α-carboxylates, depending on the chemical nature of their coordination sites with iron (Winkelmann, 1991, 2002). Several marine siderophores have been characterized (Haygood et al., 1993; Reid et al., 1993; Martinez et al., 2000, 2001), and their production is ubiquitous in marine bacterial isolates (Trick, 1989; Reid and Butler, 1991; Haygood et al., 1993; Pybus et al., 1994; Winkelmann et al., 2002). The production of siderophores by marine bacteria has been confirmed for several heterotrophic isolates from different water bodies (Reid and Butler, 1991; Pybus et al., 1994; Winkelmann et al., 2002).

When Fe(II) comes into contact with O_2 or other suitable oxidants, Fe(II) is reoxidized to insoluble Fe(III), which eventually precipitates as Fe(III) oxyhydroxides (Taillefert et al., 2000). At circumneutral pH, Fe(II) is subject to rapid chemical oxidation by dissolved O_2 (Davison and Seed, 1983; Millero et al., 1987), and the Fe(III) produced eventually hydrolyzes and precipitates through the overall reactions such

$$Fe^{2+} + 0.25\ O_2 + 2.5H_2O \rightarrow Fe(OH)_3 + 2H^+ \quad \Delta G^{\circ\prime} = -109\ kJ\ mol^{-1}.$$

The half-life of dissolved Fe(II) in air-saturated water is of the order of few minutes. Hence, bacterial Fe(II) oxidation with O_2 as an electron acceptor is usually considered doubtful from a geochemical perspective due to the speed

of the above spontaneous abiotic reaction (Davison and Seed, 1983). Paradoxically, bacteria have been linked with circumneutral Fe(II) oxidation and Fe(III) oxyhydroxide deposition for almost a century (Ghiorse, 1984; Ehrlich, 1995). A review by Emerson (2000) presents an excellent overview of the history of research on circumneutral bacterial Fe(II) oxidation, as well as the physiology and systematics of known chemolithotrophic Fe(II)-oxidizing bacteria (FeOB). Further, the potential for both anaerobic phototrophic (Widdel et al., 1993; Ehrenreich and Widdel, 1994) and chemolithotrophic nitrate-reducing bacteria (Straub et al., 1996; Benz et al., 1998; Straub and Buchholz-Cleven, 1998) to catalyze circumneutral Fe(II) oxidation is now being recognized (Straub et al., 2001).

The dominant role of microbial catalysis in Fe(II) oxidation in acidic environments (e.g. acid mine drainage and acid hot springs) is well-established (Brock et al., 1972; Singer and Stumm, 1972; Johnson et al., 1993). The biological oxidation of ferrous sulphate by acidophilic iron and sulphur oxidising bacteria has been studied extensively (Lizama and Suzuki, 1989; Jensen and Webb, 1995; Nemati et al., 1998). Comprehensive studies have focused on the rate of biological oxidation of ferrous iron and the effects of substrate Fe(II) and product Fe(III) concentrations, dissolved oxygen, CO_2 concentration, bacterial cell density, temperature, pH, etc., on the biological Fe oxidation (Liu et al., 1988; Pesic et al., 1989; Harvey and Crundwell, 1997; Malik, 2000; Dastidar et al., 2000).

Reduction of ferric iron, Fe(III) to ferrous iron, Fe(II), can be simply expressed as $Fe^{3+} + e^- \rightarrow\rightarrow Fe^{2+}$

The Fe (III) that is reduced and the Fe(II) that is produced may exist in a variety of forms. In most environments, Fe(III) is highly insoluble and occurs in a variety of amorphous and crystalline mineral forms (Nealson, 1983; Johnson, 1995). Due to its low solubility, Fe(III) tends to accumulate in particulate form, mostly as ferric (hydr)oxides (Murray, 1979), alumino-silicates (Raiswell and Canfield, 1998), and ferric phosphate phases (Buffle et al., 1989; Hyacinthe and van Cappellen, 2004). It was generally considered that Fe(III) reduction in sedimentary environments was primarily an abiotic

process (Fenchel and Blackburn, 1979; Ghiorse, 1988) and this abiotic concept dates back to some of the earliest studies on microbial Fe(III) reduction (Starkey and Halvorson, 1927). However, recent studies with environmental samples have indicated that Fe(III) reduction in soils and sediments has a biological basis. In addition to its importance in modern environments, Fe(III) respiration has also been suggested to be one of the earliest forms of respiration on ancient Earth (Lovley, 2000).

One of the recognized roles of iron is that Fe(III) serves as an electron acceptor for microbial respiration in a typical marine sediment (Ponnamperuma, 1972; Reeburgh, 1983). Respiration with ferric oxides as the electron acceptor is commonly referred to as dissimilatory ferric reduction (Lovley, 1987a,b; 1991; 2000). Fe(III) respiration is coupled to a substantial portion of organic matter remineralization in the surface sediments of marine and freshwater environments (Kostka et al., 2002). Under suboxic conditions, ferric iron is a potential electron acceptor for organic matter degradation (Froelich et al., 1979; Lovley and Phillips, 1988). Studies in freshwater (Jones et al., 1983; Jones et al., 1984; Lovley and Phillips, 1986a,b; Roden and Wetzel, 1996) and marine (Canfield, 1993; Thamdrup et al., 1994; Thamdrup, 2000; Jensen et al., 2003) habitats indicate that dissimilatory ferric reduction can contribute substantially to the oxidation of organic compounds. The extent of organic matter decomposition depends on the availability of Fe(III) for microbial reduction. The in situ dissimilatory iron reduction is influenced by a variety of factors, including the microbial community structure and biomass (Dollhopf et al., 2000), the quality and quantity of the organic matter (Chen et al., 2003), and the type and abundance of Fe(III) minerals (Bonneville et al., 2004).

Iron in sediments often exists as poorly crystalline Fe(III) oxides (Lovley, 1993; Nealson and Saffarini, 1994). In sedimentary environments, iron can be found as discrete iron minerals (Fe sulfides, carbonates, oxides) or in the form of structural iron, iron bound within the lattice of phyllosilicates (Dixon, 1998). Iron (oxyhydr)oxide minerals are a major constituent of rocks and soils, and account for approximately 40–45% of the total iron content of sediments

supplied to the global ocean (Poulton and Raiswell, 2002). Iron oxides and oxyhydroxides which may occur in marine environments are goethite (α-FeOOH), akaganéite (β-FeOOH), lepidocrocite (γ-FeOOH), feroxyhite (δ'-FeOOH), ilmenite ($FeTiO_3$), hematite (α-Fe_2O_3), magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) (Burns and Burns, 1981). These ubiquitous minerals form the reactive component of the soils, sediments and aquifers. Further, they exhibit large surface areas which bind trace metals, nutrients and organic molecules (Bonneville et al., 2004). Under suboxic conditions, they are reductively dissolved via a number of alternative abiotic and microbial pathways (Lovley, 1987; van Cappellen and Wang, 1996; Roden and Wetzel, 2002). They serve as terminal electron acceptors for the oxidation of organic matter by iron reducing bacteria. The metabolic activity of iron reducing bacteria may further enhance the natural or engineered bioremediation of contaminated sites (Lovley, 1995; McCormick et al., 2002). The rate and extent of microbial reduction of Fe(III) oxyhydroxides are influenced by a variety of factors, including the microbial community structure and biomass, the type and abundance of Fe(III) oxyhydroxides, and the sorption affinity between the oxide phases and bacteria (Caccavo et al., 1992). In addition, the Fe(III) reduction rate may be inhibited by adsorption on the Fe(III) solid (Roden et al., 2000) or cell surface (Urrutia et al., 1998) of Fe(II) formed as by-product of microbial Fe(III) reduction.

In addition to being an important oxidation pathway of organic matter degradation and generating soluble ferrous iron, microbial iron reduction has a major impact on the persistence and mobility of metals, phosphate, radionuclides and organic contaminants (Anderson and Lovley, 1999; Roden and Edmonds, 1997). Studies have demonstrated that the mechanism for microbial reduction of Fe(III) in sedimentary environments is an important process for catalyzing a large number of natural and contaminant biogeochemical cycles (Nealson and Saffarini, 1994; Lovley, 2000; Lovley and Anderson, 2000). Further, the fate of organic and inorganic contaminants have also been linked to the activities of Fe(III)-reducing bacteria in subsurface sediments (Lovley and Anderson, 2000).

Fe(III) can be present in soils in various forms (Schwertmann and Taylor, 1977). The form and concentration of reactive Fe(III) minerals are important to the environmental significance of microbial Fe(III) reduction in sedimentary environments. The most commonly occurring compounds in iron minerals are haematite (Fe_2O_3), magnetite (Fe_3O_4), limonite ($2Fe_2O_3.3H_2O$), siderite ($FeCO_3$) and pyrite (Greenwood and Earnshaw, 1984). Microbial transformations of many these have been studied in detail. The kinetics of abiotic reductive dissolution of Fe(III) iron oxyhydroxides by organic reductants have also been extensively studied (Kuma et al., 1993; Postma, 1993; Larsen and Postma, 2001). Larsen and Postma (2001) have shown that the Fe(III) reduction rate by ascorbate, normalized to the mineral surface area, decreases in the order ferrihydrite 2-lines>ferrihydrite 6-lines>lepidocrocite> goethite>hematite. Study by Brown et al., (1997) has shown that a microbial consortium was able to mediate the transformation of 11% of the Fe in a sample of magnetite into hematite. Associations of the cell with the mineral surface also have been studied. In one study using an iron-oxidizing archaea, Larsson et al., (1993) showed that close contact between microorganisms and pyrite was necessary for optimum growth and oxidation rate. Further, results of the study by Edwards et al., (1998) showed that interaction between attached cells and pyrite surface is highly specific. The less crystalline the Fe(III) form, the more readily it is microbially reduced. Ottow (1969) found that the extent of microbial reduction over a five day incubation period in bacterial cultures decreased with increasing crystallinity of the Fe(III) form and followed the sequence $FePO_4.4H_2O$> $Fe(OH)_3$> γ- FeOH > Fe_2O_3. Similar results were reported in other studies (Troshanov, 1969; de Castro and Ehrlich, 1970; Jones et al., 1983; Jones et al., 1984; Pfanneberg and Fischer, 1984; Lovley and Phillips 1986a,b; 1987b). Similarly Fe(III) bound in clay minerals is considered to be an important electron acceptor supporting the growth of bacteria in soils or sedimentary environments. Kostka et al., (2002) demonstrated that a pure culture of *Shewanella oneidensis* strain MR-1 as well as enrichment cultures of Fe(III)-reducing bacteria from rice paddy soil and subsurface sediments are capable of conserving energy for growth with

the structural Fe(III) bound in smectite clay as the sole electron acceptor. Cell counts showed that similar growth of *S. oneidensis* (10^8 cells mL^{-1}) occurred on other Fe forms [amorphous Fe(III) oxyhydroxide, and Fe citrate] or oxygen as the electron acceptor. However, cell yields of *S. oneidensis* measured as the increase in cell carbon were similar on all Fe forms tested while yields on oxygen were five times higher, in agreement with thermodynamic predictions. Over a range of particle loadings (0.5 to 4 g L^{-1}), the increase in cell number was highly correlated to the amount of structural Fe in smectite reduced. Results also indicated that the growth with smectitic Fe(III) is similar in magnitude to that with Fe(III) oxide minerals and is dependent upon the mineral surface area available.

Reduction of ferric iron may be enzymatically catalyzed (de Castro and Ehrlich, 1970). Sometimes it is coupled to electron transport chain and in the process generates ATP (Lovley, 1987a). Also ferric iron reducing organisms may metabolize fermentable substrates such as glucose, and transfer only a minor portion of the electron equivalents in the fermentable substrates to ferric iron (Lovley, 1987b). de Castro and Ehrlich (1970) demonstrated that *Bacillus* sp. reduce Fe(III), enzymatically. Fe(III) was not reduced if the glucose medium was not inoculated with the organism or if glucose was omitted from the inoculated medium. Further more Fe(III) was not reduced when uninoculated medium was acidified. This demonstrated that the decrease in pH during bacterial metabolism did not chemically reduce Fe(III). The cell free filtrate of an actively growing culture did not reduce Fe(III) and Fe(III) reduction in cell extracts was diminished if glucose was omitted. Munch and Ottow (1983) demonstrated that the reduction of Fe(III) during glucose metabolism by *Clostridium butyricum* or *Bacillus polymyxa* was not due to the production of fermentation products or a low redox potential. Reduction of Fe_2O_3 was inhibited if the Fe_2O_3 in the culture was enclosed in dialysis tubing, even though the redox potential and pH were as low as in cultures in which the organisms and the Fe_2O_3 were not segregated. These results indicated that direct contact between the bacteria and Fe(III) was required for Fe(III) reduction and suggested that microbial enzymes were necessary to catalyze the reaction. The initiation of Fe(III) reduction was associated with the

attachment of bacteria to Fe(III) particles in Fe(III)-reducing enrichment cultures (Tugel et al., 1986). The need for physical contact for Fe(III) reduction may depend upon the organism, substrate, and form of Fe(III). In a medium containing $FeCl_3$, extracellular components of a glucose-fermenting *Vibrio* sp. reduced Fe(III), at a rate that was about 30% of the rate of Fe(III) reduction in the culture (Jones et al., 1983). However, with a malate-fermenting *Vibrio* sp., there was no Fe(III) reduction in the absence of cells (Jones et al., 1984). Although it was previously considered that Fe(III)-reducing microorganisms must come into direct contact with Fe(III) oxides in order to reduce them especially due to the low solubility of Fe(III) (Munch and Ottow, 1983; Pfanneberg and Fisher, 1984), recent studies have suggested that electron-shuttling compounds and/or Fe(III) chelators, either naturally present or produced by the Fe(III)-reducing microorganisms themselves, may alleviate the need for the Fe(III) reducers to establish direct contact with Fe(III) oxides. Studies with *Shewanella alga* strain BrY and Fe(III) oxides sequestered within microporous beads demonstrated that it released compound(s) that permitted electron transfer to Fe(III) oxides when the organism cannot directly make contact. Nearly 450 μM of dissolved Fe(III) was detected in cultures of *S. alga* growing in Fe(III) oxide medium, suggesting that this organism released compounds that could solublize Fe(III) from Fe(III) oxide (Nevin and Lovley, 2002).

A wide range of substrates can potentially serve as electron donors for Fe(III) reduction by diverse organisms. Dissimilatory iron reduction is important to a number of natural processes such as the biogeochemical cycling of iron, carbon, and other elements and the weathering of ferric iron-bearing clays and minerals (Fredrickson and Gorby 1996; Kostka et al., 1996). It has been suggested that most Fe(III) reduction in anoxic freshwater and marine sediments is mediated by dissimilatory iron-reducing bacteria (Lovley, 1991). Dissimilatory iron-reducing bacteria couple the oxidation of reduced organic and inorganic compounds to the reduction of Fe(III) in energy-conserving reactions that result in cell growth (Brock and Gustafson, 1976; Lovley et al., 1997). Fe(III) respiration is an important process primarily in anoxic environments. Although some microorganisms may reduce Fe(III) in the

presence of oxygen (Arnold et al., 1990), in the aerobic environments the Fe(II) produced will react with oxygen and be converted back to Fe(III). Generally, there is also no net Fe(III) reduction in the presence of nitrate due to a combination of preferential reduction of nitrate by Fe(III)-reducing microorganisms (DiChristina, 1992) and biological oxidation of Fe(II) with nitrate (Straub et al., 1996; Straub and Buchholz-Cleven, 1998). There is a possible abiological oxidation of Fe(II) by nitrite as well as Fe(II) is rapidly oxidized abiotically with Mn(IV) (Lovley, 1991). Thus, in a typical aquatic sediments or subsurface environments, Fe(III) reduction typically takes place prior to sulfate reduction and methane production, because Fe(III) reducing microorganisms can outcompete sulfate-reducing and methanogenic microorganisms for electron donors (Lovley and Phillips, 1987a; Chapelle and Lovley, 1992).

Iron reducing bacteria belonging to various taxonomic groups have been isolated from different environments and characterized during the past two decades (Lovley, 2000). The extent of diversity among dissimilatory metal reducers has been reviewed by Lovley (1993) and Nealson and Saffarini (1994). Dissimilatory Fe(III) reducers have been isolated from deep aquifers (Lovley et al., 1990), estuarine sediments (Caccavo et al., 1992), petroleum contaminated soils (Caccavo et al., 1994), oil field fluids (Semple and Westlake, 1987), and thermal hot springs (Slobodkin et al., 1997). Majority of the dissimilatory iron reducers are placed within γ and δ subclasses of the Proteobacteria. Several other unique lineages have also been revealed using phylogeny studies inferred from 16S rRNA gene sequences (Caccavo et al., 1996; Lonergan et al., 1996; Lovley et al., 1997). Studies have shown that these organisms have the capacity to alter the chemistry of the environments in which they reside (Fredrickson and Gorby, 1996; Kostka et al., 1996). These iron reducing bacteria are usually capable of utilizing oxygen and nitrates and change to iron reduction only in the absence of more active oxidizers (Sorensen, 1982; Lovley, 1991; Propp et al., 1992). The role of nitrate-reducing bacteria in the oxidative part of the iron cycle has also been

the subject of a number of recent studies (Emerson, 2000; Straub et al., 2001).

2.6. Microbial transformations of ilmenite by microorganisms

Information on the microbial role in the reduction of ilmenite is very limited. Ilmenite has been found to stimulate the growth of lichen, *Marchantia polymorpha* (Hoffman, 1974) as well as cyanobacteria (Brown et al., 2008). However, it is still widely believed to be a recalcitrant mineral (Page and Huyer, 1984; Page and Grant, 1987).

Chapter 3

Chapter 3: Materials and Methods

3.1. Study area

Kalbadevi Bay, Ratnagiri is well known for its fishery resources and is also a potential mining site. The bay is arcuate in shape and the associated beach is nearly 5 km long and ~ 250 m wide with creeks on either ends, Are Creek at the North and Kalbadevi Creek at the south. These creeks are responsible for the deposition of major placer minerals comprising mainly of ilmenite and magnetite, which appear as black particles embedded in beach sand (Plate 3.1 a, b). Study by Siddiquie et al., (1984) has reported heavy minerals to range from 7-79 % in Kalbadevi sediments.

3.2. Location of the stations

3.2.1. Intertidal

The intertidal sampling area was located at Kalbadevi beach, Ratnagiri, India (Fig 3.1). Global positioning system (GPS Hand Module 5000) was used to determine the positions of stations in the intertidal region. The geographical locations of the sampling stations are given in Table 3.1.

1) Northern Transect (BP1): is towards the Northern side of the beach.

2) Central Transect (BP2): is located at the Center of the beach.

A study by Valsangkar (2005) has shown that heavy mineral content in the berm sediments along the studied transect is above 60% during pre-monsoon while at the other stations it is usually between 10 to 20%. The vegetation in the area comprised of sand dune, seaweeds and mangroves (Plate 3.1 c, d). The sand dune vegetation comprised of 17 species (Anon, 2004). *Ipomoea pes-caprae* was found to be the dominant species covering about 65% of the dune area.

3) Southern Transect (BP3): is located towards the southern end of the beach. The sand dune vegetation comprised of nearly 9 species and *Ipomoea pes-caprae* covers nearly 60% of the dune area (Anon, 2004). Nearly 18 species of drifted seaweed species have been recorded at this transect indicating seaweed beds in the sub tidal region (Anon, 2004).

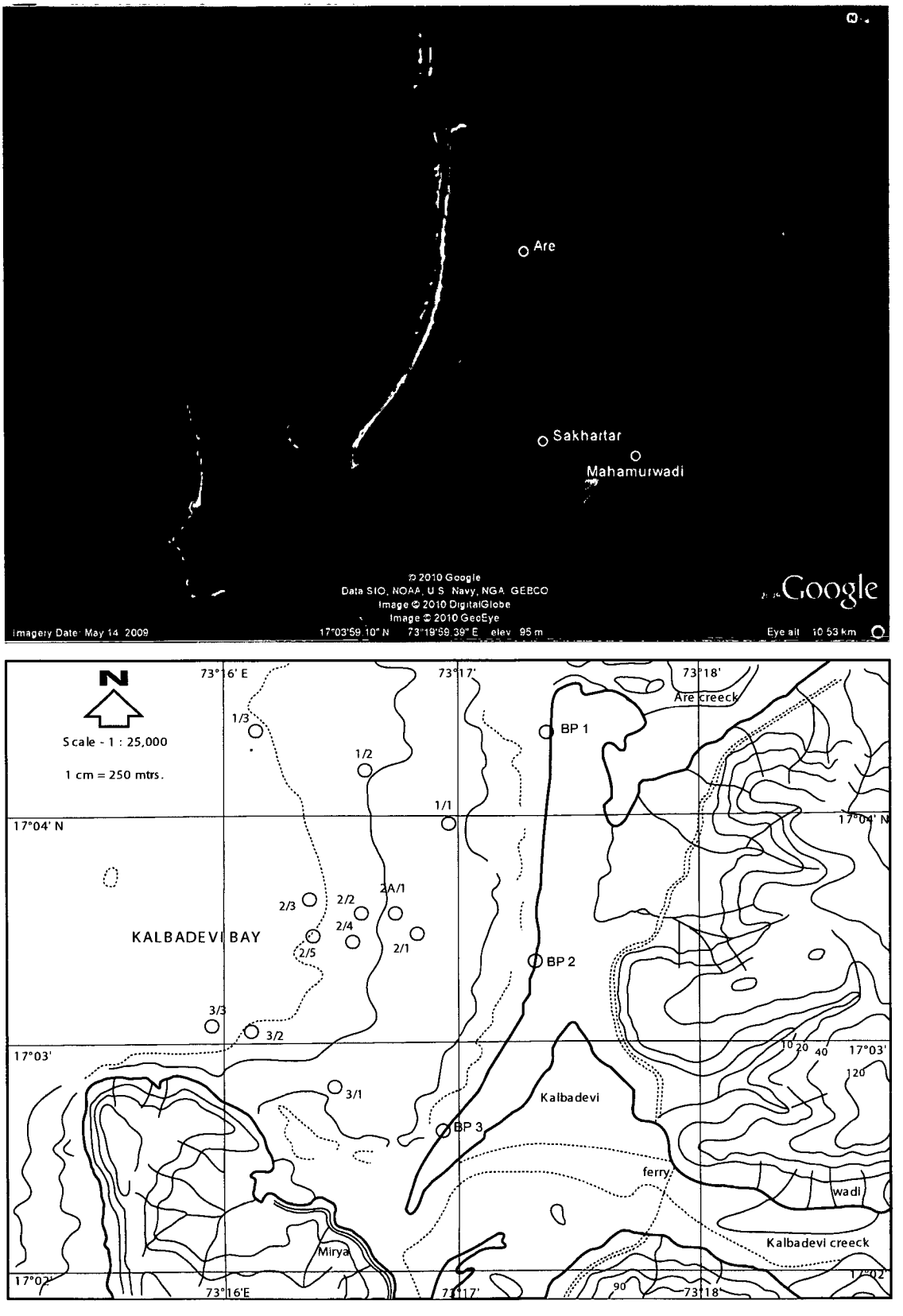


Fig 3.1: Location of sampling site in Kalbadevi

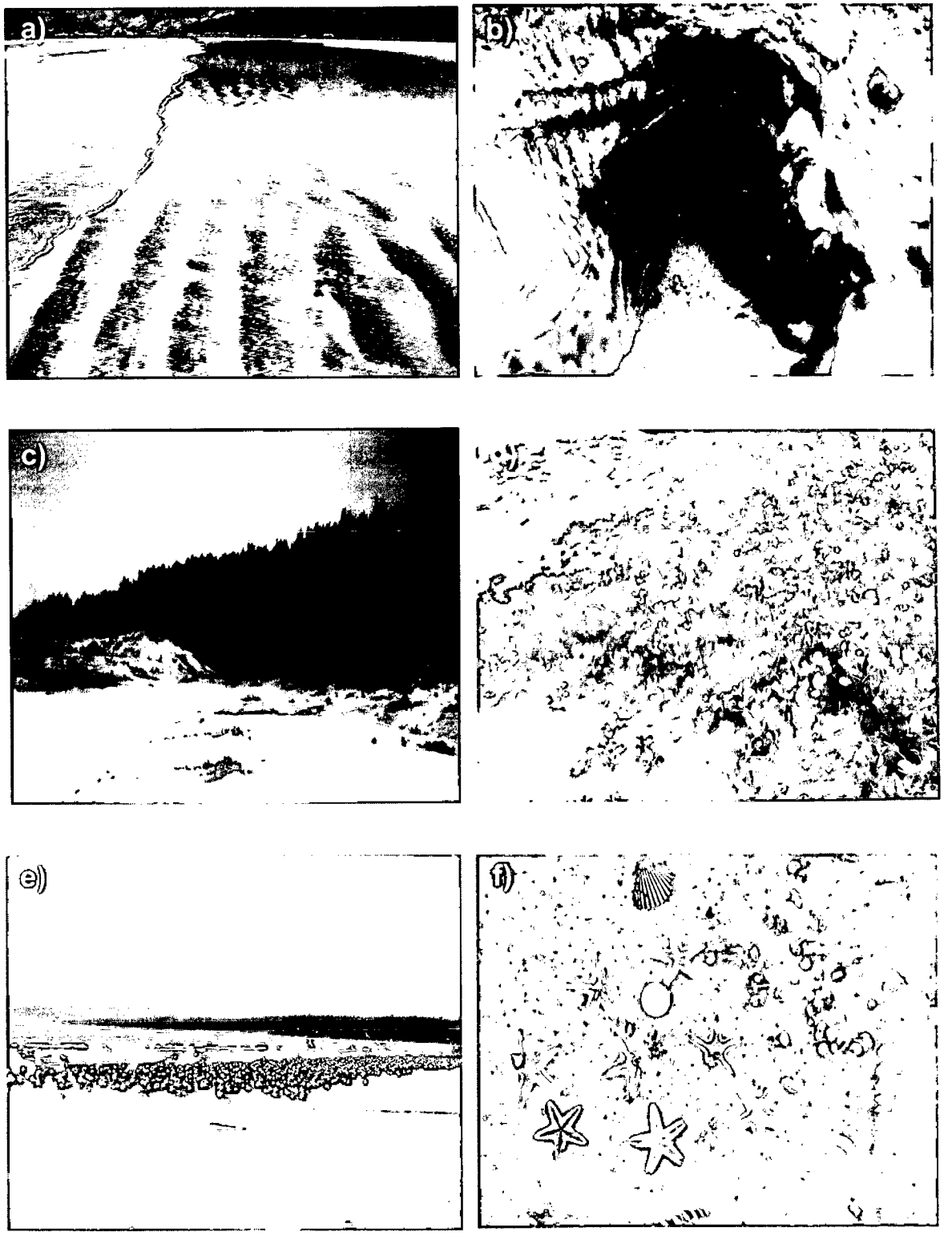


Plate 3.1: Surface deposition of ilmenite in Kalbadevi (a)
Cross-sectional view of ilmenite deposition (b)
Flora of Kalbadevi (c-d)
Fauna of Kalbadevi (e-f)

Table 3.1: Geographical location of the sampling stations in intertidal region

Transect	Latitude (N) (Deg Min)	Longitude (E) (Deg Min)	Stn No.	Station type	Distance between 2 consecutive stations (m)
Northern	17° 04.07'	73° 17.32'	BP1/1	Dune	
			BP1/2	Berm	5
			BP1/3	High tide	52.3
			BP1/4	Mid tide	51.7
			BP1/5	Low tide	33
Central	17° 03.41'	73° 17.29'	BP2/1	Dune	
			BP2/2	Berm	5.6
			BP2/3	High tide	47.5
			BP2/4	Mid tide	29.3
			BP2/5	Low tide	38.5
Southern	17° 02.68'	73° 16.93'	BP3/1	Dune	
(BP3)			BP3/2	Berm	14.2
			BP3/3	High tide	18.5
			BP3/4	Mid tide	47.6
			BP3/5	Low tide	29.8

3.2.2. Sub-tidal

The sub-tidal transects were extensions of the intertidal beach transects in the bay. The geographical locations of these stations are indicated in table 3.2.

Table 3.2: Geographical location of the sampling stations in sub-tidal region.

Transect	Stn No.	Latitude (N) (Deg Min)	Longitude (E) (Deg Min)	Water depth (m)
Northern	OP1/1	17° 03.950'	73° 16.950'	5
(OP1)	OP1/2	17° 04.166'	73° 16.583'	8
	OP1/3	17° 04.667'	73° 16.166'	10
Central	OP 2A/1	17° 03.550'	73° 16.716'	5
(OP2)	OP 2/1	17° 03.466'	73° 16.800'	5
	OP2/2	17° 03.400'	73° 16.566'	8
	OP2/3	17° 03.450'	73° 16.383'	10
Southern	OP 3/1	17° 02.798'	73° 16.450'	5
(OP3)	OP3/2	17° 03.050'	73° 16.116'	8
	OP3/3	17° 03.066'	73° 15.933'	10

3.3. Field sampling and sample collection methods

3.3.1. Sample collection

3.3.1.1. Sample collection in the intertidal zone

Sampling was conducted along three transect in the North, Central and Southern parts of the beach. Pre-monsoon sampling was carried out during February 2004, May 2004 and April 2005. Monsoon sampling was conducted during August 2004 and post-monsoon during November 2004. Perpendicular shore transect extending from the dry to surf zone was sampled. The beach was divided into five locations namely: Dune (Stn D), Berm (Stn B), High tide (Stn H), Mid tide (Stn M) and Low tide (Stn L) level (Fig. 3.2). Stn D and B comprised of the dry zone while Stn H, M and L comprised of the wet intertidal zone. Sampling for Northern and Southern transects was restricted to high tide and mid tide area from May 2004 onwards due to logistics reasons. At each location, samples were collected using push cores and auger. The sampling distance between push core and auger was 1 m. Push cores made of plexiglass (inner diameter = 10 cm) were used to collect sediment up to a depth of 20 cm. Each core sample was divided into 0-5, 5-10, 10-15, 15-20 cm using plexiglass (Fig 3.2). Sediment layer at 20-40 cm depth was collected using an auger (Plate 3.2.). Sub-samples for biological and geochemical analyses were temporarily stored at 4(±2) °C for transportation to the laboratory.

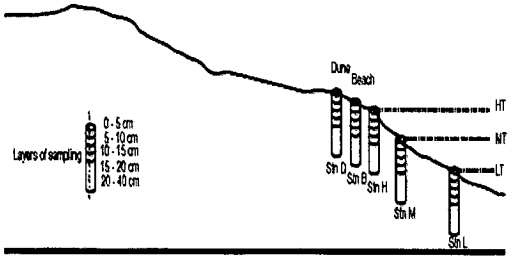


Fig 3.2: Intertidal sampling on the beach divided into five locations i.e. Dune (Stn D), Berm (Stn B), High tide (Stn H), Mid tide (Stn M), Low tide (Stn L).

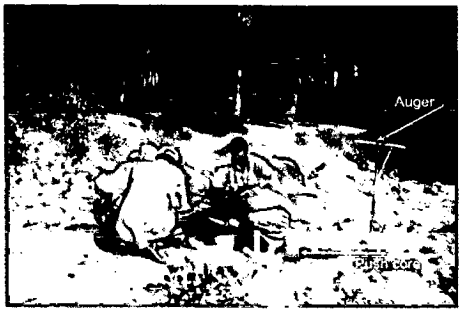


Plate 3.2: Collection of sediment samples using push core and auger

3.3.1.2. Sample collection in the sub-tidal area

Sampling was conducted along offshore extensions of the three beach transect in the North, Central and Southern parts of the beach. Sampling at each transect was carried out at water depth of 2 m, 5 m and 8 m during post-monsoon (January 2006). However sampling during pre-monsoon (May 2006) and monsoon (September 2006) was restricted to OP2 and OP3 due to sand bar formation.

Water samples were collected using Niskin water sampler. A Tygon™ tubing was attached to the outlet and aliquots of seawater for microbiological analyses was collected into clean polyethylene bottles. The samples were immediately stored at 4(±2) °C until analyses. Samples were collected for estimation of Chlorophyll *a*, primary productivity, and phytoplankton abundance and methods have been discussed in section 3.4.

Sediment samples were collected using the van Veen grab (0.04 m^2). Sub-samples for microbiological and biochemical analyses were stored at 4(±2) °C until analyses.

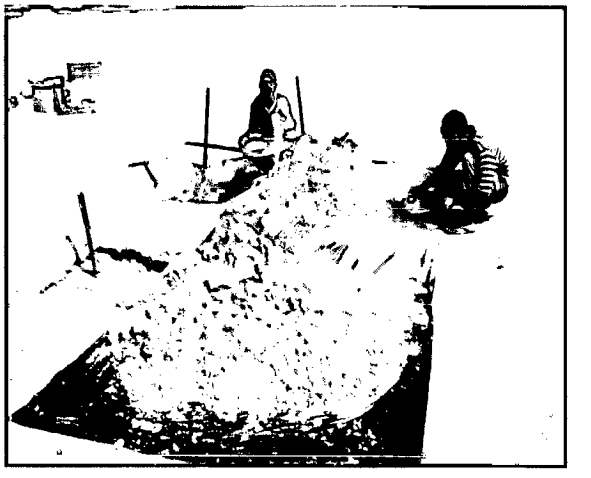
3.3.2. Sample collection during simulated mining

3.3.2.1. Simulated mining in the intertidal zone

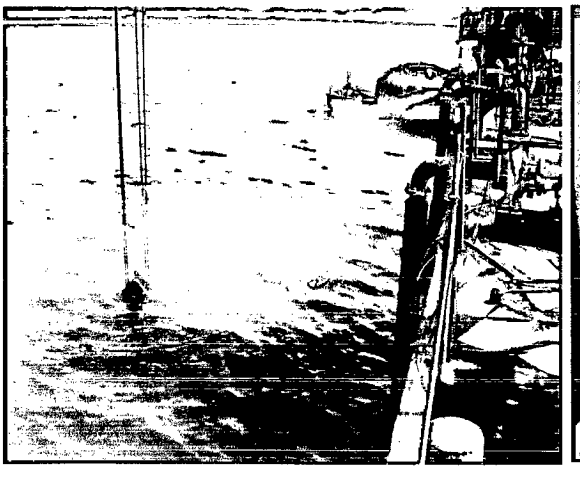
In order to understand the impact of placer mining in the study area, the central transect (BP2) was chosen for physical disturbance. A simulated disturbance was conducted by making 1 x 1 x 1 m pits, at five different locations representing dune (Stn D), berm (Stn B), high tide (Stn H), mid tide (Stn M) and low tide (Stn L) level (Plate 3.3 a, b). An effort was made to remove black sand mineral grains from the excavated part by panning and chaffing and returning the major lighter fraction to the area of collection in order to simulate the coastal mining. The experiment was conducted in February 2004 and the sampling was carried before (phase I: PI), immediately after the disturbance (phase II: PII) and 24 h after disturbance (phase III: PIII). Sediment collection up to 40 cm depth was carried out as mentioned in section 3.3.1.1. Sub-samples for microbiological and biochemical analyses were stored at 4(±2) °C for further analyses

a)

b)



c) suction



d) discharge

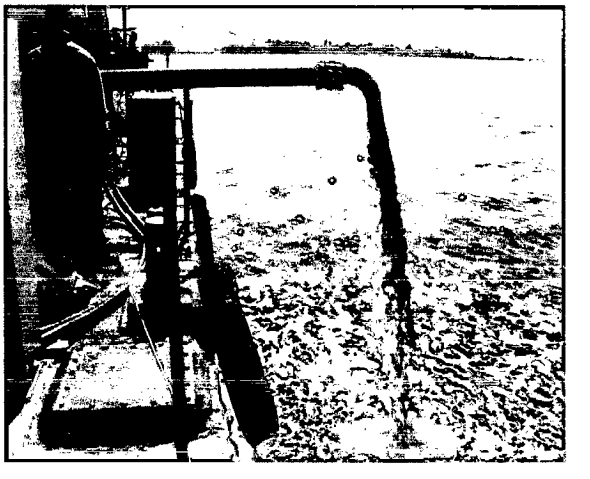


Plate 3.3: Simulated mining at Kalbadevi
(a-b) onshore beach mining
(c-d) offshore subtidal mining

3.3.2.2. Simulated mining in the sub-tidal area

Simulated mining was conducted at 6.5 m water depth along the central transect in Kalbadevi Bay and comprised of suctioning and discharging of the offshore sediments using a hydraulic jet pump that was developed by Central Mechanical Engineering Research Institute (CMERI), Durgapur. The dredging equipment on a Pontoon (40x11 m) had a pumping capacity of ~180 cu.m h^{-1} of slurry. The loosening of sand was produced by series of high-pressure water jets created by high-pressure pumps. The rotary agitators that were attached just below the pump continuously agitated the sand on the seabed and fed the dredging pumps (Samaddar et al., 2005). The dredging pumps were operated each time for duration of 20 min. The collected slurry from the input suction point was then disposed at the output ejection point (Plate 3.3. c, d). During the simulated mining, water and sediment samples were collected from three different locations namely Suction point (S), Discharge point (D) and Reference point (R).

Water samples were collected from two depths (1 m and 3 m below surface) using Niskin water sampler while surface sediment samples were collected using van Veen grab (0.04 m^2) at afore-mentioned three different locations. The experiment was conducted in February 2006 and the sampling strategy for water and sediment collection is given in table 3.3. Sub-samples for microbiological and biochemical analyses were stored at 4(±2) °C until analyses.

Table 3.3: Stages of water (W) and sediment (S) collection during simulated mining in the bay.

Time	Phase	Notation	Samples
-3 h before disturbance	Pre disturbance	PD	W/S
0 h	During disturbance	DD	W
+2 h after disturbance	2 h after disturbance	MM	W/S

3.4. Processing of samples

3.4.1. Sediment samples

3.4.1.1. Microbiological parameters

Sediment sample was thawed and a sub-sample of ~5 g wet sediment was transferred to 45 mL of filter sterilized half strength seawater (10^{-1} dilution). Aliquot of this suspension was used to enumerate total bacterial abundance, total direct viability and retrievability. Initial weight of the sediment used in the dilution was determined by drying the filtered sediment at 60 °C.

3.4.1.1.1. Direct total bacterial counts (TC)

Total direct counts were estimated by using Acridine Orange Direct Count (AODC) method (Hobbie et al., 1977; McFeters et al., 1991). Acridine orange is a fluorochrome that intercalates with nucleic acids. Bacterial cells stained with acridine orange either appear green or orange depending upon the DNA or RNA content in the cell.

An aliquot of 5 mL sample from 10^{-1} dilution was fixed with pre-filtered buffered formaldehyde (2% final concentration, FC) and stored at 4(±2) °C until analyses. One milliliter of this sub-sample was stained for 5 min with 0.1% acridine orange stain and then filtered through 0.2 μm pore size black isopore polycarbonate filter paper (Millipore). Additions were done as described in appendix I. Stained cells were counted using Nikon 80*i* epifluorescence microscope. Blanks were routinely checked for bacterial contamination. Cells were counted and the total counts (TC) were expressed as number of cells per gram.

3.4.1.1.2. Direct total viable counts (TVC)

The direct total viable counts method permits enumeration of substrate-responsive bacteria present in a sample (Kogure et al., 1979). Cell division was inhibited by the addition of nalidixic acid while growth was enhanced by the addition of yeast extract resulting in large cells that could be easily differentiated from those that do not respond to the added substrate. Elongated and enlarged cells with a clear visible invagination were counted as viable cells.

For estimation of direct viable counts under aerobic conditions (TVCa), an aliquot of 5 mL sample from 10^{-1} dilution was incubated with antibiotic cocktail and yeast extract solution (Appendix I). A reductant ($Na_2S.9H_2O$) at 0.125% (FC) was added to induce anaerobic conditions for estimating direct viable counts under anaerobic conditions (TVCan). After incubation period of 7 h (pre standardized), the sediment samples were fixed and stained as described in section 3.4.1.1.1. Numbers were expressed as number of cells per gram.

3.4.1.1.3. Retrievable counts (Colony forming units)

The 10^{-1} dilution was further serially diluted up to 10^{-6} dilution with autoclaved seawater.

3.4.1.1.3.1. Retrievable counts of heterotrophs (RC)

Retrievable heterotrophic population was assessed on plates prepared with 0.001, 0.01 and 10% strength nutrient broth (NB; 100% = 1.3g, *HiMedia Laboratories Ltd., India*) in seawater and 1.5% agar by spread plate method. A 100 µL innoculum from each dilution was used to spread plate on the different strength nutrient amended plates. The plates were incubated at room temperature (RT = 28± 2°C) for 24 h. RC was determined in terms of colony forming units (CFU) and expressed as CFU per gram.

3.4.1.1.3.2. Retrievable counts of iron bacteria (IB)

Retrievable iron bacterial population was enumerated using media described by Rodina (1972, Appendix I). An aliquot of 100 µL from each dilution was spread plated. The plates were incubated at RT and counted after 15 d. IB were determined in terms of colony forming units (CFU) and expressed as CFU per gram.

3.4.1.1.3.3. Retrievable counts of iron bacteria using agar shake method (IR)

Retrievable iron bacterial population was also enumerated using modified Winogradsky media (Rodina, 1972; Appendix I). In this method, 14 mL screw capped tubes containing 12 mL of the medium and one mL from each dilution was gently tilted to allow mixing and then allowed to set. A sterile mixture of paraffin wax and oil (2:1w/v) was then gently poured on the top to maintain

anaerobiosis. The tubes were incubated at RT. IR were enumerated after 15 d and expressed as CFU per gram.

3.4.1.2. Microbial Diversity

3.4.1.2.1. Diversity of bacterial isolates

3.4.1.2.1.1. Phylogenetic diversity of bacterial isolates

####### 3.4.1.2.1.1.1. Identification using biochemical method

Well isolated colonies were randomly isolated from plates. Nearly 800 colonies were isolated representing different concentration of media. These isolates represented different morphotypes from all the cores. The isolates were subjected to various biochemical and physiological tests (Gerhardt, 1981, Appendix I). Identification was carried out up to generic levels using phenotypic traits (Oliver, 1982; Bergey's Manual of Determinative Bacteriology, 1984).

####### 3.4.1.2.1.1.2. Phylogenetic diversity of bacterial isolates using 16S rRNA

For genotypic analysis, DNA was extracted from the isolate by the method described by Maniatis et al. (1982). The primers used for 16S ribotyping analysis were as per MicroSeq 500 kit (*Applied Biosystems, USA*). Amplification of DNA was carried out on a thermocycler (GeneAmp PCR, *Applied Biosystems, USA*). The mixture was incubated through initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30s, 54 °C for 1 min and 72 °C for 1 min. A final extension period consisted of 1 min at 72 °C. The entire 500bp long sequence generated was used for further analysis. The amplified DNA fragment was purified using the PCR purification kit (*Vivantis, Malaysia*). The purified PCR product was sequenced using the BDT v3.1 cycle sequencing PCR kit (*Applied Biosystems, USA*) on a 3130 Genetic Analyzer (*Applied Biosytems Inc, USA*) at the geneOmbio Technologies sequencing facility. A phylogenetic affiliation by establishing the closest genetic neighbours was carried out using the RDP-II program (release number 10.0) and available nucleotide databases.

3.4.1.2.1.2. Metabolic diversity of bacterial isolates

3.4.1.2.1.2.1. Ability to elaborate extracellular enzymes

The isolates ability to elaborate amylase, DNase, lipase, phosphatase and protease were checked in nutrient medium amended with starch (Amy), DNA (Dna), tween 80 (Lip), p-nitro phenyl phosphate (Phos) and casein (Prot) as substrate (Appendix I).

Capacity Index (CI) was used to measure the capacity of the group of isolates able to degrade the substrates investigated and was calculated as sum of the percentages of isolates showing positive results on substrate media divided by numbers of substrate amended media (Dählbäck et al., 1982).

Therefore CI = (Amy+Dna+Lip+Phos+Prot)/5.

3.4.1.2.1.2.2. Detection of siderophores using Chrome Azurol S assay

The Chrome Azurol S (CAS) assay is a chemical assay for the detection of siderophores. It is based on the principle that siderophores have a high affinity for ferric iron. A color change accompanies the transfer of the ferric ion from its intense blue complex to the siderophore. The blue color of the medium is due to the dye complexed with iron. When siderophore is added, the siderophore binds the ferric iron, releasing the free dye, which is orange in color. Siderophore production by the isolates was detected by the method described by Alexander and Zuberer (1991).

Briefly, isolates were incubated overnight in 0.5 mL of Modified M9 Solution containing no added ferrous (Appendix I). Cells were pelleted by centrifugation at 2500 rpm (revolutions per minute) for 10 min. In a 96 well microtiter plate, 100 µL of the supernatant was mixed with 100 µL of chrome azurol solution and the absorbance was measured at 630 nm at zero hour. After 4 h, the absorbance was measured once again with the microtiter plate reader.

3.4.1.2.2. Diversity of microbial community

3.4.1.2.2.1. Microbial diversity using molecular techniques: Pyrosequencing using 454 technology

Sediment samples belonging to beach and the bay sediment were used for analyses. Sample 1 (S1) belonged to (5-10) cm layer of the berm sediments of the onshore Kalbadevi beach while the sample 2 (S2) belonged to surface sediments of the offshore Kalbadevi bay.

3.4.1.2.2.1.1. Extraction of total DNA using PowerSoil™ DNA Isolation Kit (MoBio)

Approximately 5 g of sediment sample was lyophilized overnight and genomic DNA was extracted using a MO BIO power soil DNA isolation kit (CA, USA). This kit was used since it eliminated/minimized the interference of humic substances.

Around 0.25 g of lyophilized sediment was weighed in clean pre-weighed 2 mL eppendorf tubes. This weighed sediment was added to PowerBead tubes containing glass beads provided in the kit and gently vortexed to mix the sediment and the glass beads. A 60 μL of C1 solution containing sodium dodecyl sulfate was added to these tubes and were mixed several times by inverting followed by vortexing for 10 min at the maximum speed on a horizontal vortexer. This step dispersed sediment particles thus preventing nucleic acids from degrading while the humic acids were dissolving. The tubes were centrifuged at 13000 x g for 1 min at 4 °C and the supernatant was transferred to clean 2 mL collection tubes. A 250 μL of C2 solution was added to the tubes, vortexed for 5 s and incubated at 4 °C for 5 min. The C2 solution contained a reagent which precipitated non-DNA organic and inorganic material including humic substances, cell debris and proteins.

The tubes were centrifuged at 4 °C for 1 min at 13000 x g and 600 μL of the supernatant was collected and added to clean 2 mL collection tube by avoiding the pellet. To this supernatant, 200 μL of C3 solution was added and vortexed briefly. The tubes were incubated at 4 °C for 5 min. The solution precipitated additional non-DNA organic and inorganic material including humic acid, cell debris and proteins.

The tubes were centrifuged at 4 °C for 1 min at 13000 x g and again nearly 750 µL of supernatant was transferred to a clean 2 mL collection tube by avoiding the pellet. To this supernatant, 1200 µL of C4 solution was added and the tubes were vortexed for 5 s. The C4 solution is a high concentration salt solution and it is well known that DNA binds tightly to silica at high salt concentrations. The addition of C4 solution adjusted the DNA solution's salt concentration such that the DNA was bound tightly to the spin filters. On the other hand, the non-DNA organic and inorganic material which could be present at low levels was prevented in doing so. Approximately 675 µL was loaded onto a spin filter and centrifuged at 13000 x g for 1 min at 4 °C. The flow through was discarded and an additional 675 µL of supernatant was added to the spin filter and centrifuged at 13000 x g for 1 min at 4 °C. A total of three loads for each sample were processed. A 500 µL of C5 solution was added to the spin filters and the tubes were centrifuged at RT for 1 min at 13000 x g. The C5 solution is an ethanol based wash solution and was used to further clean the DNA that was bound to the silica filter membrane in the spin filter. This wash solution removed residual salt, humic acid, and other contaminants while allowing the DNA to stay bound to the silica membrane. The flow through was discarded and the tubes were centrifuged again at 4 °C for 1 min at 13000 x g. The spin filters were carefully placed in clean 2 mL collection tubes and care was taken to avoid splashing of any C5 solution on to the spin filter. A 100 µL of C6 solution which is a 10 mM Tris elution buffer was added to the center of the white filter membrane. When passing through the silica membrane, the C6 solution which lacks salt selectively released DNA that is bound due to the presence of high salt. The tubes were centrifuged at 4 °C for 30 s at 13000 x g and the spin filters were discarded.
The DNA samples were checked for purity using the ratio [OD_{260}/OD_{280} ~ 1.8]. Purity and quantification of 1 µL extracted DNA was carried out using Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington DE). The final volume of 100 µL of eluted DNA was concentrated by adding 4 µL of 5 M NaCl and inverting the tubes 3-5 times to mix. This was followed by the addition of 200 µL of 100 % cold ethanol. The tubes were then again mixed by inverting nearly fives times.

Samples were centrifuged at 10000 x g for 5 min at RT. All liquid was decanted and residual ethanol was removed by drying the tubes overnight in a dessicator.

3.4.1.2.2.1.2. Amplification of the V6 region of the 16S rRNA gene using high-throughput pyrosequencing

Precipitated DNA was re-suspended in sterile water. The hypervariable region of 16S rRNA gene (BV6-rRNA tags) region was amplified and subjected to high-throughput using the 454 technology using the method described by Sogin et al., (2006) and Huber et al., (2007). The region was pyrosequenced using 454 Life Science GS-FLX sequencer.

The following sequence adaptors and primers were used (vamps.mble.edu).

Roche amplicon sequencing adaptors:

A-adaptor 5'-GCCTCCCTCGCGCCATCAG-3'

B-adaptor 5'-GCCTTGCCAGCCCGCTCAG-3'

For bacterial V6 sequencing a mixture of the following 5 forward and 4 reverse primers were used.

Forward Primers (967F)

CNACGCGAAGAACCTTANC

CAACGCGAAAAACCTTACC

CAACGCGCAGAACCTTACC

ATACGCGARGAACCTTACC

CTAACCGANGAACCTYACC

Reverse Primers (1046R)

CGACAGCCATGCANCACCT

CGACAACCATGCANCACCT

CGACGGCCATGCANCACCT

CGACGACCATGCANCACCT

Thus to amplify the V6 hypervariable region of the bacterial 16S rRNA (*Eschericihia coli* positions 967-1046) (Sogin et al., 2006) and sequence it in the forward direction (relative to the 5'-3' orientation of the gene) using the Roche A primer, the forward primer consisted of the A-adaptor, 5-base key and sequence designed to bind to the 967F region of the SSU

5'-GCCTCCCTCGCGCCATCAGgatctCNACGCGAAGAACCTTANC-3'

The reverse primer consisted of the B-adaptor and a sequence designed to bind to 1046R:

5'-GCCTCCCTCGCGCCATCAG CGACAGCCATGCANCACCT -3'

3.4.1.2.2.1.3. Sequence analyses

Trimming and removal of low-quality reads was carried out as described by Sogin et al., (2006), Huber et al., (2007) and http://vamps.mbl.edu/resources. The likely low-quality sequences were identified and removed based on previous assessment of pyrosequencing error rates (Huse et al., 2007). In addition, the sequences were aligned using the NAST tool provided on the GreenGenes web site (http://greengenes.lbl.gov), and only sequences >40 base pairs were retained. Significant matches were defined as having at least 75% identity with a known sequence. Finally, those sequences that occurred only once among the dataset were removed. The 454 tags served as query to identify its closest match in a reference database (V6RefDB) containing ~40,000 unique V6 sequences (Sogin et al., 2006). Taxonomic counts from the VAMPS database (vamps.mbl.edu) were then downloaded and imported on to a MS Office excel worksheet. Sequence characteristics such as average length and tag aggregates were estimated using the R program.

3.4.1.2.2.1.4. Diversity and community structure analyses

Multiple sequence alignment was done using ClustalX 1.83 program. Based on the alignment, a distance matrix was constructed using DNAdist from PHYLIP version 3.6 with default parameters from Felsenstein (1989, 2005). These pair-wise distances served as input to DOTUR (Schloss and Handelsman, 2005) for clustering the sequences into operational taxonomic units (OTU) of defined sequence similarity. Clusters at the unique, 0.03, 0.05, and 0.10 levels were created. These OTU were used to generate rarefaction curves, and calculate diversity indices such as Chao1 (Chao, 1987) and Abundance based Coverage Estimator (ACE) (Chao and Lee, 1992) as well as the Simpson evenness index.

3.4.1.2.2.2. Metabolic profiling of microbial communities

Biolog EcoPlates^{Tm} (Biolog, Inc., Hawyard, CA, USA) were employed to determine microbial community-level physiological profiles (CLPP) through the utilization of low molecular dissolved organic carbon (LM-DOC, <1 kDa) substrates using the method of Gamo and Shoji (1999). Biolog EcoPlates^{Tm} have 96 wells, each containing a distinct DOC substrate and a redox-sensitive tetrazolium dye. Intraplate replication is procivded by thirty-one different carbon substrates in triplicate and three carbon-free control wells. These substrates include various carbohydrates, amino acids, carboxylic acids, amines and small polymers (Appendix I). Carbon substrate utilization rates (CSUR's) were generated by inoculating unamended bacterial samples into the plate wells, incubating the plates, and spectrophotometrically measuring the optical density (OD_{590}) of purple formazan dye formation from tetrazolium reduction, which is proportional to carbon substrate oxidation rate (Mills and Garland, 2002).

Briefly, two gm of sediment samples was suspended in 18 mL of autoclaved 50% SW. The microplates were inoculated with 150 µL of this dilution per well. The tests were carried out in triplicate. Plates were incubated in the dark at RT. The colour development was measured at 590 nm using a microplate reader (Bio-Rad, USA). Measurement was carried out every 24 h for a fortnight. The community metabolic diversity (CMD) was calculated by summing the number of positive responses (purple-colored wells) observed following incubation. A threshold O.D. of 0.25 for the purple coloration indicated the usage of the carbon source by the microbial community.

Community metabolic diversity (%)

$$= \frac{\text{number of positive carbon source wells} \times 100}{\text{total number of carbon source wells (31)}}$$

3.4.1.3. Sediment biochemical parameters

3.4.1.3.1. Adenosine tri phosphate (ATP)

ATP indicative of total living microbial biomass was measured using the luciferin-luciferase reaction (Holm-Hansen and Booth, 1966; Bulleid, 1977).

Sediment samples were extracted in 5.0 mL of boiling tris buffer at pH 7.7 (Appendix II). After cooling, the contents were centrifuged at about 1500 x g for 5 min. Supernatant was immediately frozen and stored at -20 °C until further analysis. A 100 µL of the extractant was analyzed using the firefly lantern bioluminescence assay (Firefly lantern FLE 50; Sigma). Counts were determined using a luminometer (LUMIstar Optima, BMG Labtech). The light emitted was proportional to the amount of ATP present and calculated against a standard curve. The concentration was expressed as ng per gram.

3.4.1.3.2. Labile organic matter in the sediment (LOM)

Labile organic matter is the sum of the total carbohydrates, total proteins and total lipids in the sediment

3.4.1.3.2.1. Total carbohydrates (CHO)

Total carbohydrates were estimated by phenol-sulphuric acid method after extraction of an aliquot of sediment sample in 5% trichloroacetic acid (Appendix II) (Gerchacov and Hatcher, 1972; Kochert, 1978). The concentration was measured at 480 nm and expressed as glucose equivalents in µg per gram.

3.4.1.3.2.2. Total lipids (LIP)

Total lipids were extracted from sediment samples by direct elution with chloroform and methanol (5:10) (Bligh et al., 1959) and oxidized with 0.15% acid dichromate (Appendix II). Absorbance was measured at 440 nm (Parsons et al., 1984). Total lipids were expressed in terms of stearic acid equivalents in µg per gram.

3.4.1.3.2.3. Total proteins (PROT)

Total proteins were estimated using Folin-phenol reagent after extraction of an aliquot of sediment sample with 1 N NaOH (Appendix II) (Lowry et al., 1951). The absorbance was determined at 750 nm. Protein concentrations are expressed as bovine serum albumin equivalents in µg per gram.

3.4.1.3.2.4. Fraction of biopolymeric carbon (BPC)

Carbon biopolymeric fraction (BPC) was calculated as the sum of carbohydrate, lipid and protein converted to carbon equivalents by assuming a conversion factor of 0.45, 0.50 and 0.70 respectively (Fichez, 1991).

3.4.1.4. Sediment geochemical parameters

3.4.1.4.1. Total carbon

Total carbon in the sediment was estimated using UIC CM 5014 coulometer.

3.4.1.4.2. Total organic carbon

Organic carbon was determined by the wet oxidation method with a precision of 0.01% (Walkley-Black, 1934; Loring and Rantala, 1992) (Appendix II).

3.4.1.4.3. Sedimentary iron concentration

The Fe(II) and hydroxylamine extractable Fe in the sediment was estimated according to Lovley and Phillips (1986a; 1987b). Ferrozine [FZ, disodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine] is a complexing agent of Fe(II) and forms a magenta complex Fe(II)(FZ)3 with a maximum absorbance at 562 nm (Stookey, 1970; Neubauer et al., 2002). This reagent is specific to Fe(II) but the use of a reducing agent can extend the method to total Fe [Fe = Fe(II) + Fe(III)].

For reagent preparation (Refer Appendix II)

3.4.1.4.3.1. Soluble Fe(II)

One gram of sediment sample was added to 2 mL of ferrozine in a pre-weighed glass vial and kept in the dark for 10 min. The amount of soluble Fe (II) was determined by measuring at 562 nm.

3.4.1.4.3.2. HCl extractable Fe (III) -FeA

One gm of sediment sample was added to 4 mL of 0.5 M HCl in a pre-weighed glass vial. The sediment sample and acid were mixed together with gentle swirling for 30 s and kept aside for 1 h at RT. One mL of the extractant

was added to 2 mL of ferrozine and again kept in the dark for 10 min. The amount of soluble Fe (II) was determined by measuring at 562 nm.

3.4.1.4.3.3. Hydroxylamine reducible Fe(III) -FeM

One gram of sediment sample was added to 4 mL of 0.25 M hydroxylamine hydrochloride in 0.25 M HCl in a pre-weighed glass vial. The sediment sample and acid were mixed with gentle swirling for 30 s and kept aside for 1 h at RT. One mL of the extractant was added to 2 mL of ferrozine and kept in the dark for 10 min. The amount of soluble Fe (II) was determined by measuring at 562 nm.

3.4.1.4.3.4. Standard preparation

The standard curve was prepared by using $FeSO_4.7H_20$ in 0.1 N HCl

3.4.1.4.4. Ilmenite concentration

Sediment samples were washed repeatedly with distilled water to remove the salt. The carbonates, organic matter and ferruginous coatings were removed by treating with 1:10 HCl, H_2O_2 (30% by vol.) and $SnCl_2$ respectively. The treated samples were dried in the oven at 40 °C, and known quantity of representative samples were used for dry and wet sieving separately. Different size fractions were obtained from sieves of 0.5 phi mesh by dry sieving and grouping to determine the heavy mineral content using bromoform (specific gravity 2.89), following standard procedures (Milner, 1962). The separated heavy minerals were further subjected to magnetic separation using hand magnet and S.G. Frantz isodynamic separator.

3.4.2. Water samples

Water samples at different water depths were collected using Niskin bottle.

3.4.2.1. Microbiological parameters

3.4.2.1.1. Direct total bacterial counts (TC)

An aliquot of 5 mL water sample was fixed and processed as described in section **3.4.1.1.1.** Numbers were expressed as cells per mL.

3.4.2.1.2. Direct total viable counts (TVC)

Water samples were incubated under aerobic and anaerobic conditions as described in section 3.4.1.1.2. Further analysis of samples was carried out as described in section 3.4.1.1.1. Numbers were expressed as cells per mL.

3.4.2.1.3. Retrievable counts of heterotrophs (RC)

Water samples for retrievable heterotrophic population was assessed on plates prepared with 10% strength nutrient broth (NB; 100% = 1.3g) in seawater and 1.5% agar by spread plate method as described in section **3.4.1.1.1.3.** Numbers were expressed as CFU per mL.

3.4.2.2. Biological parameters

3.4.2.2.1. Chlorophyll *a* (Chl *a*) and phaeophytin

Water samples were collected from Niskin sampler into clean polyethylene bottles with Tygon™ tubing and immediately stored at 4(±2) °C till further analyses in the onshore laboratory. In the laboratory, 500 mL of the water samples were filtered through 47 mm GF/F filter paper (Millipore, 0.7 µm pore size). The pigments were extracted by immersing the filter papers in 10 mL of 90% acetone at 4(±2) °C for 18 h (Appendix I) (Holm-Hansen et al., 1965). Following extraction, filter papers were centrifuged at 1500 x g for 5 min and fluorescence was measured using a fluorometer (Trilogy, Turner Design).

Phaeophytin, the degraded product of chlorophyll was estimated after acidifying the extract with 90% HCl and measuring the treated extract on the fluorometer (Trilogy, Turner Design).

Chl *a* and phaeophytin concentration was expressed as mg m^{-3}.

3.4.2.2.2. Phytoplankton abundance and composition

For numerical analysis and species composition, 250 mL of water samples from each depth was fixed in 0.5 mL Lugol's iodine solution (Appendix I). Formalin (3% FC) was added as a preservative. The samples were kept undisturbed in the laboratory for a week till complete sedimentation was achieved. The samples were then concentrated to a known volume and one mL (in triplicate) of the concentrated sample was counted using a Sedgwick-Rafter slide at 100x magnification on an inverted microscope. An average of

triplicate value was used to calculate the number of cells per liter. The phytoplankton cells were identified according to Tomas (1997).

3.4.2.2.3. ATP content

ATP content was analyzed using luciferin-luciferase reaction after filtering the water sample through a 0.22 μm pore size filter paper. The ATP content was extracted into the solution by extracting it in boiling 0.1 mM Tris buffer (pH 7.8) for 5 min. The ATP content in the extract was estimated as described in section 3.4.1.3.1. (Appendix II)

3.4.3. Microcosm experiments

3.4.3.1. Microcosm experiments to determine the ability of native bacteria to release iron in soluble form from ilmenite

Sediment samples were collected from berm station of the central transect at Kalbadevi beach, divided into three parts and each set was run in triplicates. One part was used to detect the release of iron from ilmenite sediment and two parts were used as controls. Controls were in the form of heat and azide inactivated sediments. The experimental set up is described in table 3.4

Table 3.4: Experimental set up for microcosm studies with whole sediments to determine the ability of native bacteria to remove iron in soluble form from ilmenite.

Experimental set up	Set I	Set II	Set III
Kalbadevi sediment with native bacteria	√	√	√
Ferrozine medium I	50 mL	50 mL	50 mL
Autoclaving at 121 °C for 15 min (heat killed)	--	√	--
Sodium azide (20 mM)	--	--	√

Approximately 1 g of sediment was weighed and added to 50 mL of Ferrozine medium I. Individual sets were maintained and sacrificed for each monitoring day. The experiment was run for 15 d. The bottles were incubated in the dark at RT to avoid photo-oxidation. Aliquots of the inoculated media as well as the control medium was taken from each tube and amount of soluble Fe(II) in the media was determined by measuring OD at 562 nm.

Simultaneously, bacteria capable of reducing iron were enumerated according to a modification of the most-probable number (MPN) assay supplied with

ferrozine (ferrozine-MPN) (Appendix I) (Nielsen et al., 2002). Ferrozine-MPN assay medium was used and serial dilutions of beach sediment suspensions were used as inoculum. Tubes were incubated in the dark for 3 d at RT. MPN values were calculated from standard MPN tables. The results were scored as positive if the absorbance at 562 nm, determined directly by the modified ferrozine-MPN assay, was more than twice the value of a negative control (Nielsen et al., 2002).

3.4.3.2. Microcosm experiments to determine the effect of varying concentration of ilmenite on heterotrophs

Beach sediment samples were mixed with sterile ilmenite from Kalbadevi in the ratio 1:1, 1:2, 1:3, 1:4 to monitor the influence of black sand on the native flora.

Sediment samples were mixed with varying concentration of ilmenite and slurry prepared using artificial sea water (ASW). For the experiment, sediment samples were collected from Dona Paula Beach using an acrylic corer and stored at 4(±2) °C until analysis. Experiments were set up within 1-2 h. The experimental set up is described in table 3.5. Retrievability was enumerated from these flasks by spread plating 100 µL of the sample from the respective flask on 0.01% nutrient broth amended agar as described in section 3.4.1.1.3.1. The retrievability was monitored on 3, 5 and 15 d. Following 24 h incubation at RT, the colony forming units (CFU) were counted and the counts are expressed as CFU per gram.

Table 3.5: Experimental set up for microcosm studies with whole sediments to determine the effect of varying concentration of ilmenite on heterotrophs.

Sr. No.	Experimental flasks	ASW (50 mL)	Approx. % of ilmenite	Proportion of sand	
				Beach sand (g)	Ilmenite (g)
1	Control (C)	√	0	1.0	--
2	1F	√	50	0.5	0.5
3	2F	√	66	0.33	0.67
4	3F	√	75	0.25	0.75
5	4F	√	80	0.2	0.8

3.4.3.3. Microcosm experiments to determine the effect of iron released from ilmenite at bacterial level on abundance and activity at primary level

The natural phytoplankton community that was present in the Kalbadevi Bay waters during sample collection was used to set up laboratory microcosm experiments. The seawater used to set up the experiment was filtered through 200 µm mesh to avoid loss of phytoplankton due to grazing by zooplankton and other higher organisms.

Four different experiment combinations were set up with appropriate controls. Each combination consisted of 12 flasks of 1 L capacity containing 0.5 L of the media. The experimental design consisted of two controls one containing only filtered seawater and the other containing 10 µM $FeSO_4.7H_2O$ in filtered seawater. The treatments Exp-1 contained only ilmenite and Exp-2 in addition to ilmenite contained 10 µM $FeSO_4.7H_2O$ in filtered seawater (Table 3.6). For analysis, one flask from each set was sacrificed at the onset and on day 1, 2, 3, 4, 7, 14, 21, 28, 42, 56 and 84.

Table 3.6: Experimental set up for microcosm studies to determine the effect of iron released from ilmenite at bacterial level on abundance and activity at primary level.

			Seawater	Ilmenite (100mg)	$FeSO_4.7H_2O$ (10µM)
1	**Control 1**	**C1**	√	-----	-----
2	**Control 2**	**C2**	√	-----	√
3	**Experiment 1**	**Exp-1**	√	√	-----
4	**Experiment 2**	**Exp-2**	√	√	√

The phytoplankton community used in the experiment was the natural community present in the Kalbadevi Bay waters at the time of sampling. In each sample, Chl *a* concentration, phytoplankton density, and change in generic composition of the assemblage was estimated as described in section 3.4.2.2. TC was estimated after fixing water sample with pre-filtered formaldehyde (2.5% FC) as described in section 3.4.1.1.1. ATP content was analyzed as described in section 3.4.2.2.3. Changes in concentration of

ferrous [Fe(II)] and hydroxylamine extractable Fe (FeM) was periodically sampled and quantified spectrophotometrically at 562 nm by the ferrozine assay (Stookey, 1970; Lovley and Phillips, 1987b; Neubauer et al., 2002) as described in section 3.4.1.4.3. Briefly, for determination of Fe(II) concentration, aliquots of one mL of filtered suspension were extracted with 2 mL of the ferrozine and measured spectrophotometrically at 562 nm wavelength. The hydroxylamine reducible Fe (FeM) was measured in unfiltered samples after one mL of the unfiltered sample containing Fe(II) and FeM was digested in 5 mL of 0.25 M hydroxylamine hydrochloride in 0.25 M HCl solution for 1 h. FeM was calculated as the difference in the Fe concentration measured between unfiltered and filtered samples. All analyses were done in triplicate. Care was taken to acid-wash all glassware used for iron analysis. The data was subjected to statistical analyses using Statistica version 6 to test the correlation among various parameters. Diversity indices were analyzed using Primer ver. 6. The extent of surface colonization and microbial dissolution of ilmenite was assessed using Scanning Electron Microscopy (SEM). Briefly, smears were prepared on glass pieces and dehydrated by immersing them for 15 min in increasing concentration of ethanol. Slides were air-dried, mounted on a stub, sputter coated with Au/Pd to a thickness of 100A° and visualized at 8000x using a scanning electron microscope (JEOL JSM-5800).

3.4.4. Laboratory experiments with isolates

Bacterial isolates from the berm region of Kalbadevi beach were used for the experiments. The identity of the isolates was established by 16S rDNA sequencing.

3.4.4.1. Laboratory experiments to determine the ability of the isolates to release soluble iron from ilmenite

Initially, the isolates were maintained in ASW medium containing 0.01% glucose to increase biomass yield. The cells were harvested by centrifugation at 8000 rpm for 10 min when the cell density reached an OD_{600} of 1.5. Cell pellets were washed twice with sterile saline, re-suspended in a known volume and used as innoculum for the experiment.

Before the experiment, the Ferrozine medium I was mixed with the mineral and incubated for ca. 3 d to check for chemical leaching of iron from ilmenite. About 100 mg of sterile ilmenite obtained from the Kalbadevi beach was added to flasks containing 10 mL of Ferrozine medium I (Appendix I). 100 µL of the re-suspended pellet was added to this media. Control was maintained with the addition of sterile ilmenite to the Ferrozine mediun I but without the addition of cell pellet. Flasks were incubated in the dark at RT. Monitoring of the flasks was carried out on different time intervals for a period one month. Three mL of the media was drawn from each flask to detect the amount of soluble Fe (II) as described in section 3.4.1.4.3.1. Cell numbers were enumerated with the help of a Neubauer-counting chamber using the light microscope. About twenty fields were counted on an average to assess the numbers. SEM images were captured and analyzed according to section 3.4.3.3. Samples for transmission electron microscopy (TEM) were prepared and analyzed at All India Institute of Medical Sciences, New Delhi (AIIMS).

Mean generation time (g) was calculated using the equation from Brock and Madigan (1991). $g = \frac{\ln 2}{\mu}$

where μ_g = growth rate constant = $\frac{(\ln(N_t) - \ln(N_0)}{\Delta T}$

N_t = Number of cells at the end of time at which subsequent sample was taken

N_0 = Number of cells at the start of initial time

$\Delta T = T_t - T_0$, i.e., the length of the time interval, where T_0 is the initial start time and T_t is the time at which a subsequent sample is taken

3.4.4.2. Laboratory experiments to determine the effect of varying concentration of chemical form of iron, $FeSO_4.7H_2O$ on a bacterial isolate

3.4.4.2.1. Determination of cell viability in iron amended media

For the experiment, isolate Fe13, was harvested in seawater (SW) containing 0.01% glucose (Glu) to stimulate growth till the mid-exponential phase. The

cells were collected from the medium, washed thrice in 0.85% NaCl solution and concentrated by centrifugation at 10000 x g for 10 min for use in the incubations.

In order to assess the response of the cells to varying concentrations of iron, two series were run. The experimental medium - control series (EM-C series) consisted of the cell free sterile control and was maintained without the addition of inoculum to check for auto-oxidation and –reduction. The experimental medium - experimental series (EM-E series) consisted of the medium suspended with a fixed cell density of isolate Fe13 ($0.084 \pm 0.022 \times 10^{12}$ cells L^{-1}). The EM medium contained SW and 0.01% Glu. Filter sterilized stock solution of Fe in the form of 10 mM $FeSO_4.7H_2O$ was added to the EM medium to provide an initial concentration of 1, 10, 100 or 1000 μM above an ambient concentration of 4 μM present in the SW used for medium preparation. Five combinations were tested in each series, details of which are presented in Table 3.7.

Table 3.7: Experiment to measure changes in the rate of iron uptake and increase in cell density.

	EM medium containing ASW + 0.01% Glucose (50 mL)	**$FeSO_4.7H_2O$ (final concentration)**	**Test innoculum (100 μL)**
Control series (EM-C)			
EM-C1	√	X	X
EM-C2	√	√ (1 μM)	X
EM-C3	√	√ (10 μM)	X
EM-C4	√	√ (100 μM)	X
EM-C5	√	√ (1000 μM)	X
Experimental series (EM-E)			
EM-E1	√	X	√
EM-E2	√	√ (1 μM)	√
EM-E3	√	√ (10 μM)	√
EM-E4	√	√ (100 μM)	√
EM-E5	√	√ (1000 μM)	√

The sets were (1) EM-C1 and EM-E1 had EM medium alone, (2) EM-C2 and EM-E2 had EM medium + 1 μM $FeSO_4.7H_2O$, (3) EM-C3 and EM-E3 had EM medium + 10 μM $FeSO_4.7H_2O$, (4) EM-C4 and EM-E4 had EM medium + 100 μM $FeSO_4.7H_2O$, (5) EM-C5 and EM-E5 had EM medium + 1000 μM $FeSO_4.7H_2O$ (Table 3.7). Test tubes of 50 mL capacity were filled to the brim to simulate microaerophilic → anaerobic conditions. The initial pH of the medium was adjusted to 8(±0.2) using sterile 0.1 N NaOH solution. All the tests were done in triplicate at 28(±2) °C. Sets of tubes were sampled on different days during the 37 d experiment. The pH was monitored using a pH meter (Thermo Orion 3 star) during the course of the experiment. Cell densities were measured by counting the cells with a Neubauer-counting chamber at the specified intervals during the 37 d experiment. Number of cells was expressed as cells per litre. Mean generation time (g) was calculated according to calculation given in section 3.4.4.1.

Concentration of total cellular proteins was used as an indicator of cell growth and was estimated spectrophotmetrically at 750 nm by Lowry's method using Folin-phenol reagent after cells from a 5 mL aliquot of sample were pelleted (Lowry et al., 1951). Protein concentrations were given as bovine serum albumin equivalents (Appendix II). Cell ATP content was analyzed as described in section 3.4.2.2.3.

3.4.4.2.2. Determination of cell morphology and intracellular metal accumulation studies

To detect changes in the cell morphology brought about by increasing concentration of iron, images of cells from iron amended and unamended seawater were captured and analyzed by scanning electron microscopy (SEM) according to section 3.4.3.3. An energy-dispersive X-ray spectrometer (EDS) was carried out in conjunction with the SEM on selected cells and on metal precipitates at an accelerating voltage of 15keV.

Additionally, powder X-ray diffraction (XRD) scans were performed to distinguish the mineral type contained in the sample. The bacterial cells were harvested at the end of the experimental period, by centrifuging the medium at 10000 x g for 10 min. Cell pellet was washed thrice with distilled water

followed by acetone and then air-dried and crushed (Deplanche and Macaskie 2008). The crushed dried samples were scanned using a Rigaku X-Ray powder diffractometer using monochromatic Cu $K\alpha_1$ radiation (λ = 1.54050 Å). The diffraction spectrum was recorded from 10° to 100° (2θ) with a step size of 0.02° (2θ). The d values were determined with the aid of charts prepared by Brown (1980).

3.4.4.2.3. Analysis of soluble and insoluble phases of iron

Changes in concentration of ferrous [Fe(II)] and hydroxylamine extractable Fe (FeM) was periodically sampled and quantified spectrophotometrically at 562 nm by the ferrozine assay as described in section 3.4.3.3. Oxidation of iron was monitored by measuring the decrease of iron concentration relative to the fall in concentration in sterile cell-free controls.

Rate of Fe(II) oxidation (sFe, μM^{-1} day^{-1}) was calculated using the equation

$$sFe(II) = \frac{Fe(II)_t - Fe(II)_0}{\Delta T}$$

Dividing the rate of Fe(II) oxidation with bacterial numbers gave cell specific activity (sp_s; M $cell^{-1}$ day^{-1}).

Specific activity (sp_s, $cell^{-1}$ day^{-1}) was calculated as $\frac{sFe}{N_t}$

where $Fe(II)_0$ = Soluble Fe at the start of incubation time

$Fe(II)_t$ = Soluble Fe at the end of incubation time

ΔT = Incubation time in days, $(T_t - T_o)$

N_t = Number of cells at time T_t

The rate constants ($\mu_{Fe(II)}$) was derived from the plots by calculating the slopes for each data set (Ahonen and Tuovinen, 1989).

3.4.4.2.4. Determination of Fe(II) and Fe(III) using voltammetry

The speciation of Fe was carried out using a voltammeter in interface with 797VA computrace (Metrohm, Switzerland). The potential was measured against an Ag/AgCl reference electrode and a platinum rod as a counter electrode. The Fe determination was carried out with SMDE (static mercury drop electrode) in the differential pulse mode. The speciation of iron was carried out at pH 7 in a measuring cell containing 10 mL of supporting

electrolyte (0.1 M Sodium pyrophosphate). The blank was registered before sample addition after 300 s degassing with 99.9995% Nitrogen (Medgas and Equipments). Sample volume (1 mL) depending on the concentration was appropriately diluted with charcoal treated (to reduce background), filtered (0.22 µm pore size membrane) Milli-Q water (18.2 Ω resistance) prior to analysis. The sample was directly added to the measuring vessel containing the electrolyte and was degassed for another 180 s prior to deposition. The analysis was carried out with 30 s deposition time, 6 s equilibration time, 5 mV voltage step and 0.5 s voltage step time. The potential was scanned in three replications from 0 to -1.3V with sweep rate of 10 mV s^{-1}. The analysis were quantified with two standard additions of Fe(II) ($FeSO_4.7H_2O$, 1 g l^{-1}) stock solution prepared in 0.1 M HCl and Fe(III) ($NH_4Fe(SO_4)_2 \cdot 12H_2O$, 0.5 g l^{-1}) in 0.1 M phosphoric acid. The determination of iron was carried out following modified Metrohm VA application work DE4-0191-032007.

3.5. Statistical analyses

Differences in investigated parameters between stations and correlation among variables were tested using parametric analyses. Bacterial data were log (x+1) transformed before analyses. The factorial Analysis of Variance (ANOVA) was used to test for variation in bacterial and biochemical parameters among different sampling events. The Tukey test was used for post hoc comparisons. All statistical tests and correlation analyses were performed using the Statistica ver 6.0 and Primer 6.0 softwares. The Spearman rank (rs) correlation analysis was carried out to test correlation among microbial and environmental parameters. Significant correlations were plotted using the software Cytoscape ver. 2.6.3. This software helped in visualizing, illustrating, and analyzing the complex interrelationship patterns. The relationship between the sediment characteristics and biological parameters was further investigated using Principal Components Analysis (PCA) (Jongman et al., 1987). The link between the biotic pattern and abiotic variables was explored using the biological environmental gradients (BIO-ENV) procedure, which aimed to select the environmental variables subset that maximized the rank correlation (ρ) between biotic and abiotic similarity matrix.

Chapter 4

Chapter 4: Spatio-temporal variation of bacterial and biochemical parameters

This chapter covers the crucial data required to understand the community assembly and ecological processes. This base line information is essential if mining of ilmenite rich sands from the sandy beaches is undertaken.

The results is divided into two sections,

Section 4.1.1 deals with Northern, Central and Southern transects on onshore beach system. The emphasis and detailed study was laid more on the Central transect which was more moderate in its trophic status as compared to the relatively more oligotrophic Northern transect or relatively more eutrophic Southern transect.

Section 4.1.2 deals with the offshore offshoots of the Northern, Central and the Southern transect.

4.1. Results

4.1.1. Onshore beach system – Kalbadevi beach

4.1.1.1. The whole study area in general – Baseline data

Results of the bacterial and biochemical parameters presented in this section have been integrated over a depth of 40 cm sediment cores.

4.1.1.1.1. Bacterial parameters:

TC in the sediments ranged from 2.16 x 10^7cells m^{-2} at Stn BP1/3 in May to 1.61 x 10^9 cells m^{-2} at Stn BP2/1 in April (Fig. 4.1a). Maximum TVCa count of 8.08 x 10^8 cells m^{-2} was recorded at Stn BP2/1 in April while a minimum of 2.31 x 10^6 cells m^{-2} was recorded at Stn BP2/5 station in August (Fig. 4.1b). High TVCan population of 4.77 x 10^8 cells m^{-2} was estimated at Stn BP1/4 in February while a low count of 1.61 x 10^6 cells m^{-2} was recorded at Stn BP2/5 station in August (Fig. 4.1c). Lowest retrievability was observed on all the three concentrations of nutrients, in August along the Northern transect while maximum number was obtained in April (Fig. 4.2 a, b and c).

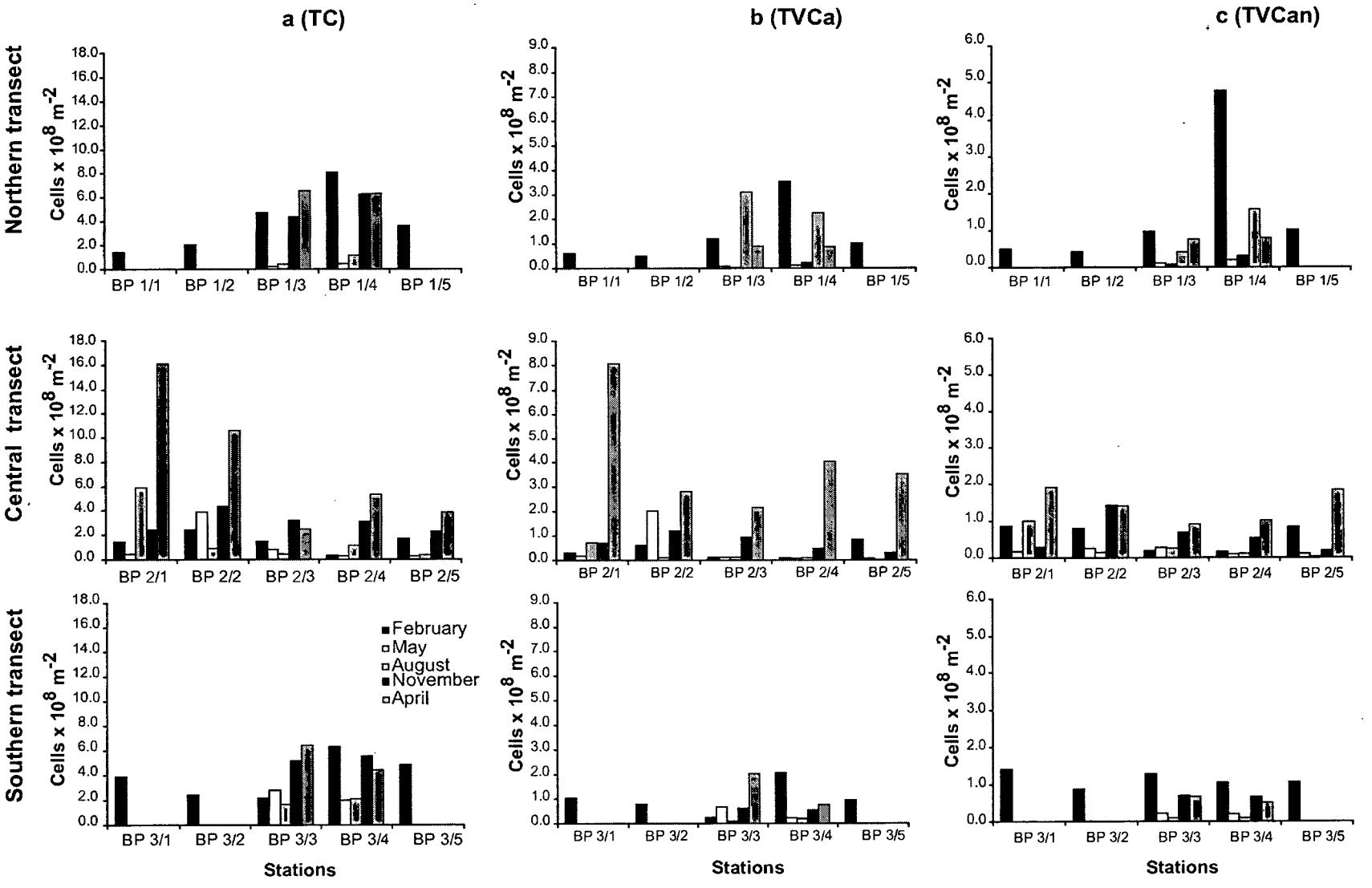


Fig 4.1: Seasonal variation in (a) TC, (b) TVCa and (c) TVCan integrated over a sediment depth of 40 cm at the three studied transects: Northern, Central and Southern

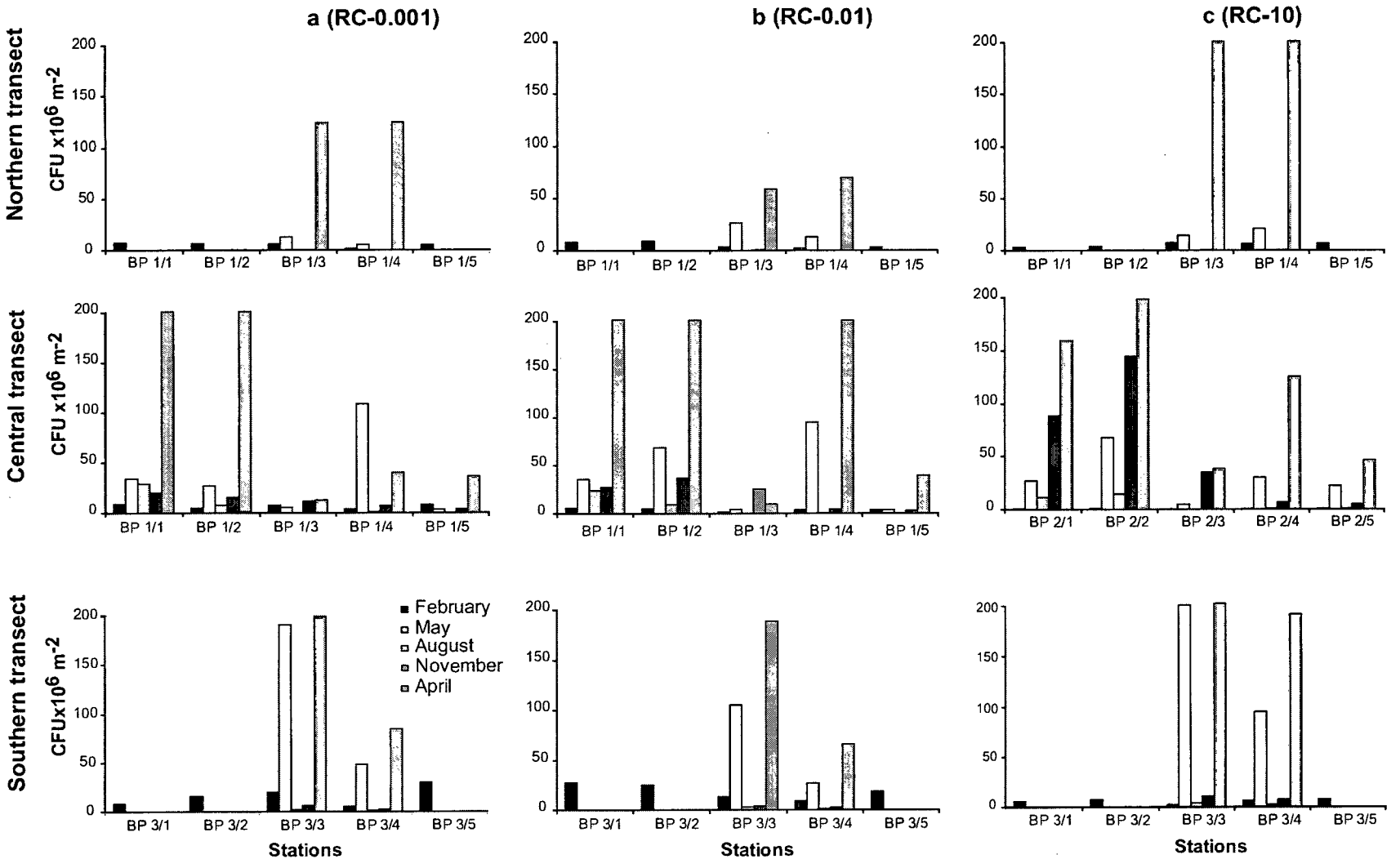


Fig 4.2: Seasonal variation in RC for (a) 0.001%, (b) 0.01% and (c) 10% nutrient concentration integrated over a sediment depth of 40 cm in the three studied transects: Northern, Central and Southern

4.1.1.1.2. Biochemistry of the sediments:

Sedimentary ATP varied from 0.8 μg m^{-2} at Stn BP3/4 in May to 140 μg m^{-2} at Stn BP3/4 during August (Fig. 4.3). Carbohydrates ranged from 1.2 mg m^{-2} at Stn BP2/4 during November to 89 mg m^{-2} at Stn BP1/2 in February. Lipids ranged from 1.5 mg m^{-2} at Stn BP2/5 in April to 48 mg m^{-2} at Stn BP1/3 in April while proteins varied from 1.2 mg m^{-2} at Stn BP3/3 in May to 69 mg m^{-2} at Stn BP2/1 in November (Fig. 4.4 a, b and c).

4.1.1.1.3. Comparative study between of the three transects:

The average sedimentary bacterial population and biochemical concentration along the three transects were compared. TC along the Southern transect (3.85×10^8 cells m^{-2}) was 8 and 21% higher than the Northern and the Central transect respectively. This did not reflect in the viable fraction. TVCa population (1.20×10^8 cells m^{-2}) at the Central transect was 8 and 35% higher than the Northern and the Southern transect respectively. The contribution of this fraction of viability to TC was 31, 40, 20% along the Northern, Central and Southern transect respectively. On the other hand, TVCan population (0.92×10^8 cells m^{-2}) of the Northern transect sediments was 33% and 26% higher than the Central and the Southern transect respectively and this population contributed between 18 to 26% to TC. The Southern transect showed maximal retrievability at 0.001% and 10% nutrient amended media while the maximum retrievability on 0.01% nutrient amended media was recorded at the Central transect. On an average, the contribution of RC to TC varied from 6 to 14% on 0.001% nutrient broth amended media, 4 to 25% on 0.01% nutrient broth amended media and 11 to 22% on 10% nutrient broth amended media. The average sedimentary ATP along the Southern transect (29 μg g^{-1}) was 52 and 57% higher than the Northern and the Central transect respectively. Overall the contribution of different sedimentary LOM constituents was in the sequence CHO (42%) > LIP (32%) > PROT (26%) along the Northern transect, CHO (42%) = PROT (42%) > LIP (16%) along the Central transect and CHO (42%) > PROT (30%) > LIP (28%) along the Southern transect.

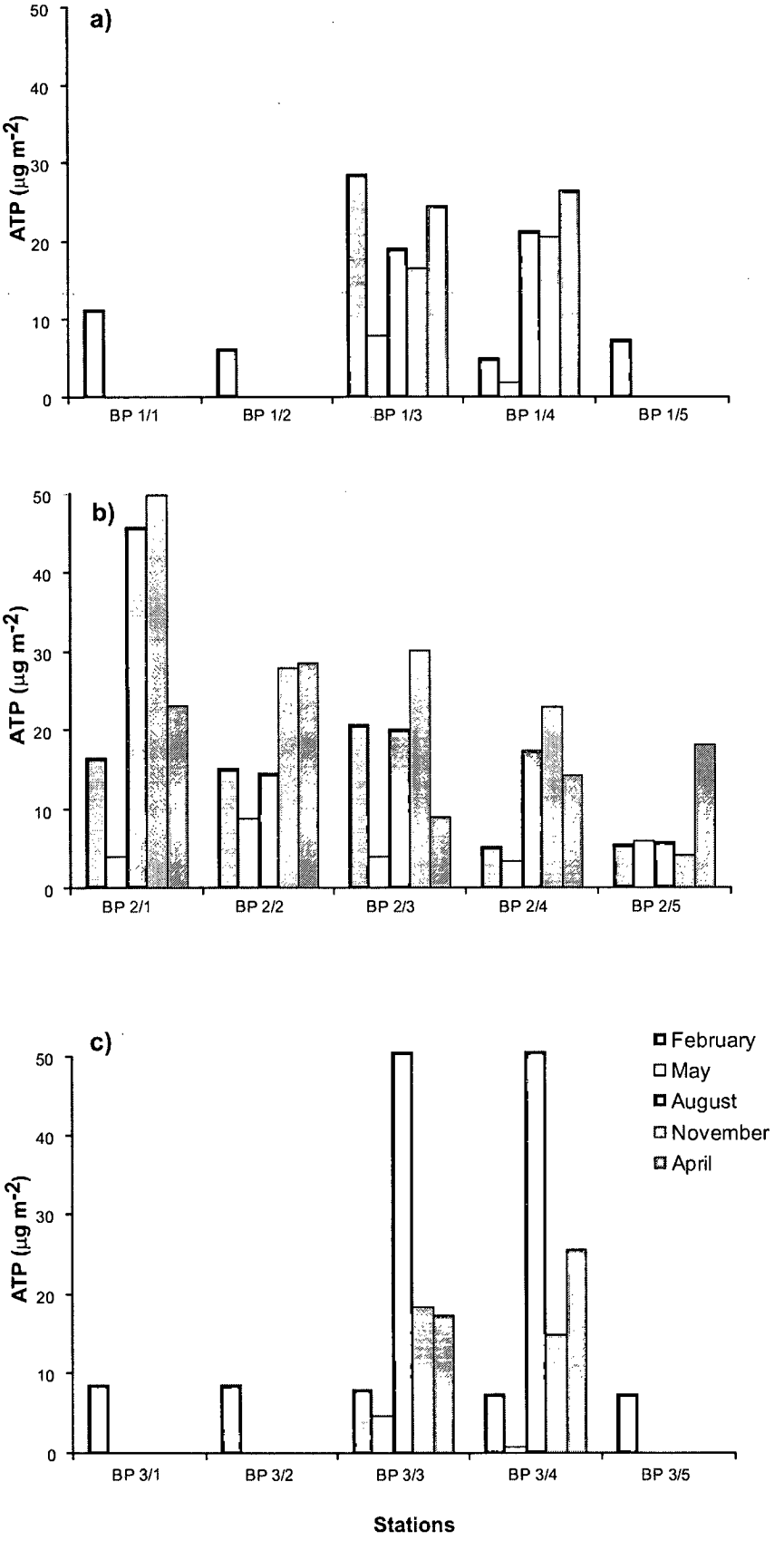


Fig 4.3: Seasonal variation in ATP integrated over a sediment depth of 40 cm at the three studied transects: (a) Northern, (b) Central and (c) Southern

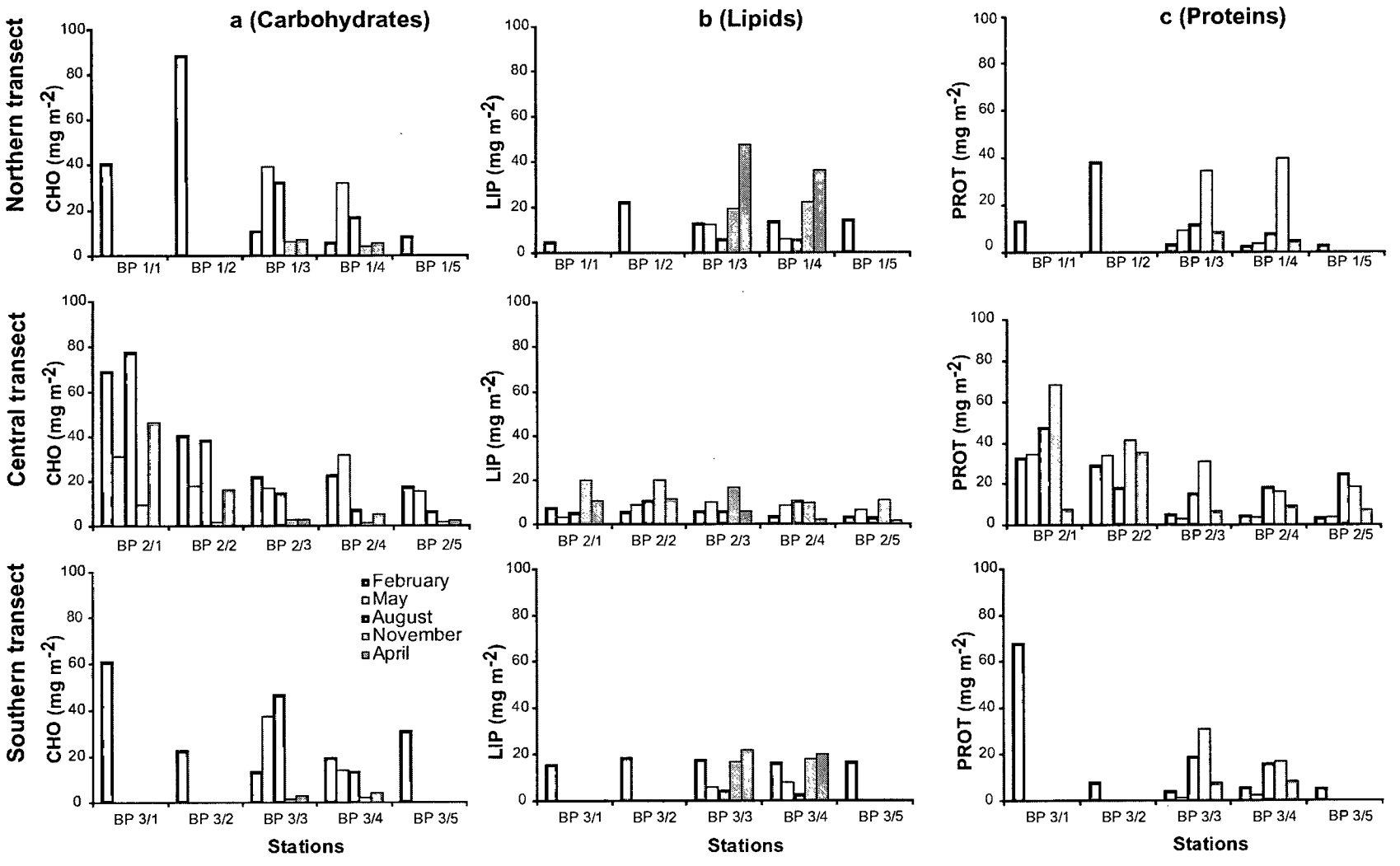


Fig 4.4: Seasonal variation in (a) Carbohydrates, (b) Lipids and (c) Proteins integrated over a sediment depth of 40cm at the three transects: Northern, Central and Southern

4.1.1.1.4. Statistical analyses

Interrelationships between the different parameters along the three transects was investigated statistically.

4.1.1.1.4.1. Northern transect

TC abundance was positively and significantly correlated to TVCa population ($n = 183$, $r = 0.66$) and TVCan ($r = 0.74$). Further, nearly 44% ($r = 0.67$) of the variation in TVCa population caused a significant variation in the TVCan population. Overall, TC abundance and viability did not display significant correlation with the retrievable population. However, variation in RC-0.001 population contributed to a significant >80% variation in RC on 0.01% strength and RC on 10% strength population ($p \leq 0.001$). Variation in living biomass as measured by ATP contributed nearly 6% variation in the bacterial parameters. LOM correlated significantly with CHO ($r = 0.78$) and PROT ($r = 0.66$). Moreover, most of the bacterial parameters correlated significantly with biochemical parameters along the Northern transect (Table 4AT1, Appendix III). TC correlated positively with PROT ($r = 0.25$), and LIP ($r = 0.58$) and negatively with CHO ($r = -0.49$). Similarly, TVCa correlated positively to LIP ($r = 0.44$) and PROT ($r = 0.22$) and negatively to CHO ($r = -0.44$). CHO also contributed negatively to the variation in TVCan population at nearly 19% ($r = -0.44$, $p \leq 0.001$). The variation in the PROT concentration brought about a significant negative variation ($p \leq 0.001$) in all the RC populations, while variation in the LIP concentration contributed to a significant positive relationship ($p \leq 0.001$) (Table 4AT1, Appendix III).

4.1.1.1.4.2. Southern transect

TC correlated significantly to TVCa ($n = 179$, $r = 0.44$) and TVCan ($r = 0.35$). Though TVCa population correlated significantly with RC population, TC and TVCan population did not show any significant correlation (Table 4AT1, Appendix III). Variation in RC-001 population contributed significantly to the variation in RC-0.01 ($r = 0.9$) and RC-10 population ($r = 0.78$). Further, the bacterial parameters also correlated significantly with biochemical parameters along the Southern transect (Table 4AT1, Appendix III). Variation in ATP contributed to <3% variation in the RC population. LOM correlated

significantly to CHO (r = 0.71) and PROT (r = 0.85). Among the LOM constituents, variation in the LIP contributed a significant >10% variation in all the bacterial parameters. The variation in the other biochemical constituents contributed between 2-5% variation in the bacterial parameters (Table 4AT1, Appendix III).

4.1.1.1.4.3. Central transect:

Abundance in TC displayed significant correlation with TVCa (n = 354, r = 0.81) and TVCan population (r = 0.74) (Table 4AT1, Appendix III). The variation in RC population contributed significantly to the variation in the TC population (> 16%, $p \leq 0.001$). TVCa and TVCan population displayed strong interrelationship (r = 0.75). RC-0.001 related significantly with RC-0.01 (r = 0.9) and RC-10 (r = 0.66). LOM significantly correlated with CHO (r = 0.86) and PROT (r = 0.74). The variation in LOM constituents contributed to <10% variation in the bacterial parameters, while variation in ATP contributed to <5%. Depth played a significant role in the distribution of the bacterial and biochemical parameters.

4.1.1.2. Spatio temporal variability in the central transect

Vertical distributions of bacterial and biochemical parameters were characterized by a significant decrease with depth in the sediment ($p \leq 0.001$) (Fig 4.5). Bacterial and biochemical parameters also displayed a significant variation between month and station (Table 4AT2, Appendix III). TC, TVCa, TVCan, RC-0.001 were generally high in the dune sediments. Similarly with the exception of lipids, all the biochemical parameters were high in the dune sediments (Table 4.1).

With the exception of sedimentary lipids and proteins, all bacterial and biochemical parameters were high during April. TC within the sediment ranged between 7.37×10^4 cells g^{-1} at (20-40) cm layer, Stn BP2/3 in February to 3.77×10^7 cells g^{-1} at (15-20) cm layer, Stn BP2/1 in April. Viability under aerobic condition varied from 1.14×10^4 cells g^{-1} at (20-40) cm layer, Stn BP2/4 in February to 2.29×10^7 cells g^{-1} at (15-20) cm layer, Stn BP2/1 in April (Table 4.2). Also, viability under anaerobic conditions ranged from 1.66×10^4

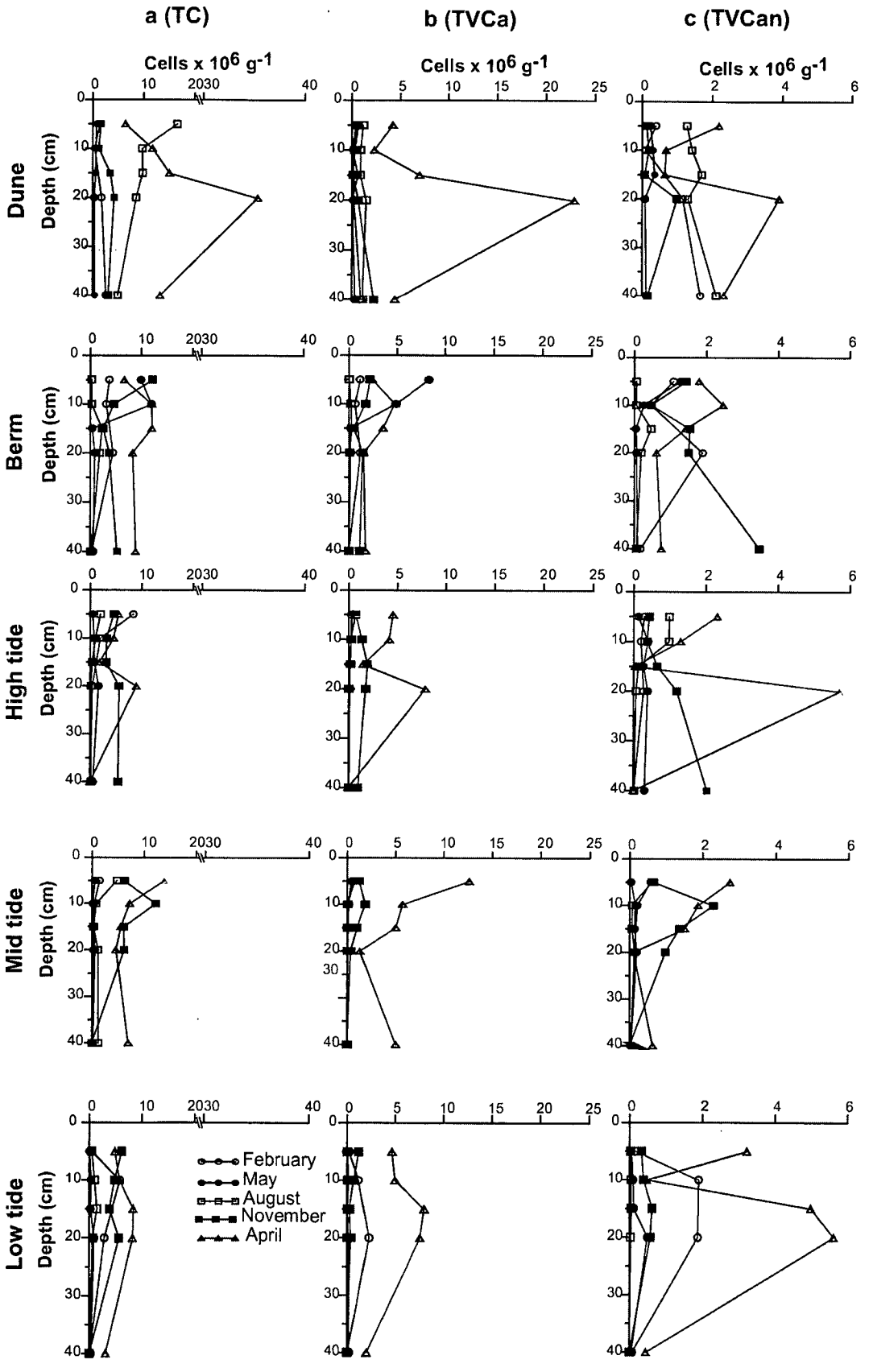


Fig 4.5: Spatio-temporal variation of (a) TC, (b) TVCa and (c) TVCan in dune, berm, high tide, mid tide and low tide sediments of the Central transect

cells g^{-1} at (20-40) cm layer, Stn BP2/4 in February to 5.70 x 10^6 cells g^{-1} at (15-20) cm layer, Stn BP2/3 in April. RC-0.001 population varied from 5.7 x 10^2 CFU g^{-1} at (10-15) cm layer, Stn BP2/4 in August to 6.1 x 10^6 CFU g^{-1} at (0-5) cm layer, Stn BP2/1 in April while those retrieved on 0.01% nutrient amended media ranged between 5.3 x 10^2 CFU g^{-1} at (15-20) cm layer, Stn BP2/5 in August and 2.4 x 10^7 CFU g^{-1} at (5-10) cm layer, Stn BP2/4 in April. RC-10 varied from 5.1 x 10^2 CFU g^{-1} at (5-10) cm layer, Stn BP2/3 in February to 5.6 x 10^6 CFU g^{-1} at (15-20) cm layer, Stn BP2/4 in April (Fig 4.6). Percentage contribution of direct viability to TC was in the range of 12 to 59% while retrievability contributed between 0.2 to 29% (Table 4.3).

Table 4.1: Average depth integrated values of the parameters studied in the sediments throughout the year along the central transect at different locations.

Parameters	Station				
	BP 2/1	BP 2/2	BP 2/3	BP 2/4	BP 2/5
Bacterial parameters					
TC (cells x 10^8 m^{-2})	5.27 (±6.40)	4.45 (±3.69)	1.68 (±1.15)	2.03 (±2.17)	1.68 (±1.50)
TVCa (cells x 10^8 m^{-2})	2.01 (±3.40)	1.36 (±1.09)	0.70 (±0.89)	0.97 (±1.73)	0.96 (±1.47)
TVCan (cells x 10^8 m^{-2})	0.84 (±0.70)	0.80 (±0.61)	0.50 (±0.32)	0.38 (±0.39)	0.60 (±0.76)
RC-0.001 (CFU x 10^8 m^{-2})	1.01 (±1.75)	0.60 (±1.02)	0.08 (±0.05)	0.32 (±0.46)	0.10 (±0.15)
RC-0.01 (CFU x 10^8 m^{-2})	1.33 (±2.47)	0.74 (±1.03)	0.08 (±0.10)	1.61 (±3.06)	0.10 (±0.18)
RC-10 (CFU x 10^8 m^{-2})	0.57 (±0.67)	0.85 (±0.85)	0.16 (±0.19)	0.33 (±0.53)	0.15 (±0.20)
Biochemical parameters					
ATP (μg m^{-2})	28 (±20)	19 (±9)	17 (±10)	13 (±8)	8 (±6)
CHO (mg m^{-2})	47 (±28)	23 (±16)	12 (±9)	14 (±13)	9 (±7)
LIP (mg m^{-2})	9 (±6)	11 (±5)	9 (±5)	7 (±4)	5 (±4)
PROT (mg m^{-2})	38 (±22)	32 (±9)	12 (±11)	10 (±7)	11 (±10)
LOM (mg m^{-2})	94 (±28)	66 (±7)	33 (±13)	31 (±10)	25 (±9)

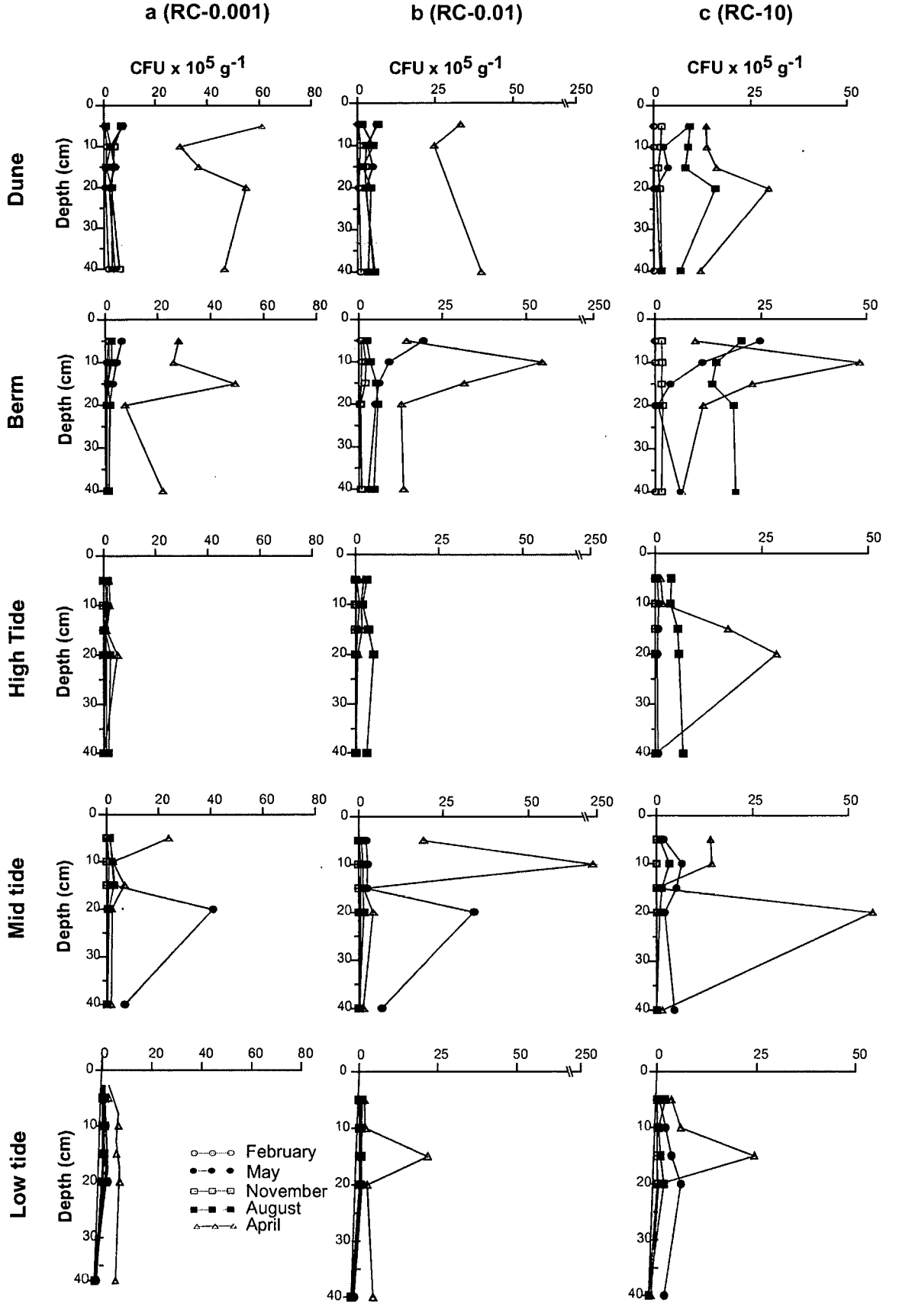


Fig 4.6: Spatio-temporal variation of RC at (a) 0.001%, (b), 0.01% and (c) 10% nutrient concentration in dune, berm, high tide, mid tide and low tide sediments of the Central transect

Sedimentary ATP ranged between 5 ng g^{-1} at (20-40) cm layer, Stn BP2/4 in February to 3060 ng g^{-1} at (20-40) cm layer, Stn BP2/1 in August (Fig 4.7). Carbohydrates in sediments varied from 10 μg g^{-1} at (10-15) cm layer, Stn BP2/1 in November to 3135 μg g^{-1} at (10-15) cm layer, Stn BP2/4 in May, lipids between 9 μg g^{-1} at (20-40) cm layer, Stn BP2/5 in April to 261 μg g^{-1} at (10-15) cm layer, Stn BP2/1 in November and protein from 12 μg g^{-1} at (0-5) cm layer, Stn BP2/3 in May to 1713 μg g^{-1} at (0-5) cm layer, Stn BP2/1 in August (Fig 4.8). Generally, the contribution of different LOM constituents to LOM was in sequence CHO > PROT > LIP (Table 4.4).

Table 4.2: Average depth integrated values of the parameters studied in the sediments during different months along the central transect.

Parameters	Sampling months				
	February	May	August	November	April
Bacterial parameters					
TC (cells x 10^8 m^{-2})	1.49 (±0.76)	1.13 (±1.58)	1.76 (±2.35)	3.05 (±0.81)	7.68 (±5.62)
TVCa (cells x 10^8 m^{-2})	0.41 (±0.31)	0.51 (±0.86)	0.22 (±0.29)	0.73 (±0.36)	4.13 (±2.32)
TVCan (cells x 10^8 m^{-2})	0.56 (±0.36)	0.17 (±0.09)	0.30 (±0.39)	0.63 (±0.49)	1.41 (±0.46)
RC-0.001 (CFU x 10^8 m^{-2})	0.07 (±0.02)	0.36 (±0.43)	0.08 (±0.12)	0.12 (±0.06)	1.49 (±1.75)
RC-0.01 (CFU x 10^8 m^{-2})	0.04 (±0.02)	0.41 (±0.40)	0.07 (±0.10)	0.19 (±0.15)	3.16 (±3.13)
RC-10 (CFU x 10^8 m^{-2})	0.004 (±0.002)	0.30 (±0.23)	0.05 (±0.07)	0.56 (±0.60)	1.13 (±0.70)
Biochemical parameters					
ATP (μg m^{-2})	13 (±7)	5 (±2)	21 (±15)	27 (±16)	19 (±8)
CHO (mg m^{-2})	34 (±21)	23 (±8)	29 (±30)	3 (±3)	15 (±19)
LIP (mg m^{-2})	5 (±2)	7 (±3)	7 (±3)	15 (±5)	6 (±5)
PROT (mg m^{-2})	15 (±15)	16 (±17)	25 (±13)	35 (±21)	13 (±12)
LOM (mg m^{-2})	55 (±37)	46 (±19)	61 (±42)	54 (±29)	34 (±27)

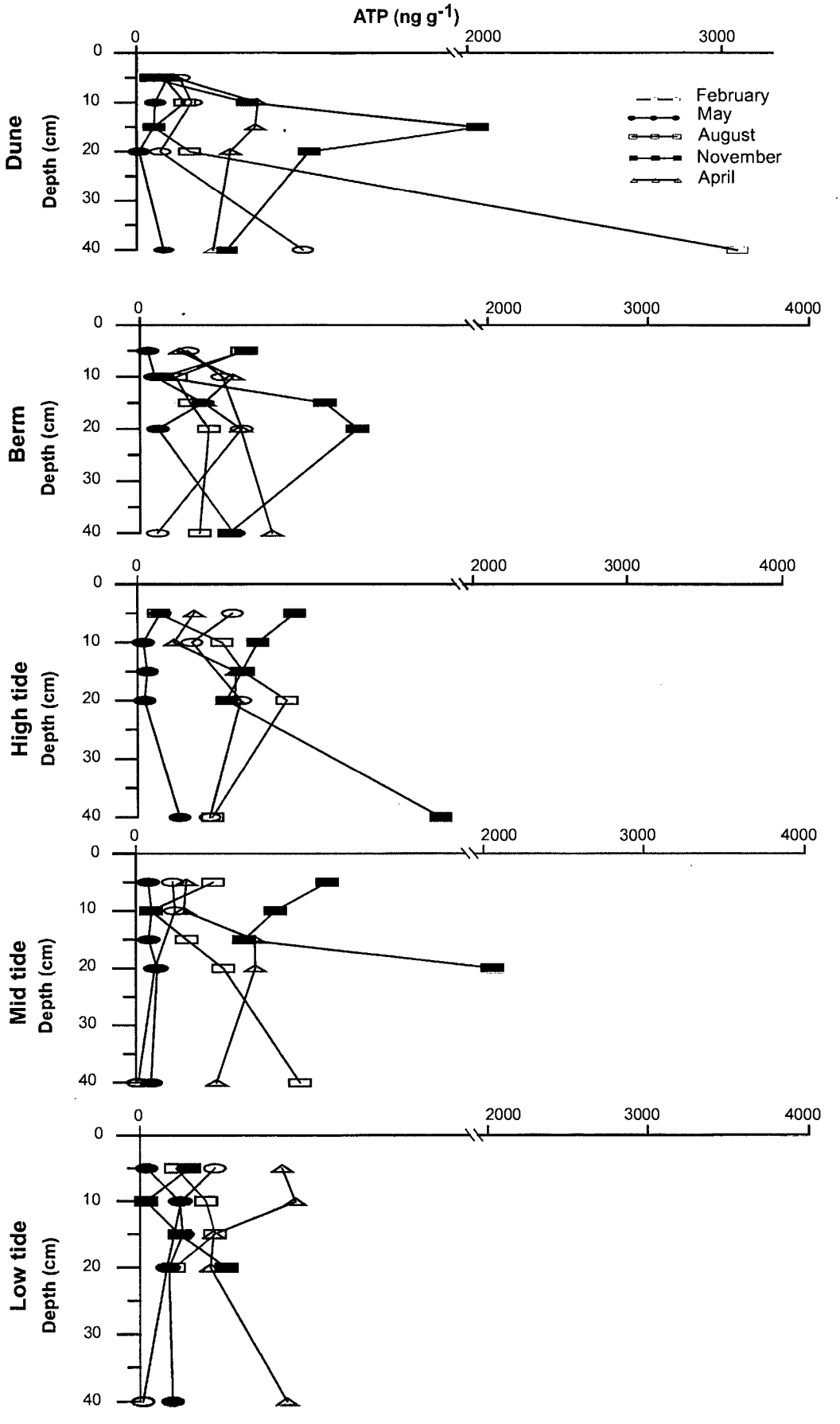


Fig 4.7: Spatio-temporal variation of ATP in dune, berm, high tide, mid tide and low tide sediments of the Central transect

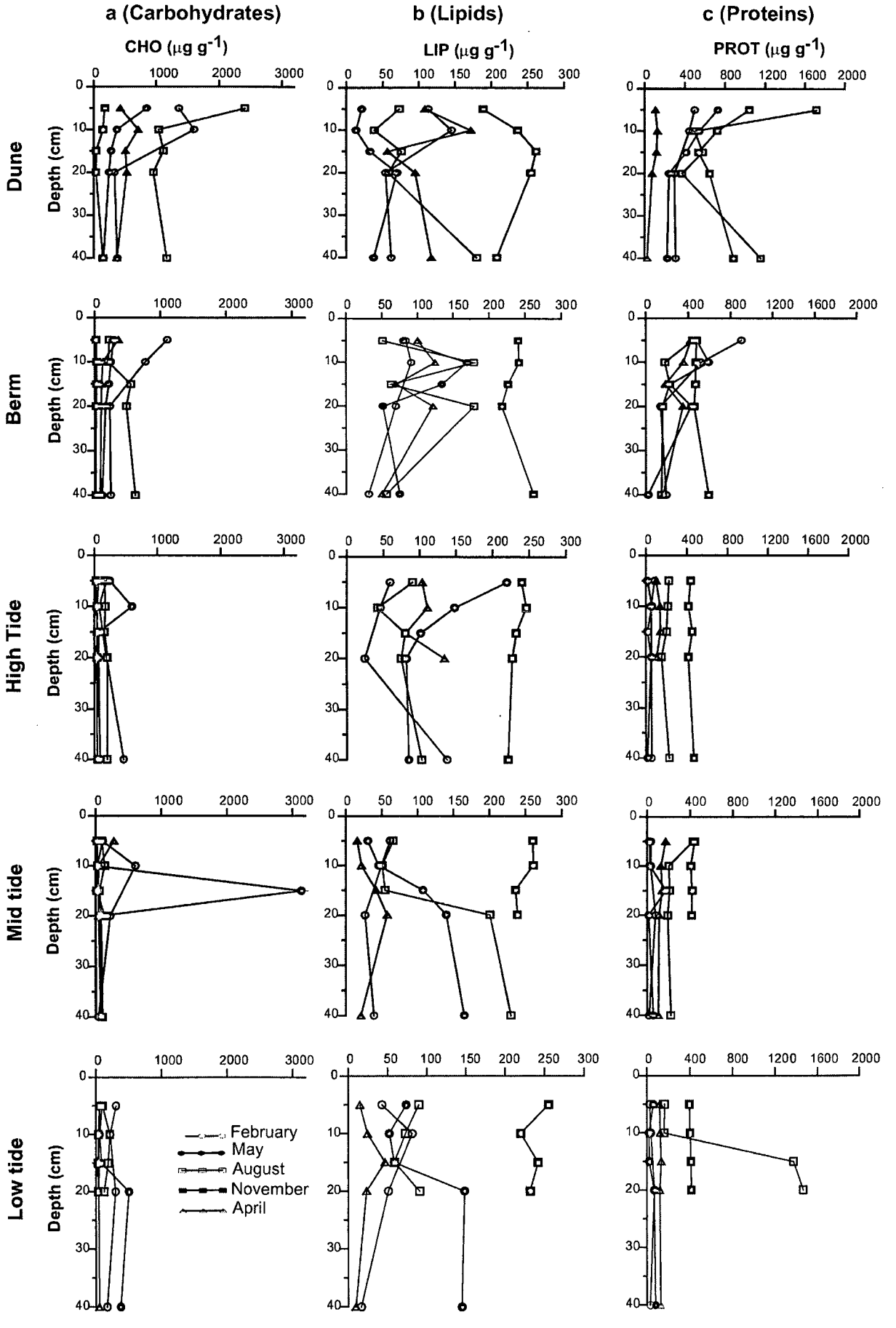


Fig 4.8: Spatio-temporal variation in (a) Carbohydrates, (b) Lipids and (c) Proteins in dune, berm, high tide, mid tide and low tide sediments of the Central transect

Table 4.3: Percentage contribution of different bacterial parameters to total bacterial abundance during the different months sampled at central transect

Sampling month	TC ($x10^5$ cells g^{-1})	% contribution of different bacterial parameter to TC				
		TVCa	TVCan	RC-0.001	RC-0.01	RC-10
February	2.00	25	32	4	2	0.2
May	1.36	50	17	29	34	30
August	2.92	12	17	4	4	2
November	5.00	21	19	3	5	15
April	9.23	59	21	18	33	16

Table 4.4: Percentage contribution of LOM constituents to LOM during the different months sampled at central transect.

Sampling month	LOM ($\mu g\ g^{-1}$)	% contribution of LOM constituents to LOM		
		CHO	LIP	PROT
February	192	63	9	27
May	182	55	15	30
August	453	45	9	46
November	511	6	30	64
April	154	42	18	40

Examination of around 825 isolates from the different depths revealed that the isolates were gram-negative and affiliated to β-Proteobacteria, γ-Proteobacteria and Bacteroidetes. The β-Proteobacteria *Alcaligenes* and γ-Proteobacteria *Pseudomonas* were well represented along the whole transect during all the seasons. The β-Proteobacteria *Chromobacterium* and other groups belonging to class γ-Proteobacteria viz *Aeromonas, Vibrio, Acinetobacter, Moraxella, Enterobacter* were also represented. *Flavobacteria* of phylum Bacteroidetes were also encountered (Table 4.5). Highest number of genera were retrieved during the pre-monsoon month of February. The dune and the berm area had higher population of oxidase positive isolates, while the intertidal region had a higher abundance of catalase positive forms.

Table 4.5: Distribution of various bacterial genera in sediments of different locations along the central transect

Sampling station		Sampling month			
		February	August	November	April
Stn D	*Acinetobacter*			√	
	Alcaligenes	√	√	√	√
	Alteromonas	√	√	√	√
	Chromobacterium	√			√
	Cytophaga		√	√	
	Flavobacterium		√	√	√
	Moraxella	√			
	Pseudomonas	√	√	√	√
	Vibrio				
Stn B	*Acinetobacter*	√	√		
	Alcaligenes	√	√	√	√
	Alteromonas	√	√	√	√
	Chromobacterium	√		√	√
	Cytophaga				
	Flavobacterium	√	√	√	√
	Moraxella	√			
	Pseudomonas	√	√	√	√
	Vibrio	√			
Stn H	*Acinetobacter*				
	Alcaligenes	√		√	√
	Alteromonas				
	Chromobacterium	√			√
	Cytophaga				
	Flavobacterium	√		√	
	Moraxella		√		
	Pseudomonas	√	√	√	√
	Vibrio		√		
Stn M	*Acinetobacter*				
	Alcaligenes	√	√	√	√
	Alteromonas				
	Chromobacterium	√			
	Cytophaga				
	Flavobacterium			√	
	Moraxella	√		√	
	Pseudomonas	√	√	√	√
	Vibrio	√	√	√	√
Stn L	*Acinetobacter*				
	Alcaligenes	√		√	
	Alteromonas				
	Chromobacterium	√			
	Cytophaga				
	Flavobacterium	√		√	
	Moraxella	√	√		√
	Pseudomonas	√	√	√	√
	Vibrio	√	√	√	√

* √ signifies the presence at the sampling station

Over 60% of the isolated strains were capable of expressing amylase, protease and lipase enzymes. The ability of isolated bacterial strains capable of decomposing macromolecular organic compounds is summarized in the figure 4.9. Isolates capable of hydrolyzing DNA were <50%. Least abundant were bacteria expressing phosphatase enzyme. There was no well described pattern of horizontal distribution along the studied transect. Amylase positive isolates dominated the beach ecosystem especially the dune and berm while the low tide region was marked by higher abundance of lipase positive isolates. The percentage of bacteria expressing enzymes showed seasonal variation. The average occurrence of bacteria hydrolyzing macromolecular compounds increased during post-monsoon season of November and pre-monsoon season of April. The capacity index which is a measure of the ability of bacterial isolates to decompose macromolecular compounds varied between 13% at berm during February and 78% at the dune region during November.

4.1.2. Offshore system – Kalbadevi bay

4.1.2.1. Sediment:

Generally the total bacterial abundance in the sediment was 10^7 cells g^{-1} during the entire sampling period. High abundance was estimated during May. The TVCa population ranged between 10^{6-7} cells g^{-1} while the TVCan population varied between 10^{5-7} cells g^{-1}. Like TC, the TVCa and TVCan population, recorded maximal values during May 2006. Retrievability on 10% nutrient amended plates was in the range of 10^{5-6} CFU g^{-1} and like the other bacterial parameters high values were enumerated during May. The Southern transect had relatively high load of bacteria as compared to Northern and Central transects (Table 4.6). Like the bacterial parameters, biochemical parameters in the sediments generally displayed high concentrations in May. The sediments of the Southern transect was relatively rich in the LOM constituents (Table 4.6).

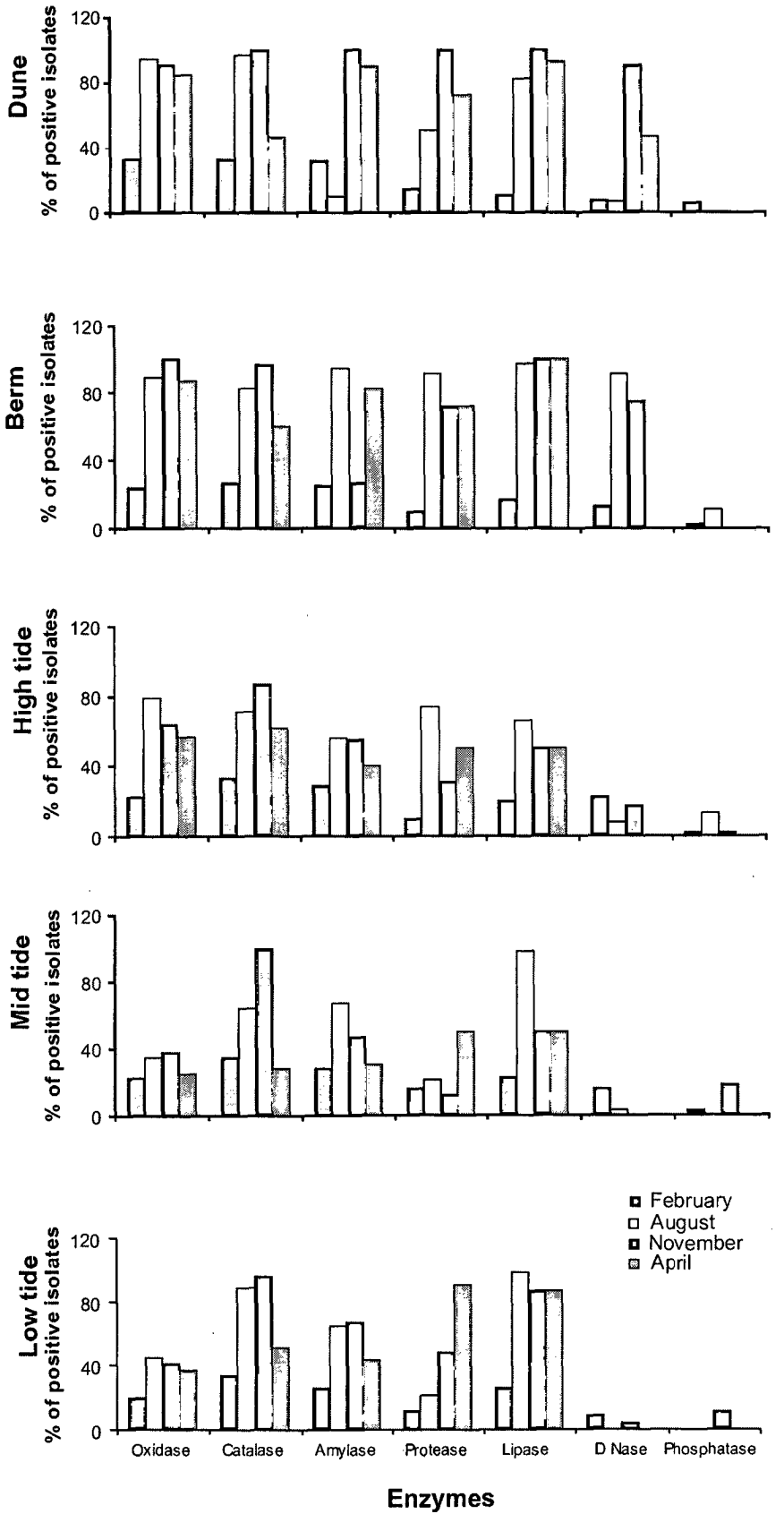


Fig 4.9: Spatio-temporal distribution of elaboration of enzymes by bacterial isolates from dune, berm, high tide, mid tide and low tide sediments of the Central transect

Table 4.6: Average values of bacterial and biochemical parameters covering the sampling year along the different transects in the offshore Kalbadevi sediments

Parameters	Sampling months	Transect		
		Northern	Central	Southern
Bacterial parameters				
TC (cells x 10^7 g^{-1})	January	1.78 (±0.37)	1.92 (±0.43)	1.89 (±0.36)
	May	*	3.49 (±1.09)	7.17 (±0.55)
	October	*	3.28 (±1.66)	1.05 (±0.39)
TVCa (cells x 10^7 g^{-1})	January	0.68 (±0.17)	0.35 (±0.23)	0.52 (±0.39)
	May	*	1.70 (±1.05)	3.01 (±2.63)
	October	*	1.02 (±1.06)	0.09 (±0.08)
TVCan (cells x 10^7 g^{-1})	January	0.19 (±0.05)	0.12 (±0.09)	0.23 (±0.04)
	May	*	1.90 (±0.76)	3.39 (±0.91)
	October	*	0.56 (±0.73)	0.15 (±0.09)
RC-10 (CFU x 10^7 g^{-1})	January	0.09 (±0.01)	0.07 (±0.04)	0.18 (±0.15)
	May	*	0.57 (±0.60)	1.33 (±0.47)
	October	*	0.37 (±0.30)	0.06 (±0.04)
Biochemical parameters				
ATP (ng g^{-1})	January	384 (±69)	453 (±42)	522 (±52)
	May	*	502 (±43)	476 (±62)
	October	*	370 (±90)	595 (±265)
CHO (μg g^{-1})	January	314 (±238)	309 (±134)	334 (±89)
	May	*	313 (±4)	220 (±33)
	October	*	355 (±193)	299 (±142)
LIP (μg g^{-1})	January	138 (±73)	146 (±103)	221 (±41)
	May	*	163 (±26)	196 (±30)
	October	*	116 (±84)	217 (±26)
PROT (μg g^{-1})	January	834 (±343)	980 (±268)	1227 (±1107)
	May	*	1739 (±1263)	1037 (±508)
	October	*	1928 (±2351)	515 (±279)

* Transect not sampled

4.1.2.2. Water:

Unlike the bacterial parameters, in the sediment, maximal abundance in TC was estimated during October. However, concentration of other biological parameters such as ATP and Chl *a* concentration was high in May. The Southern transect, however, was relatively rich in bacterial abundance, Chl *a* and ATP concentrations (Table 4.7).

Table 4.7: Average values of bacterial and biochemical parameters covering the sampling year along the different transects in the off shore Kalbadevi waters

Parameters	Sampling months	Transect		
		Northern	Central	Southern
TC (cells x 10^7 mL^{-1})	January	1.16 ±(0.36)	0.65 (±0.49)	1.19 (±0.47)
	May	*	1.19 (±0.42)	1.19 (±0.78)
	October	*	2.56 (±1.76)	1.42 (±0.60)
TVCa (cells x 10^7 mL^{-1})	January	0.72 (±0.11)	0.31 (±0.55)	0.52 (±0.53)
	May	*	0.29 (±0.26)	0.23 (±0.15)
	October	*	1.36 (±0.96)	0.47 (±0.43)
TVCan (cells x 10^7 mL^{-1})	January	0.86 (±0.34)	0.03 (±0.01)	0.40 (±0.44)
	May	*	0.18 (±0.15)	0.16 (±0.19)
	October	*	0.77 (±0.90)	0.49 (±0.66)
RC-10 (CFU x 10^6 mL^{-1})	January	0.61 (±0.22)	0.03 (±0.03)	2.61 (±2.77)
	May	*	0.29 (±0.20)	0.47 (±0.33)
	October	*	4.28 (±4.02)	1.79 (±1.65)
ATP (ng mL^{-1})	January	4.4 (±2.8)	2.2 (±0.5)	2.3 (±0.1)
	May	*	2.9 (±0.5)	3.2 (±0.2)
	October	*	3.7 (±0.9)	2.9 (±0.3)
Chl a (mg m^{-3})	January	2.9 (±0.5)	3.8 (±1.9)	3.8 (±1.4)
	May	*	5.2 (±0.7)	5.9 (±1.0)
	October	*	0.2 (±0.09)	0.2 (±0.07)
Phaeo a (mg m^{-3})	January	2.2 (±0.4)	2.5 (±0.9)	2.5 (±0.9)
	May	*	2.1 (±1.5)	1.9 (±1.0)
	October	*	0.1 (±0.06)	0.1 (±0.09)
Phytoplankton cells (x 10^3 counts L^{-1})	January	3.7 (±1.6)	4.2 (±1.9)	5.7 (±1.4)
	May	*	18.8 (±6.2)	26.8 (±9.1)
	October	*	21.4 (±3.2)	17.2 (±8.5)

* Transect not sampled

Diatoms dominated during different sampling periods in the sequence; May (97%) > October (90%) >January (65%). A clear succession of species was recorded in the distribution of diatom species. During January, the phytoplankton community was dominated by *Thalassionema* spp., *Thalassiothrix* spp. in May and in October by *Nitzschia* spp. Among dinoflagellates, *Ceratium* spp. dominated during January and October while *Dissodinium* spp. predominated in May (Fig. 4.10)

4.2. Discussion

4.2.1. Common spatio temporal patterns of the Kalbadevi beach and bay

Though the current study was undertaken spatially for all transects along the Northern, Central and Southern transect, both onshore and offshore, detailed study was carried out on the onshore Central transect.

Sediment labile organic matter and bacterial abundance showed clear differences between the three transects both spatially and temporally. Although the overall quantitative distribution of LOM among the three transects was comparable, there was difference in the qualitative distribution among the three transects. The Northern transect had higher concentrations of sedimentary carbohydrates and lipids while the Central transect had higher concentration of sedimentary proteins. Further the average PROT:CHO ratio of 0.6 indicated that the Northern transect was relatively more oligotrophic than the Central and Southern transect which had a ratio of 1 and 0.7 respectively. Also, the sediments of the Northern transect tended to harbour higher population of viable anaerobic bacteria [0.92 (±1.23) x 10^8 cells m^{-2}]. In the similar line of argument, the Southern transect, both offshore and onshore, tended to be more eutrophic in nature. Also the degree of interdependence between the bacterial and the biochemical parameters was more at the Northern and Southern transect. Study by Deming and Yager (1992) has demonstrated that bacterial abundance and distribution is dependent on the availability of labile organic compounds. Further, the morphodynamic and physicochemical characteristics of the position of the transect may be a determining factor for the presence of organic matter which in turn could

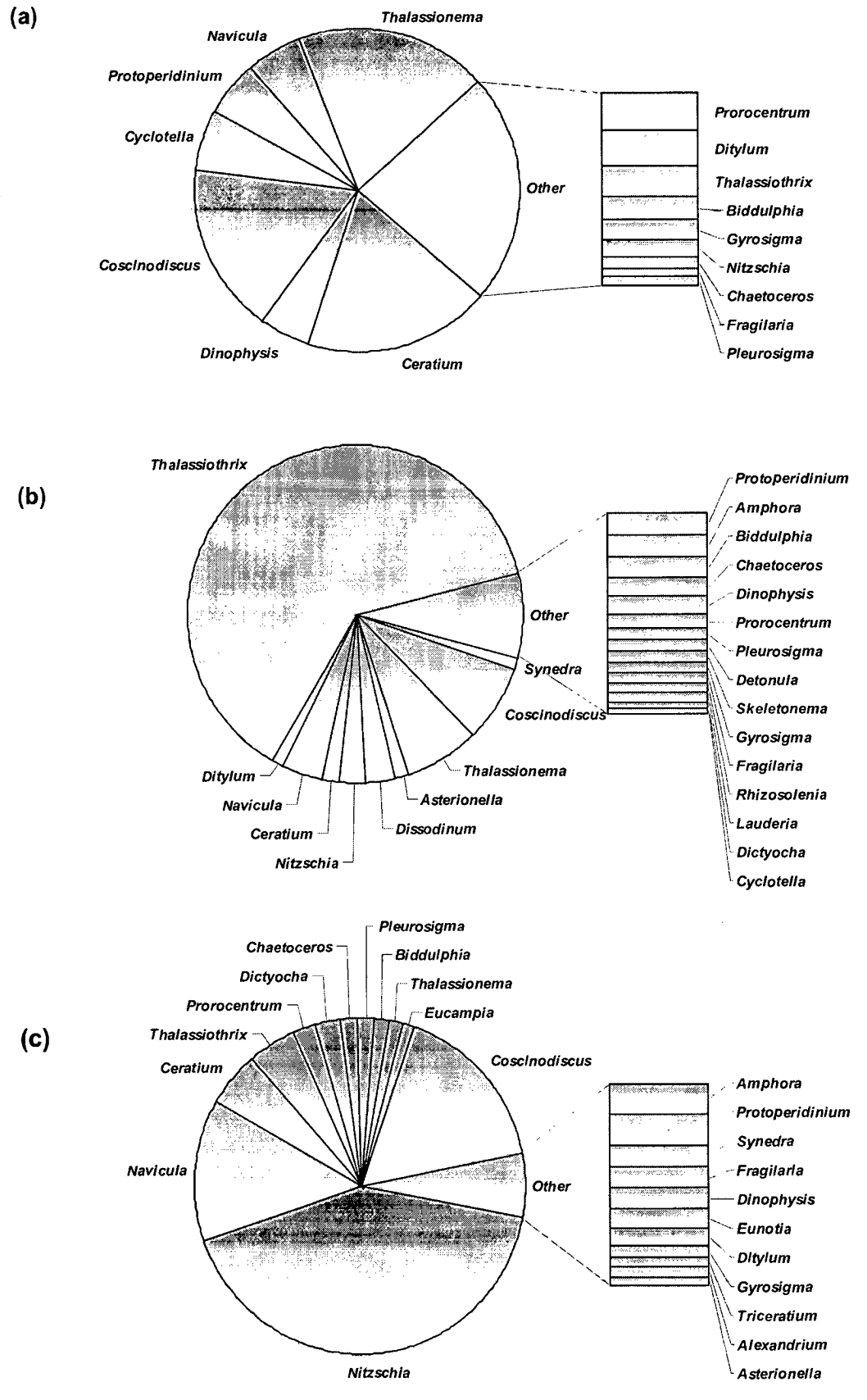


Fig 4.10: Percent composition of phytoplankton genera in Kalbadevi water during (a) January, (b) May and (c) October

translate into higher or lower bacterial load. The Are creek and Kalbadevi creek lie at close proximity along the Northern and the Southern transects. Both these creeks are lined by mangrove vegetation and feed the associated bay waters with huge amount of plant litter. The decomposition and litter fall may contribute immensely to the increased nutrient levels (Dham et al., 2002). The Northern transect is influenced by this huge amount of plant litter. On the other hand, the Southern transect not only receives huge deposition of terrestrial plant litter but also is rich in benthic biomass deposition and drifted seaweeds (Sivadas, 2009).

Moreover, in the offshore Kalbadevi bay sediments, the high bacterial load was coupled with high sedimentary protein concentration during pre-monsoon month of May and synchronized with high phytoplankton abundance and Chl *a* maxima during the same month in Kalbadevi Bay waters. Thus it may suggest a strong benthic pelagic coupling. The increased protein concentration in the sediment during May may indicate that the protein content is more related to phytoplankton distribution in the water column. Further, the increase in the bacterial load suggested that the rapid increase may be a response to pelagic production. Study by Danovaro et al., (1999) has shown that the sedimentary protein was dependent upon the downward flux of phytoplankton pigments. Bacterial parameters like total abundance, viability and retrievability in the Kalbadevi bay waters were high during post-monsoon month of October. On the other hand, an increase in bacterial and biochemical parameters in Kalbadevi beach sediments during November was also evident. This increase may be attributed to allochthonous inputs from the hinterland. As in other ecosystems, diatoms were a dominant community in Kalbadevi Bay and in May constituted nearly 97% of the phytoplankton community. Dinoflagellates were generally <10% but increased levels of 35% were recorded in January. This may suggest increased input of organic matter into the system. Generally, the heterotrophic dinoflagellates increase with the increase in input of organic matter, while the diatoms are more autotrophic.

4.2.2. Spatial variability in the bacterial and biochemical parameters in the Kalbadevi beach along the Central transect

At the Central transect, the different spatial patterns of bacterial and biochemical parameters suggested a distinct composition of the microflora and/or origin of the organic matter inputs during the different sampling periods.

The supra-tidal region of the study site in Kalbadevi beach comprised predominantly of *Casuarina* tree (25% m^{-2}) at the dune and *Ipomoea pescaprae* shrubs (65% m^{-2}) in the berm (Anon, 2004). The beach has a gradual slope harboring *Casuarina* plantation followed by a steep gradient from supra-littoral zone to the low tide level. The dune and the berm region is covered by sand dune vegetation in the supra-littoral zone. Nearly 65% of the berm area is covered by *Ipomoea pes-caprae*, while *Cyperus arenarius* and *Launaea pinnatifida* forms a dense cover separating the backshore zone of *Casuarina* plantation. Other plants such as *Clerodendrum inerme, Calotropis gigantea, Tephrosia purpurea, Physalis minima, Acrocephalus capitatus, and Spinifex littoreus* form an aggregate of mixed species. Also, the beach sediments of Kalbadevi bay contain ca 60% ilmenite on an average. During the sampling period, the intertidal region was covered with plant litter deposited by the tidal currents. It is possible that this vegetation as well as the plant litter contribute to the sedimentary organic matter.

The composition of sedimentary organic matter in the present study is comparable with other beach areas (Meziane et al., 1997; Cividanes et al., 2002; Incera et al., 2003; Fabiano et al., 2004). Naturally, higher sedimentary protein concentration in the dune and berm region could not only be due to the greater abundance of bacteria but also due to the proteinaceous exudates that could emanate from the roots of the existing vegetation (Hertenberger et al., 2002). This high protein concentration also implied that there was no constraint for heterotrophic metabolism in the sediments (Fabiano et al., 1995). The maximum carbohydrate concentration at the dune could be attributed to the dune vegetation. Vascular plants are known to contribute to

sedimentary carbohydrates (Bhosle and Dhople, 1988). Among the biochemical variables contributing to sedimentary labile organic matter (LOM), the carbohydrates were the dominant class. This usually signifies oligotrophic type of sediments. According to Danovaro et al. (1993), high carbohydrate concentrations in the sediments are characteristic of highly oligotrophic or detritic environments. Nevertheless, in the present study the carbohydrate concentrations are linked to contribution by the dune vegetation. Overall, carbohydrates, proteins and lipids contributed to LOM in the sequence CHO > PROT > LIP at 42, 41, 16% respectively. Similarly statistical analysis showed that carbohydrates, proteins and lipids contributed significantly to the variation in LOM in the sequence CHO > PROT > LIP at 73, 54, 3% respectively. This suggests that the input rate of carbohydrate is greater than its degradation rate. On the other hand, low contribution of protein and lipids may also indicate preferential utilization of these biochemical components. The CHO > PROT > LIP pattern of sedimentary concentration for Kalbadevi sediments may suggest the utilization in the sequence CHO < PROT < LIP. Previous study by Bhosle and Dhople (1988) has shown that favorable utilization of energy rich lipids and easily hydrolysable protein could contribute to lower these values in the sediment. The carbohydrate concentrations could be a contribution from vegetation, which in turn could contribute to total organic carbon (TOC) and biopolymeric carbon (BPC). BPC that is derived from the sum of protein, carbohydrate and lipid is assumed to be a reliable estimate of the labile fraction of the total sedimentary organic matter (Danovaro et al., 1993). It gives the holistic status of the available nutrients in the sediment (Fichez, 1991). In the present study, there is a marked decrease in BPC with depth and from the dune to the low tide level. The lower levels of LOM and nearly homogenous distribution of LOM and thus BPC in the intertidal region could be due to the effect of the wave action. It is well known that strong hydrodynamism permits deposition of coarser sediments through which water runs easily preventing the accumulation of dead organic matter (Langezaal et al., 2003). In contrast, living organic matter measured in terms of ATP does not get dispersed but is reflected in the accumulation at the high tide. It is known that ATP is used to understand the distribution and dynamics of living biomass (Stoeck et al., 2000). Higher ATP values at high tide sediments

suggest that there could be entrapment of small meiobenthic/zooplankton organisms at this level. Reichgott and Stevenson (1978) have shown that copepods are major contributors to sediment ATP. In the present study too relatively higher meiobenthic population was recorded in the intertidal sediments (Anon, 2005).

The natural abundance of bacteria in the present study area is generally an order of magnitude less than reported elsewhere (Schmidt et al., 1998; Luna et al., 2002; Moreno et al., 2006). Bacteria are found commonly attached to the grains in sandy beach sediment and their abundance is controlled by various factors having complex relationships (Meadows and Anderson, 1968; Yamamoto and Lopez, 1985). Studies have shown that total abundance ranged from 10^{7-10} cells per gram and increase with finer sediment as it provides with a greater surface area (Dale, 1974; Meyer-Reil et al., 1978; Schmidt, et al., 1998). Lower bacterial abundance at mid tide level could be accredited to the concentration of both meio and macrofauna at this level (Anon, 2005). It is well known that sedimentary benthic bacteria are considered as a major food source for benthic organisms (Boucher and Chamroux, 1976; Sundbäk et al., 1996; Moreno et al., 2006) and perhaps this low abundance at this station is due to the feeding and grazing pressure by higher benthic organisms. Nevertheless, the higher numbers at certain locations of the studied transect could be attributed either to silt content or the labile organic matter derived from the associated vegetation comprising of dominant *Ipomoea pes-caprae* shrubs in the berm and *Casuarina* tree plantation in the dune. Similar observations have been made by several workers (Webley et al., 1952, Andrade et al., 1997; Whipps, 2001). The rhizosphere of sand dune plants has been shown to contain abundant bacteria and this has been accredited to the root exudates and root-deposits that form the substrates for the bacteria. In turn, the plants derive benefits from bacterial association. Interactions with microbes are crucial in obtaining inorganic nutrients and growth-influencing substances for the plants, especially in the terrestrial environment (Vancura, 1986). However, in the present study area, statistical analyses have revealed that the bacterial

abundance, viability and retrievability were not constrained by LOM or its constituents.

On an average, the potentially viable cells encountered formed a significant 33% of the total abundance. The direct viable counts could control up to 65% ($p \leq 0.001$) of variation in TC while the viable cells enumerated after anaerobic incubation could control up to 54% ($p \leq 0.001$). Higher aerobic viability encountered at the low tide could be due to diffusion of oxygenated waters to the deeper layer brought about by the churning movement of the sediment or the continuous nutrient replenishment from the surf waters at the low tide region. The variability ranged from 10^4 to 10^6 cells per gram in the dune and the berm. This could be due to less diffuse nutrients distributed generally in patches/pockets at the berm and the dune. However, the variability persists at the low tide in spite of diffusion of surf waters at deeper depths. Thus the variability across the whole transect was over two orders. Overall, viability measured under anaerobic conditions was higher at 21% of the total counts and like aerobic viability varied over two orders. The tendency to flourish under anoxic conditions could be a measure of their facultative nature and/or the frequency of encountering anaerobic regimes in space or in time. The intertidal region is under influence of surf water only during the high tide and is drained off during low tide. This tidal cycle could influence the physiology of the microbial communities present therein particularly at the low tide region where the frequency of influence is higher and longer. This inference is further corroborated by strong relationship between the aerobic and anaerobic forms. The strong relationship between TVCa and TVCan ($n = 354$, $r = 0.75$, $p \leq 0.001$), suggests that there is higher abundance of facultative anaerobes in the bacterial community with ability to adapt to oxic and suboxic conditions during high and low tide respectively.

Like viability, the retrievability i.e colony forming units was high. Though culturable counts from environmental samples, usually represent only a small fraction (0.1% ± 10%) of the active microbial community (White et al., 1998), the counts from the Kalbadevi accounted for 13% of TC on 10%NB, 16% of

TC on 0.01% NB media and 12% of TC on 0.001% NB media. The fraction of retrieved percentages thus suggests that the active microbial community could be high. RC retrieved from different concentrations of nutrient amended media had a significant influence on the variation of TC ($\sim r = 0.4$, $p \leq 0.001$) suggesting that it could control about 16% of the TC. The 12% culturable fraction on 10% NB and 0.001% NB media implies that a considerable fraction of sedimentary bacteria find these widely different concentration optimal. These observations suggest that the bacteria are highly resilient or the ecosystem is heterogeneous in nature in the trophic requirement. Presence of significant interrelationship between the populations is supportive of the latter.

Although the retrievability was high, the diversity was restricted to only eight groups of culturable bacteria. β-Proteobacteria *Alcaligenes spp* and the nutritionally and metabolically more versatile group, *Pseudomonas* of γ-Proteobacteria were well represented along the Central transect. Usually, γ-Proteobacteria is more predominant in other ecosystem (Musat et al., 2006). In addition to γ-Proteobacteria, β-Proteobacteria also predominates in this ecosystem. Further, both the groups found in the beach sediments are usually known to be associated with the rhizospheres of plants. 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, 1992) and are also adapted to low nutrient levels (Höfle, 1983; Stoeck et al., 2002). However, it is pertinent to note that most of the groups encountered at the study site are also involved in iron reduction. The area is rich in ilmenite which may act as source of iron for these iron reducers. Further, it is well known that *Pseudomonas* sp. play an important role in Fe(III) reduction especially in the rhizophere (Ottow and Glathe, 1971).

Expression of enzymes such as catalase and oxidase is encountered where catalase is normally involved in the resistance and survival upon exposure to H_2O_2 (Gu, 2004) whereas oxidase is an enzyme that catalyzes the reactions of molecular oxygen. The dune and the berm which were the comparatively

dry zones than the intertidal area had higher population of oxidase positive isolates while the intertidal had high abundance of catalase positive isolates. The higher population of oxidase positive isolates at dune and the berm area could be in response to the high oxygen evolution generally encountered at rhizospheres of vegetation.

The distribution of metabolic diversity of bacteria could be deduced by the ability of the bacterial isolates to express enzymes to hydrolyze different types of macromolecular compounds such as lipids, carbohydrates, fats and nucleic acids. In sandy beaches, most of the organic matter breakdown is carried out by microorganisms, mainly by bacteria (Jedrzejczak, 1999; Koop et al., 1982). Overall, the studied isolates were able to express enzymes in the sequence lipase > amylase > protease> DNase > phosphatase. Further, the isolates from areas with the lowest sedimentary LOM had apparently highest metabolic versatility. Similar differences in response have been encountered when comparing estuarine with relatively more oligotrophic isolates from offshore waters (Goltekar et al., 2006). On an average, lipolytic bacteria accounted for 65% of all the studied isolates. Frequency of isolates expressing lipolytic activity was also maximum at low tide perhaps due to inputs from surf waters. High lipase positive strains in the low tide region indicated that the organisms prefer lipid as their substrate in this region. High number of isolates expressing this enzyme is also found in other beaches (Podgórska and Mudryk, 2003) as well in marine systems (Bölter and Reinheimer, 1987; Krstulović and Solić, 1988; Mudryk et al., 1991; Mudryk, 1998). Studies have also shown that lipolytic bacteria play an important role in the modifying and transforming lipid compounds in water bodies (Wakeham, 1995; Martinez et al., 1996; Gajewski et al., 1997). 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, 1987). Considerable amounts (i.e. 2 to 45%) of lipids are accumulated by ciliates, zooplankton, phytoplankton, benthos and detritus hence the source of lipids could include dead phytoplankton, zooplankton, meiobenthos, macrobenthos and detritus (Siuda et al., 1991; Albers et al., 1996; Harvey et al., 1997). The

expression of amylolytic activity was well represented all over the beach especially at the dune and the berm region. Amylolytic bacteria accounted for nearly 51% of the total isolates studied. This may be attributed to the abundance of the substrate carbohydrates which made up nearly 42% of the total LOM. Protease is relatively uniformly distributed across the transect suggesting its ubiquity in this ecosystem. Nearly 46% of the bacteria were capable of hydrolyzing proteins. The source of proteins may include dead bodies of phytoplankton, zooplankton and benthos (Billen and Fontigny, 1987). Extracellular proteases hydrolyze proteins into mono- or oligomers, mainly peptides and amino acids (Pantoja and Lee, 1994). These low molecular weight organic compounds are immediate precursors in the synthesis of proteins and participate in many pathways of microbial cell metabolism (Simon, 1998; Mudryk and Podgórska, 2005). In the Kalbadevi beach, bacteria capable of DNA-hydrolysis accounted for nearly 25%. DNase expression increased towards the drier zones perhaps helping to meet their nitrogen requirements. DNA is the basic biopolymer occurring in the cells of all living organisms, and released after their death to the environment. Heterotrophic bacteria can utilize DNA in their metabolic processes as a source of carbon, nitrogen, and phosphorus (Jørgensen et al., 1993, Dell'Anno et al., 1999). On the other hand, bacteria capable of expressing phosphatase were generally rare in the sand of the Kalbadevi beach. It is well known that extracellular phosphatases can play an important role in supplying phosphorus to heterotrophic microorganisms and to autotrophic algae and incidence of higher phosphatase activity is indicative of phosphate limitation (Marxsen and Schmidt 1993). This implied that the bacteria in these placer rich beach ecosystems were apparently not limited by this nutrient.

Thus the dune and the berm sediments of the placer rich Kalbadevi beach promoted high abundance, culturability and taxonomic diversity.

Chapter 5

Chapter 5: Comparative study of abundance, activity and diversity of culturable bacteria in ilmenite-rich supra-littoral berm with ilmenite poor dune and intertidal region

5.1. Introduction

This chapter presents a comparative study between three different sites on the beach transect. These sites were chosen based on the sedimentary LOM content and ilmenite concentration. Bacterial parameters covered total as well as culturable abundance, culturable phylogenetic and metabolic diversity. Geochemical and biochemical nature of the sediments are related to biotic properties. Statistical analyses between the three sites was used to delineate the influence of ilmenite on the bacterial community.

5.2. Results

5.2.1. Geochemistry of the sediments

Generally, the heavy mineral, ilmenite decreased with depth except at Stn B where the mid depth accumulation increased at 20 cm depth. Percentage of ilmenite in the beach sediment ranged from 1.55 (Stn M, 20-40 cm depth) to 28.42% (Stn B, 10-20 cm depth). Average concentration of ilmenite (Ilm) at Stn B. was significantly higher ($p \leq 0.001$) than Stn M. It was nearly 7.5 times more than Stn M and 2.5 times more than Stn D (Table 5AT1, Appendix III, Fig 5.1a)

Total carbon (Tcarb) ranged from 0.71% (Stn D, 20-40 cm depth) to 4.7% (Stn M, 20-40 cm depth) and the average concentration was in the sequence Stn M > Stn B > Stn D (Fig 5.1b). Thus, average Tcarb concentration in the Stn B sediment was 71% lower than Stn M sediments. On the other hand, total organic carbon (TOC) ranged from 0.01% (Stn M, 0-5 cm depth) to 0.08% (Stn D, 20-40 cm depth). The average concentration of TOC was in the sequence Stn D > Stn B > Stn M and the average TOC values in the Stn B sediment was 76% higher than Stn M sediment (Table 5.1., Fig 5.1c).

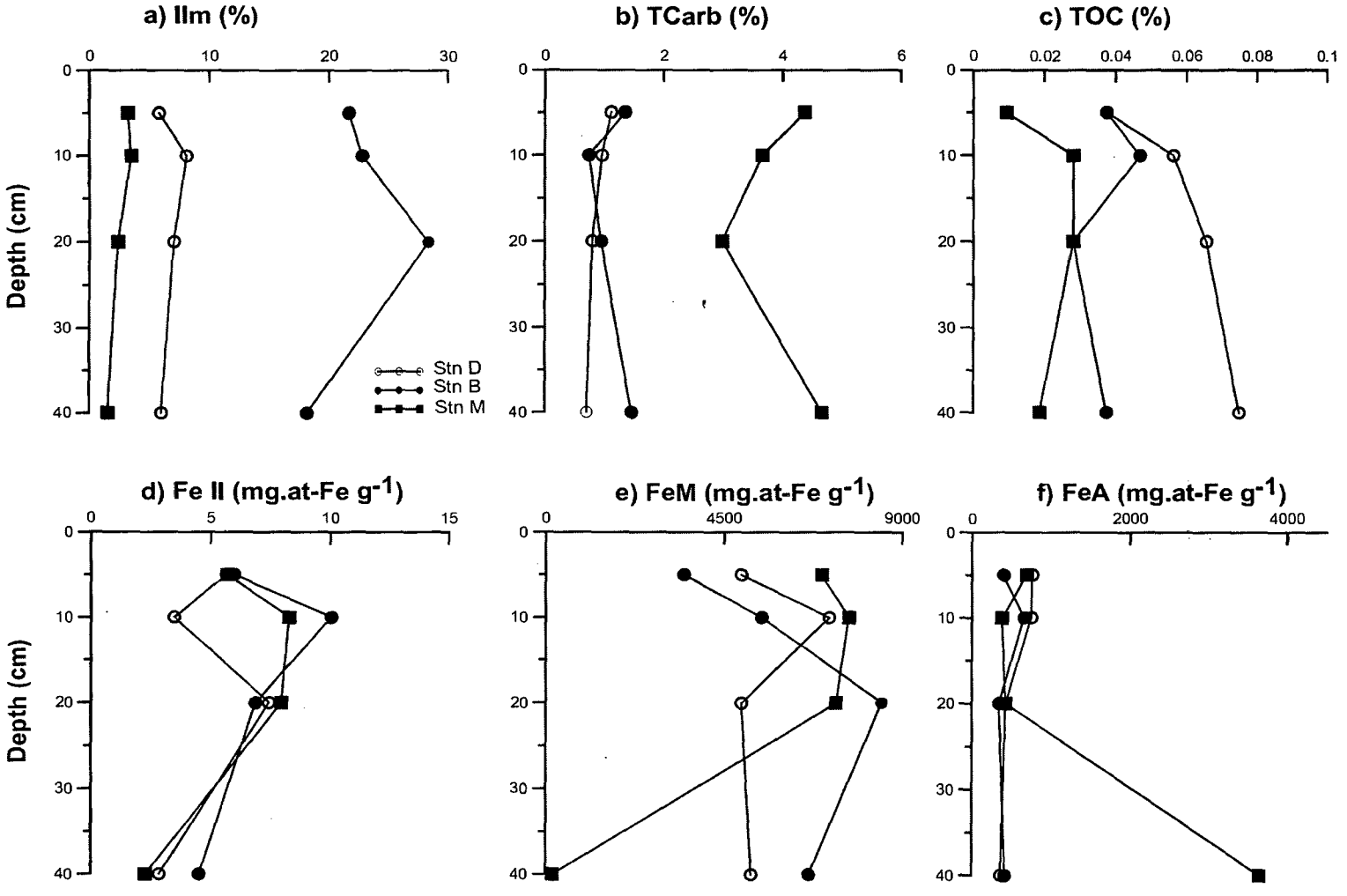


Fig 5.1: Down core variation in sedimentary geochemical parameters at Dune (Stn D), Berm (Stn B) and Mid tide (Stn M) stations.

*(a) Ilm - Ilmenite, (b) Tcarb - Total carbon, (c) TOC - Total organic carbon, (d) Fe(II), (e) FeM- Microbially reducible iron, (f) FeA- Acid soluble iron

The sedimentary Fe(II) concentration ranged from 2.28 mg-at.Fe g^{-1} (Stn M, 20-40 cm depth) to 10.04 mg-at.Fe g^{-1} (Stn B, 5-10 cm depth) while the microbially reducible Fe(III) (FeM) concentration varied from 171 mg-at.Fe g^{-1} at Stn M, 20-40 cm to 8487 mg-at.Fe g^{-1} at 10-20 cm layer of Stn B. The average concentration of Fe(II) and FeM was in the sequence Stn B > Stn M > Stn D. On the other hand, the HCl extractable Fe(III) (FeA) concentration ranged from 344 mg-at.Fe g^{-1} (Stn B, 10-20 cm depth) to 3643 mg-at.Fe g^{-1} (Stn M, 20-40 cm depth) and the average concentration was in the sequence Stn M > Stn D > Stn B (Table 5.1., Fig 5.1 d-f).

Table 5.1: Average values of bacterial and biochemical parameters at the dune, berm and mid tide region during relatively stable month of February as compared to other seasons.

	Stations		
Parameters	**Stn D**	**Stn B**	**Stn M**
TC (x 10^6 cells g^{-1})	1.55 (±1.21)	2.90 (±1.81)	0.54 (±0.60)
TVCa (x 10^6 cells g^{-1})	0.42 (±0.33)	0.74(±0.54)	0.18(±0.24)
TVCan (x 10^6 cells g^{-1})	0.82 (±0.68)	0.90 (±0.73)	0.24(±0.22)
RC-0.001 (x 10^6 CFU g^{-1})	0.10 (±0.06)	0.07 (±0.04)	0.04 (±0.04)
RC-0.01 (x 10^6 CFU g^{-1})	0.06 (±0.03)	0.06 (±0.01)	0.04 (±0.02)
RC-10 (x 10^6 CFU g^{-1})	0.006 (±0.003)	0.004 (±0.002)	0.004 (±0.004)
IB (x 10^6 CFU g^{-1})	0.002 (±0.0007)	0.001 (±0.001)	0.0006 (±0.0004)
IR (x 10^6 CFU g^{-1})	0.0026 (±0.002)	0.0007 (±0.0003)	0.0005 (±0.0004)
ATP (ng g^{-1})	191 (±163)	165 (±114)	64 (±45)
CHO (μg g^{-1})	917 (±656)	585 (±427)	238 (±264)
LIP (μg g^{-1})	93 (±40)	69 (±26)	44 (±17)
PROT (μg g^{-1})	385 (±99)	438 (±320)	39 (±24)
LOM (μg g^{-1})	1396 (±769)	1091 (±749)	321 (±265)
Tcarb (%)	0.90 (±0.16)	1.13 (±0.30)	3.92 (±0.68)
TOC (%)	0.06 (±0.01)	0.04 (±0.01)	0.02 (±0.01)
Fe(II) (mg-at.Fe g^{-1})	4.9 (±2.0)	6.9 (±2.4)	6.0 (±3.2)
FeA (mg-at.Fe g^{-1})	577 (±217)	456 (±142)	1286 (±1428)
FeM (mg-at.Fe g^{-1})	5548 (±1043)	6012 (±1912)	5528 (±3261)
Ilm (%)	6.7 (±1.1)	22.8 (±4.3)	2.7 (±0.88)
Community metabolic diversity (substrates utilized)	13 (±7)	26 (±3)	12 (±10)
Capacity index (%)	40	37	48
Phylogenetic diversity	6	8	5
Siderophore producers (%)	59	87	27

5.2.2. Biochemistry of the sediments:

The depth profiles of sedimentary protein, carbohydrates, lipids and LOM as well as ATP showed a subsurface maximum (Fig 5.2a). Average concentration of LOM was highest at Stn D (1396 ± 769 $\mu g\ g^{-1}$) followed by Stn B (1091 ± 749 $\mu g\ g^{-1}$) and Stn M (321 ± 265 $\mu g\ g^{-1}$). At Stn B, the average concentration of protein, carbohydrate, lipid and ATP was 10.4, 1.5, 0.5 and 1.5 times higher than Stn M (Table 5.1, Fig 5.2 b-e).

5.2.3. Bacterial abundance

5.2.3.1. Total bacterial abundance (TC)

TC ranged from 10^5 to 10^6cells g^{-1} and showed subsurface maxima (Fig 5.3a). Stn B recorded the highest abundance (2.9 ± 1.81 x 10^6 cells g^{-1}) while Stn M recorded an order less (Table 5.1). At Stn B, the abundance was significantly higher (5 times) than Stn M ($p \leq 0.002$) (Table 5AT1, Appendix III).

5.2.3.2. Total direct viable counts (TVC)

The average viable population at Stn B was nearly three times higher than Stn M and the difference was significant (TVCa: $p \leq 0.01$; TVCan: $p \leq 0.01$). The viability of bacteria ranged from 26 to 34% of TC under aerobic conditions. However, under anaerobic conditions the viability was still higher at 31% at Stn B, 44% at Stn M and 53% at Stn D (Table 5.1, Fig 5.3b and c).

5.2.3.3. Culturable counts (CFU)

When media was amended with 0.001 and 10% nutrients, average retrievability was highest at the dune while at 0.01%NB, the number of CFU retrieved were high at berm. Further, the contribution of the retrievable population to TC was highest on 0.01% nutrient amended plates and contributed to nearly 9% at Stn B, 15% at Stn D and 20% at Stn M (Table 5.1., Fig 5.3 d-f). The average retrievability of iron utilizing bacteria (IB) was higher at Stn D than Stn B by 28% while iron reducing bacteria (IR) was relatively equally distributed between Stn D and B. (Table 5.1., Fig. 5.3 g,h).

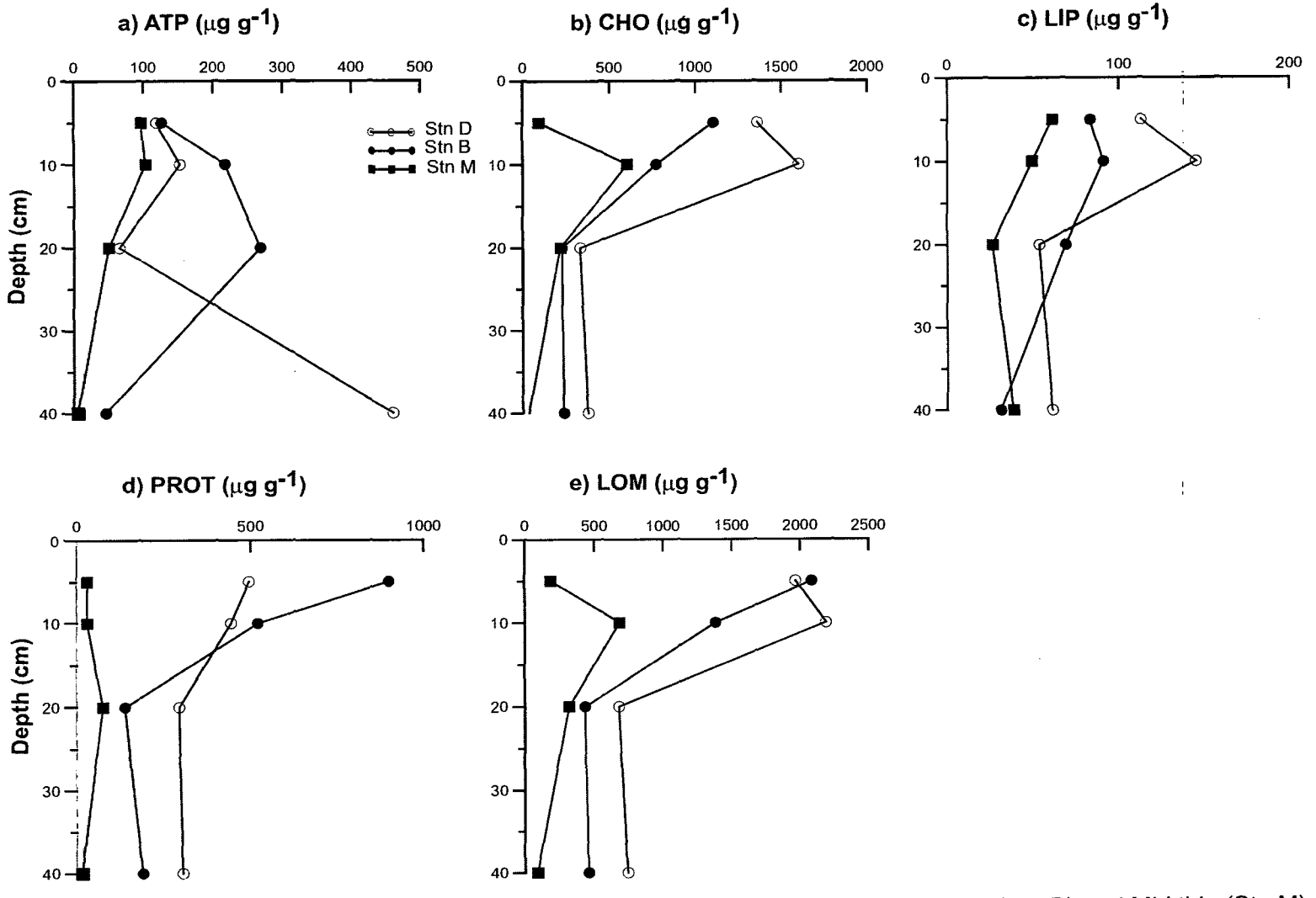


Fig 5.2: Down core variation in sedimentary biochemical parameters at Dune (Stn D), Berm (Stn B) and Mid tide (Stn M) stations
*(a) ATP, (b) CHO - Carbohydrates, (c) LIP - Lipids, (d) PROT- Proteins, (e) LOM - Labile organic matter

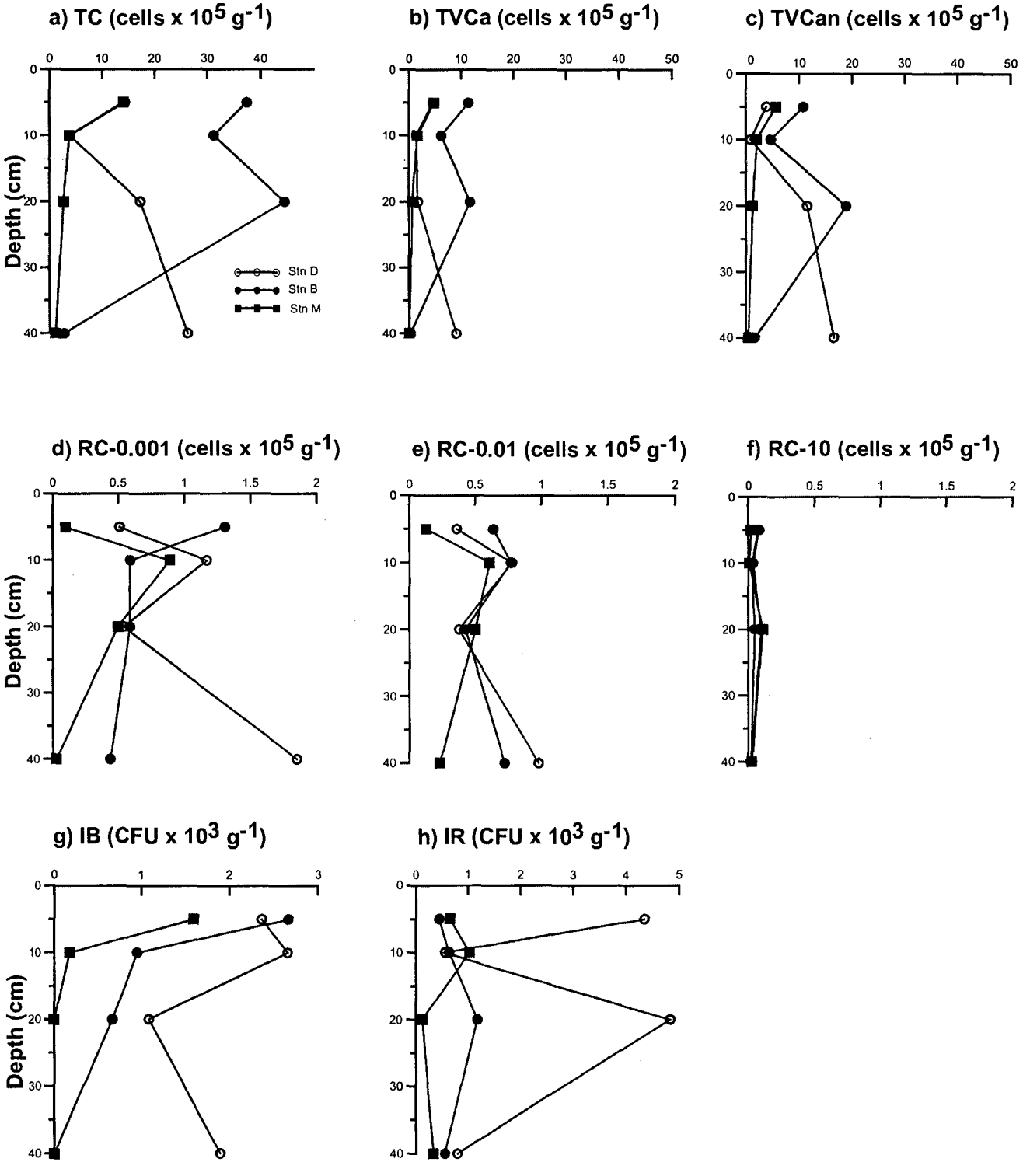


Fig 5.3: Down core variation in sedimentary bacterial parameters at Dune (Stn D), Berm (Stn B) and Mid tide (Stn M) stations

* TC - Total counts, Total viable counts - aerobes (TVCa); - anerobes (TVCan), Retrievable counts on 0.001% (RC-0.001); on 0.01% (RC-0.01); on 10% (RC-10) nutient amended media, IB - iron bacteria, IR - iron reducing bacteria.

5.2.3.4. Phlyogenetic diversity of bacterial isolates

Biochemical characterization of nearly 262 isolates retrieved on different media revealed that they were affiliated to β-Proteobacteria, γ-Proteobacteria and Bacteroidetes. β-Proteobacteria was represented by *Alcaligenes and Chromobacterium.* γ-Proteobacteria was represented by *Aeromonas, Acinetobacter, Moraxella, Pseudomonas and Vibrio. Flavobacterium* of phylum Bacteroidetes was also encountered. The number of genera encountered at Stn B was highest (8) followed by Stn D (6) and Stn M (5) (Fig 5.4).

5.2.3.5. Metabolic diversity of bacterial isolates

In general, the frequency of bacterial isolates testing positive for the elaboration of hydrolytic enzymes was higher at Stn B than the other two sites. Amylase positive isolates dominated the beach ecosystem. Stn M was marked by higher incidence of lipase, DNAse and protease positive isolates. Stn B had higher population of oxidase positive isolates, while the Stn M had a higher abundance of catalase positive forms (Fig 5.5). The average capacity index measuring the occurrence of bacterial isolates elaborating enzymes for hydrolyzing macromolecular compounds on different substrate amended nutrient medium was 37% for isolates from Stn B, 40% for Stn D and 48% for Stn M. The siderophore producers were higher at Stn B followed by Stn D and Stn M (Table 5.1).

5.2.3.6. Community level physiological profiling (CLPP)

Community substrate utilization pattern using ECOLOG plates indicated that the Stn B community could utilize wide spectrum of substrates. On an average, 26 of the substrates were utilized at Stn B, 13 at Stn D and 12 at Stn M. At Stn B, the utilization pattern was oriented towards sugars. Use of miscellaneous substrates such as tween was maximal (Fig 5.6, Table 5.2).

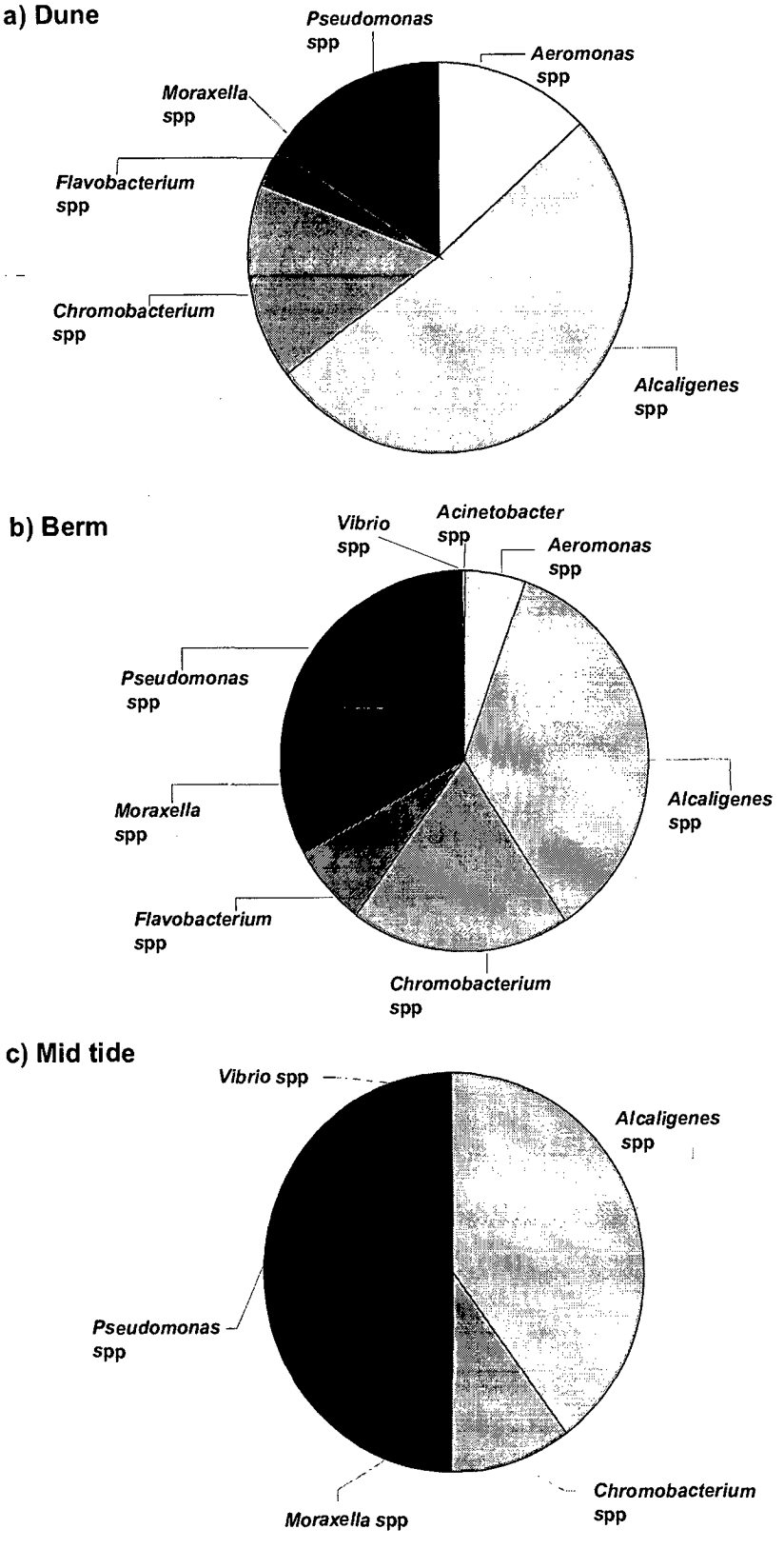


Fig 5.4: Phylogenetic diversity of bacterial isolates at the three different sites

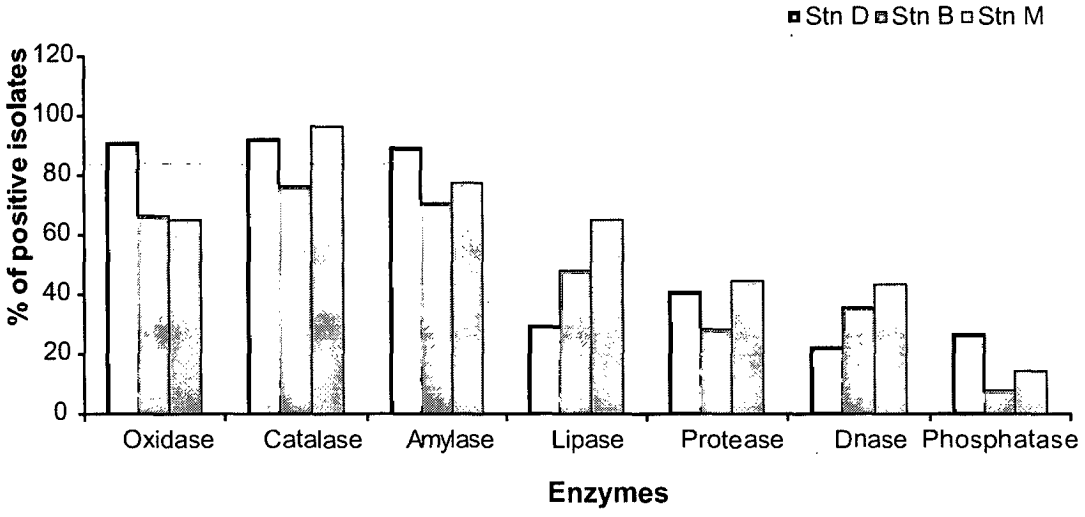


Fig 5.5: Enzymes elaborated by bacterial isolates at the three different sites: Stn D - Dune, Stn B - Berm, Stn M - Mid tide

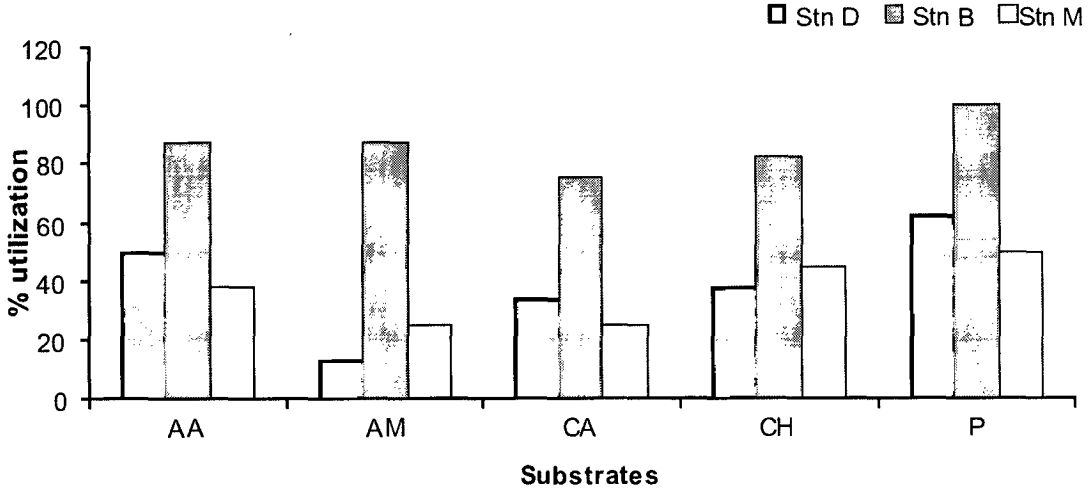


Fig 5.6: Substrate utilization patterns of the bacterial community at the three different sites: Stn D - Dune, Stn B - Berm, Stn M - Mid tide

*AA - Amino acids, AM - Amines and amides, CA - Carboxylic acids, CH - Carbohydrates, P - Polymers

Table 5.2: Average of substrate utilization pattern by microbial communities in percentage at the three sites (100%= 4 depths).

		% utilization		
Guild (Abbrev.)	**Substrate**	**Stn D**	**Stn B**	**Stn M**
Amino acids	L-Arginine	50 (±58)	100	25 (±50)
(AA) (n=6)	L-Asparagine	50 (±58)	100	50 (±58)
	Glycyl-L-glutamic acid	50 (±58)	100	50 (±58)
	L-Phenylalanine	25 (±50)	50 (±58)	25 (±50)
	L-Serine	75 (±50)	100	50 (±58)
	L-Threonine	50 (±58)	75 (±50)	25 (±50)
Amines & amides	Phenylethylamine	0	75 (±50)	25 (±50)
(AM) (n=2)	Putrescine	25 (±50)	100	25 (±50)
Carboxylic	D-Galacturonic acid	50 (±58)	100	50 (±58)
acids (CA) (n=9)	D-Glucosaminic acid	33 (±58)	100	25 (±50)
	2-Hydroxybenzoic acid	0	25 (±50)	0
	4-Hydroxybenzoic acid	25 (±50)	50 (±58)	0
	γ-Hydroxybutyric acid	50 (±58)	25	25 (±50)
	Itaconic acid	50 (±58)	100	50 (±58)
	α-Ketobutyric acid	0	100	0
	D-Malic acid	25 (±50)	100	25 (±58)
	Pyruvic acid methyl ester	75 (±50)	100	50 (±58)
Carbohydrates	N-Acetyl-D-glucosamine	50 (±58)	100	50 (±58)
(CH) (n=10)	D-Cellobiose	50 (±58)	100	75 (±50)
	i-Erythritol	50 (±58)	100	25 (±50)
	D-Galactonic acid γ-lactone	50 (±58)	100	50 (±58)
	Glucose-1-phosphate	0	50 (±58)	75 (±50)
	D,L-α-Glycerol phosphate	0	50 (±58)	25 (±50)
	α-D-Lactose	50 (±58)	100	25 (±50)
	D-Mannitol	75 (±58)	100	50 (±58)
	β-Methyl-D-glucoside	50 (±58)	25 (±50)	50 (±58)
	D-Xylose	0	100	25 (±50)
Polymers	α-Cyclodextrin	25 (±50)	100	50 (±58)
(P) (n=4)	Glycogen	50 (±58)	100	0
	Tween 40	75 (±50)	100	75 (±50)
	Tween 80	100	100	75 (±50)

5.2.3.7. Statistical analysis:

One way ANOVA detected significant differences between the berm and mid tide stations (Table 5AT1, Appendix III). Examination of the correlation between the various bacterial and biochemical parameters showed interesting relationships (Table 5AT2, Appendix III). The significant interrelationships were further analyzed using "*Cytoscape*". This software helped in bringing into focus the evolution of interrelationship with increasing concentration of ilmenite (Table 5AT2 - Appendix III, Figs 5AF1, 5AF2, 5AF3 - Appendix IV). The constituents of LOM correlated significantly with the bacterial parameters at Stn D. The variation in the aerobically viable microorganisms contribute to more than 77% ($p \leq 0.001$) of the variation in total bacterial population in these sediments. Another salient relationship was between ilmenite and TC. Nearly 59% ($r = 0.77$, $p \leq 0.01$) of the variation in TC is controlled by the variation in ilmenite. Further this ilmenite also seemed to positively influence viability at Stn B and Stn M. However, there was a negative correlation of ilmenite with viability at Stn D (Fig 5.7). To corroborate the correlation analysis, PCA analysis was done using 20 parameters and variation assigned to three principal components (Table 5.3).

At the dune station, the first principal component (PC) described 54% of the total variance in the data and had significant loadings (>±0.2) on depth, TC, TVCa, TVCan, ATP, TOC, CHO, LIP, PROT, LOM, Tcarb, FeA (Fig 5.8). The second PC explained 33.3% of the total variance and interrelated to ATP, RC-0.001, RC-0.01, IB, FeM, (positive loadings) RC-10, IR, Fe(II) (negative loadings). TC and TVCa were inversely related to LOM and ilmenite at the dune station. The first 2 PC at the berm station explained 79.2% of the total variance in the data. The first PC accounted for 43.2% of the variation and had positive significant loadings (>0.2) on depth, FeM while the biological parameters such as TC, TVCa, CHO, LIP, PROT, LOM, RC-0.001, RC-10, IB displayed negative loadings. Further, the second PC which explained 36% of the variation correlated positively to RC-0.01, TOC, and negatively to Ilm, TC,

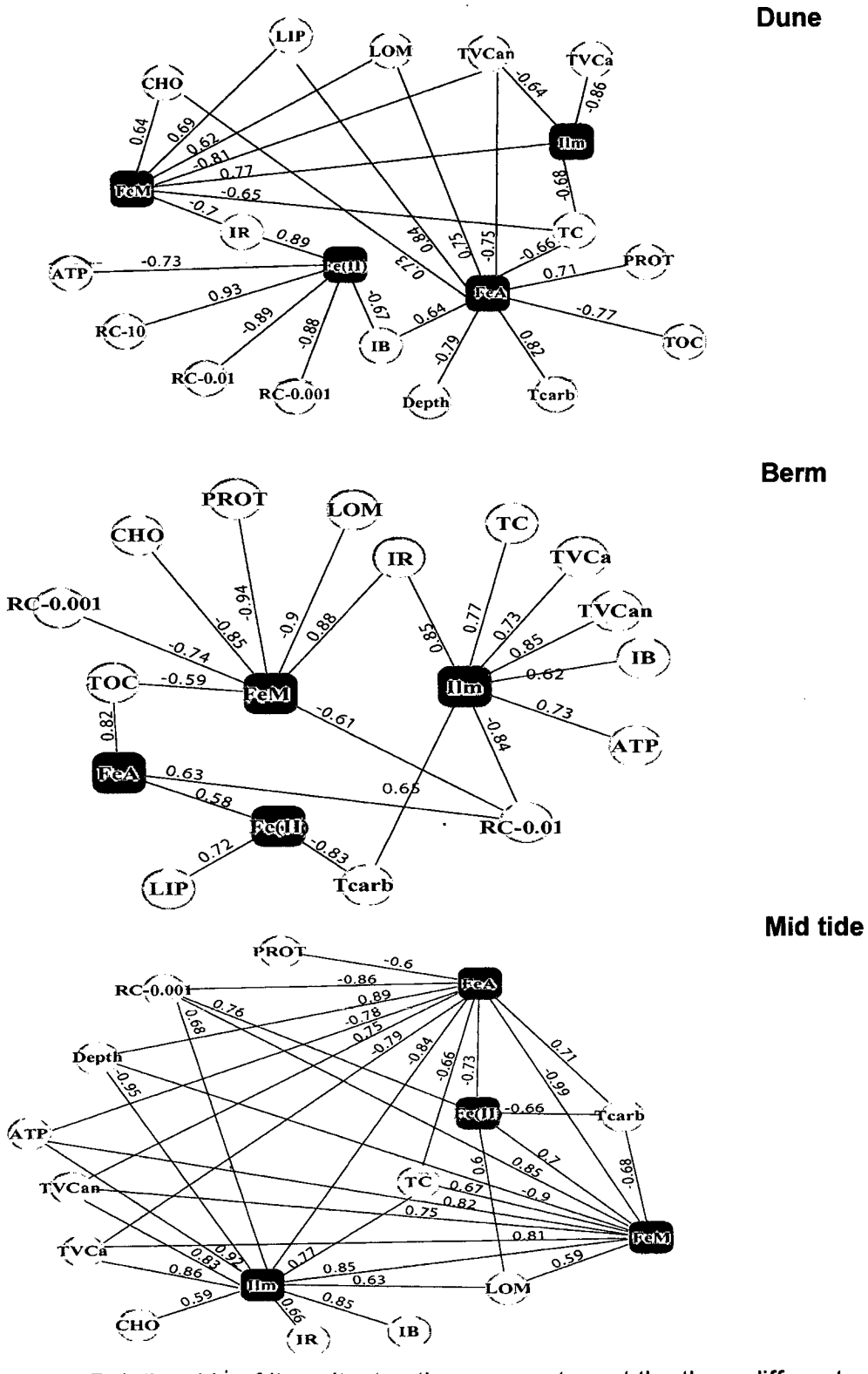


Fig 5.7: Relationship of Ilmenite to other parameters at the three different sites, showing simpler and more direct relationships at Berm

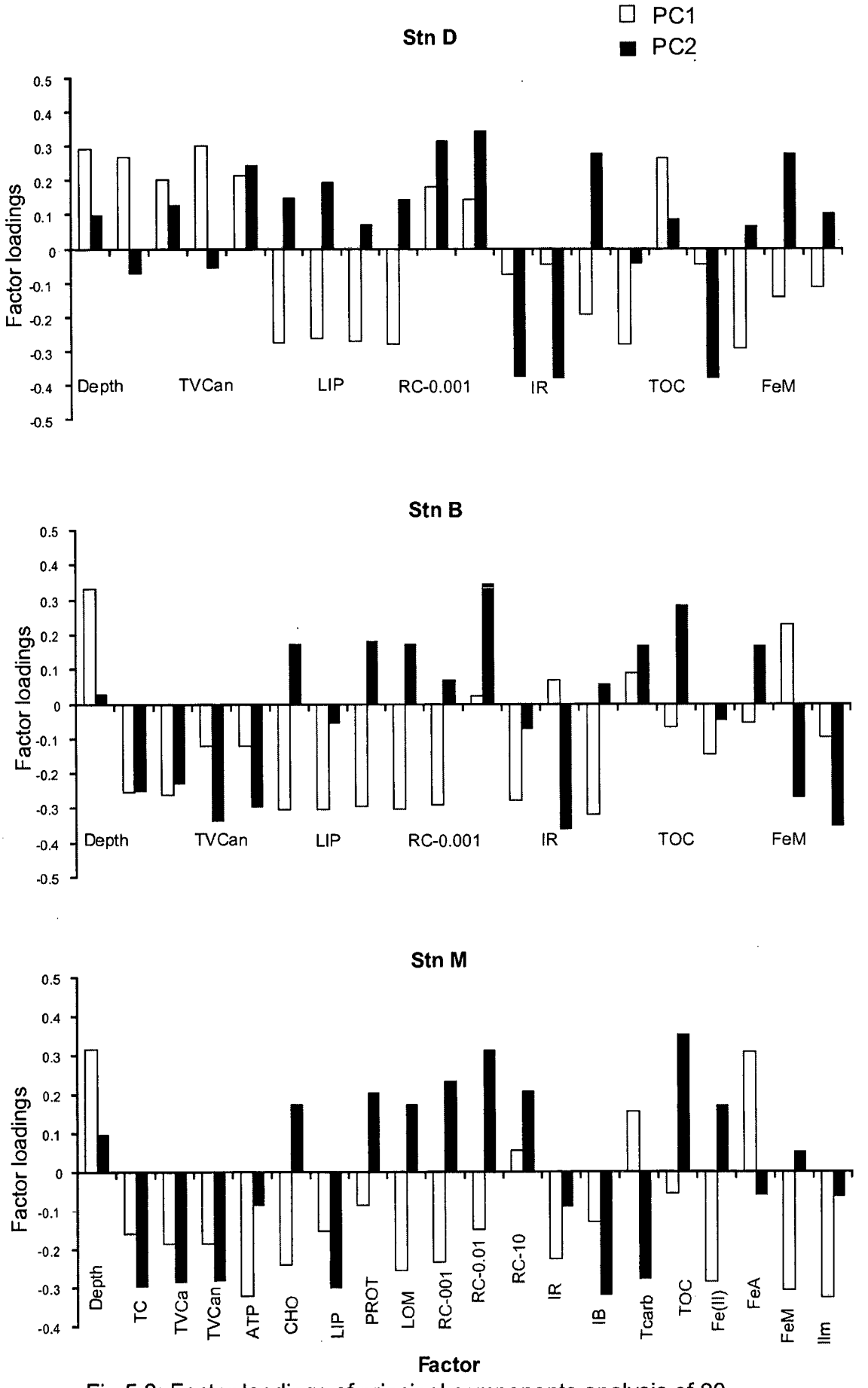


Fig 5.8: Factor loadings of principal components analysis of 20 environmental parameters for the three different sites: Stn D - Dune, Stn B - Berm, Stn M - Mid tide

Table 5.3: Principal component analysis of the parameters at the three different stations.

	Dune			Berm			Mid tide		
Factor	Eigen value	% Variation	Cum.% Variation	Eigen value	% Variation	Cum. % Variation	Eigen value	% Variation	Cum. % Variation
1	10.9	54.4	54.4	8.63	43.2	43.2	9.09	45.5	45.5
2	6.59	33.0	87.3	7.2	36.0	79.2	7.72	38.6	84.1
3	2.53	12.7	100.0	4.17	20.8	100.0	3.18	15.9	100.0

	Factor	Factor	Factor	Factor	Factor	Factor	Factor		Factor
Variables	1	2	3	1	2	3	1	Factor 2	3
Depth	**0.294**	0.096	-0.007	**0.333**	0.028	0.095	**0.313**	0.098	-0.1
TC	**0.269**	-0.073	0.263	**-0.253**	**-0.248**	-0.041	-0.158	**-0.296**	0.175
TVCa	**0.203**	0.125	0.422	**-0.261**	**-0.229**	0.089	-0.187	**-0.286**	0.126
TVCan	**0.299**	-0.055	0.054	-0.122	**-0.334**	0.128	-0.187	**-0.28**	0.153
ATP	**0.214**	**0.244**	0.208	-0.123	**-0.294**	-0.242	**-0.322**	-0.084	-0.032
CHO	**-0.278**	0.149	0.071	**-0.302**	0.171	0.005	**-0.241**	0.174	-0.273
LIP	**-0.263**	0.193	0.001	**-0.304**	-0.055	-0.209	-0.153	**-0.299**	-0.174
PROT	**-0.271**	0.069	0.261	**-0.294**	0.179	0.075	-0.087	**0.205**	0.437
LOM	**-0.279**	0.143	0.091	**-0.302**	0.169	0.029	**-0.255**	0.174	-0.235
RC-0.001	0.18	**0.313**	0.02	**-0.289**	0.069	0.243	**-0.232**	**0.235**	-0.164
RC-0.01	0.142	**0.343**	-0.045	0.021	**0.342**	-0.192	-0.149	**0.311**	-0.123
RC-10	-0.076	**-0.377**	-0.013	**-0.277**	-0.073	0.267	0.057	**0.208**	0.447
IR	-0.048	**-0.38**	0.093	0.068	**-0.361**	-0.069	**-0.226**	-0.09	-0.386
IB	-0.193	**0.276**	0.192	**-0.318**	0.057	0.16	-0.128	**-0.318**	0.15
Tcarb	**-0.28**	-0.043	0.232	0.09	0.168	0.418	0.154	**-0.278**	-0.242
TOC	**0.262**	0.087	-0.283	-0.069	**0.28**	-0.307	-0.054	**0.351**	-0.087
Fe(II)	-0.048	**-0.379**	-0.103	-0.146	-0.047	-0.438	**-0.284**	0.169	0.117
FeA	**-0.294**	0.066	0.109	-0.057	0.168	-0.43	**0.306**	-0.06	-0.195
FeM	-0.144	**0.276**	-0.329	**0.229**	**-0.27**	-0.071	**-0.309**	0.052	0.184
Ilm	-0.113	0.101	-0.56	-0.098	**-0.351**	-0.088	**-0.325**	-0.063	-0.06

TVCa, TVCan, ATP, IR, FeM. Although on the second PC, Ilm correlated positively to TC, TVCa, TVCan, ATP and IR, it interrelated negatively with ilmenite and RC-0.01 on the first PC. At the mid tide station, the first 2 principal components explained 84.1% of the total variance in the data. The first principal component which explained 45.5% included depth, FeA (positive loadings), ATP, RC-0.001, IR, CHO, LOM, FeM, Fe(II), Ilm (negative loadings). The second factor which included RC-0.001, RC-0.01, RC-10,

PROT, TOC (positive loadings), TC, TVCa, TVCan, IB, LIP, Tcarb (negative loadings) accounted for 38.6% of the variation. Ilm related negatively to most of the biological parameters.

According to BIO-ENV (Table 5.4), at the dune station, the best correlations (ρs) were found with depth and Fe(II), FeA, FeM, Tcarb, TOC, Ilm and biochemical parameter such as LIP and PROT. At the berm station, the best correlations were found with depth, Tcarb, TOC, ilmenite and protein. At mid tide, the best correlation was found with depth, Tcarb, TOC and Ilm.

Table 5.4: BIO-ENV analyses showing the influence of abiotic parameters on the bacterial parameters.

K*	Best variable combinations (ρ_s)		
	Dune	**Berm**	**Mid tide**
1		Tcarb (0.484)	D (0.895)
2		Tcarb, TOC (0.483)	D, Tcarb (0.895) D, TOC (0.895) D, Ilm (0.895)
3		D, PROT, Ilm (0.332)	D, Tcarb, TOC (0.895) D, Tcarb, Ilm (0.895) D, TOC, Ilm (0.895)
4	D, LIP, PROT, FeM (0.761)	Tcarb, D, PROT, Ilm (0.332) TOC, D, PROT, Ilm (0.332)	D, Tcarb, TOC, Ilm (0.895)
5	D, LIP, Fe(II), FeA, Ilm (0.763) D, LIP, PROT, Fe(II), FeM (0.763) D, LIP, PROT, Tcarb, FeM (0.761) D, LIP, PROT, TOC, FeM (0.761) D, LIP, PROT, FeM, Ilm (0.761)	Tcarb, TOC, D, PROT, Ilm (0.332)	

* K = number of independent abiotic components required to influence the dependent bacterial parameters.

ρ_s = Spearman correlation; Number in parenthesis gives correlation value.

D = Depth

5.3. Discussion:

The interaction between biotic and abiotic factors show certain amount of differences and similarities between dune, berm and mid tide station. The *Casuarina* plantation stabilizes the backshore zone while *Ipomoea pes-caprae* dominates the berm vegetation. These along with the associated vegetation contribute to the sedimentary organic matter which could dictate the abundance and diversity of microbes. The general abundance of bacteria i.e TC is low by one or two orders than the other beach sediments. TC can get lowered by intense grazing by meiofauna population. However, in this study area, the general abundance of meiofauna population was low (Anon, 2005). On the other hand, TC may be less perhaps because of the inhibitory effect of high heavy mineral concentration. Paradoxically, however, in this study we have found that the percentage of total, viable and culturable counts to be the highest in the berm where the ilmenite concentrations are the maximum (60% of the total heavy minerals). Ilmenite at the dune and mid tide region is found at the deeper depths of > 40 cm while at the berm region the ilmenite seems to accumulate at 15-20 cm depth layer (Valsangkar, 2005). Comparing the diversity of the culturable bacteria between the supralittoral and intertidal zones show that this parameter is affected by the ilmenite concentrations. Diversity in terms of number of genera is maximum in the berm at eight followed by dune and mid tide. There was a distinct difference in the culturable metabolic diversity between culturable bacteria from relatively low LOM harboring Stn M and higher LOM containing berm and dune region. Isolates from Stn M could elaborate more types of enzymes than those from Stn D and B. Conversely, substrates utilization patterns of the communities suggested that Stn B was more diverse. It may be noted that the ECOLOG substrates used by the resident microflora especially at the berm region is high. It is well known that bacterial communities prefer carbohydrate substrates under oxic conditions as well as under pre-anoxia. The preferred catabolic pathway of many aerobic and facultative heterotrophs involves oxidation of a simple or complex carbohydrate and oxygen as a terminal electron acceptor (Madigan et al., 1997; Rosenstock and Simon, 2003). Also

input of carbon fixed in the form of carbohydrates synthesized via photosynthesis and terrestrial carbohydrate-rich allochthonous organic matter flushed into the sediments may cause such a behaviour. This trend has been seen in lake sediments (Vreča, 2003; Heinen and McManus, 2004); hence conditions may select for bacterial populations that readily utilize carbohydrates.

Amino acid substrates were also used in appreciable amounts by the berm communities. It is may be noted that amino acids are nitrogen-rich (Madigan et al., 1997). Hence this community may be perhaps utilizing amino acids as a source of nitrogen, in addition to a carbon source by assimilating the ammonium side-chain (Pettine et al., 1999). In turn, the ammonium may be included into organic molecules such as other amino acids and proteins (Hollibaugh and Azam, 1983). High use of amino acids suggests that bacteria may be nitrogen deprived during this pre-monsoon season of February. However, due to the opposing catabolic and anabolic processes of amino acid utilization, it is quite possible that there are two separate groups of bacterial taxa involved in the breakdown of extracellular amino acids and incorporation into cellular amino acids and they may be acting in a synergistic fashion rather than both processes being conducted simultaneously by the same taxon (Atlas and Bartha, 1998). The berm communities also could utilize carboxylic acids. The knowledge of free carboxylic (organic) acids in aquatic and sediment systems is very limited. Naturally occurring organic acids such as carboxylic acids are often a product of bacterial fatty acid catabolism, photochemical degradation of high molecular weight organic carbon, or as an end product of fermentative metabolism, which occurs independent of dissolved oxygen and redox potential (Bertilsson and Tranvik, 2000; Ding and Sun, 2005). It may be noted that the concentration of ilmenite is high in the berm region. Precursors of siderophore was utilized relatively more in the berm sediments than the other two stations. The different forms of hydroxy benzoic acids were utilized more at the berm station than the other station (Table 5.2). Laboratory experiments have demonstrated that *in vitro* system 4-hydroxybenzoic acid is hydroxylated to 3,4-dihydroxybenzoic acid (protocatechuic acid) and decarboxylated to hydroquinone. Similarly 2-

indicated that at the dune station, the most important factor in the first component is viability under anaerobic conditions followed by FeA, Tcarb and CHO. High carbohydrate could lead to intense bacterial activity that would contribute to decrease in pH in the sediment. This in turn would make the contribution from acid soluble iron very important especially under acidic conditions. Further, PCA analyses suggested that Fe(II) correlates with RC-10 population which may indicate that the increase in the Fe(II) concentration may influence the increase in RC-10. Microbial growth may be enhanced in the presence of bioavailable iron ie Fe(II). In general, Fe(III) usually exists as a oxyhydroxide mineral at circumneutral pH. This becomes soluble under low-pH conditions consequently acting as an electron acceptor for microbial growth (Blöthe and Roden, 2009). Further, the dune environment is rich in LOM and concentration of sedimentary carbohydrates is high (917 ± 655 $\mu g\ g^{-1}$). At the berm, ilmenite clusters together with aerobes, anaerobes and ATP indicating that the growth of mixotrophs may be stimulated in the presence of ilmenite. The increase in carbon dioxide and volatile /fatty acids produced together with high concentration of lipids could possibly lower the pH of the sediment thereby further increasing the anaerobic population. This increase could also be in terms of rise is ATP level in the sediment. At the mid tide station, ilmenite, microbially reducible iron and ATP cluster together. The ATP found at the mid tide region could be from sources other than the bacteria, such as phytoplankton and zooplankton and phytoplankton in this region may be dissolving/utilizing the iron released from ilmenite by bacterial action.

Although the BIO-ENV procedure does not give the direction of correlations, it can indicate variables that possibly influence the differences in community structure. BIO-ENV analyses of the data revealed that ilmenite in combination with other parameters such as depth, lipids, proteins and microbially extractable iron could possibly affect the distribution of all bacterial parameters at the dune station. Correlation analyses indicated that microbially extractable iron had a negative relationship with most of the bacterial parameters. The BIO-ENV analyses shows that at the mid tide station, depth plays an important role in controlling the distribution of bacterial parameters.

At this region, water logging during high tide allows ilmenite to sink at depths >40 cm. Hence there is an influence of both ilmenite and depth on the bacterial parameters. Though total carbon content is very high, and total organic carbon content is very low both seem to influence bacterial distribution to same extent. In the simple correlation matrix too, the influence of Tcarb on the culturable forms of bacteria was significantly negative showing that when Tcarb is high, organic carbon is low thus affecting the numbers of culturable forms. In contrast, the BIO-env analyses at the berm station showed that influence of total carbon and organic carbon played a relatively lower role. The influence of depth was relatively low and was significant only in combination with protein and ilmenite. Correlation analyses also illustrated that the Tcarb had a negative relationship with TC distribution.

It is well known that organisms associate with minerals to derive energy from inorganic substrates (Berthelin, 1983; Chapelle, 1993; Lovely and Chapelle, 1995; Stevens and McKinley, 1995; Ehrlich, 1996). Studies in the laboratory as well as in acidic sediments have shown that heterotrophic microorganisms especially iron reducers are capable of metabolizing substrates such as glucose and malate with the reduction of Fe(III) (Lovley, 1987a,b; Küsel et al., 1999). Further, microbes can cause low pH microenvironments at mineral surfaces. Barker et al., (1998) while studying dissolution of biotite found pH values of 3–4 in proximity of bacterial cells which resided within cleavages in biotite when the bulk solution pH was 7.0. The rates of silicate mineral dissolution is known to increase as acidity decreased below pH 5.5. (Blum and Lasaga, 1988). It is also known that although only the outer surface of a given mineral grain is colonized by microorganisms, interior surfaces also show signs of enhanced dissolution. This dissolution is brought about by organic acids of plant and microbial origin. Bacterial and fungal involvement is organic matter degradation could result in elevated carbonic acid levels (Chapelle et al., 1987; Chapelle and Lovley, 1990). Very high concentrations of aconitate, acetate, citrate, formate, isocitrate, lactate, malate, oxalate, pyruvate, propionate and succinate have been detected in rhizosphere soil rich in minerals (Fox and Comerford, 1990; Grierson, 1992; Shen et al., 1996).

Similarly, it is probable that Fe from the ilmenite mineral is brought into the solution through the generation of acids which is outcome of organic matter degradation. The utilization of more substrates by the resident community at the berm and the dune may clearly imply that the resident microflora is capable of utilizing the acids, sugars etc that is produced. It is also pertinent to note that culturable forms capable of utilizing iron as well as reducing iron was high in dune and berm system. The hydroxylamine extractable Fe(III) i.e FeM, which is assumed to be microbially reducible (Lovley and Phillips, 1987b) is very high in the berm sediments followed by dune and midtide region. This clearly implies that the microflora are actively involved in the solubilization of iron from ilmenite. It is known that when the oxidative and reductive parts of the Fe redox cycle come together with ongoing input of energy, a self-sustaining microbial community based on Fe redox cycling may develop (Blöthe and Roden, 2009). This sustained microbial Fe redox cycling has been proposed in various redox interfacial environments like groundwater Fe seeps (Emerson and Revsbech, 1994.), plant roots (Emerson et al., 1999), the sediment-water interface in circumneutral pH (Sobolev and Roden. 2002; Roden et al., 2004) and acidic (Peine, et al., 2000) aquatic ecosystems, and hotsprings and hydrothermal vents (Pierson et al., 1999; Kashefi, et al., 2003).

Thus, in this study of estuarine beach of Kalbadevi rich in ilmenite placer deposits, a distinct influence of the heavy mineral on distribution, diversity and activity of culturable bacterial flora has been shown.

Chapter 6

Chapter 6: Microbial interaction with ilmenite

6.1. Introduction

Though ilmenite is known to be a recalcitrant mineral, the iron component is readily released by bacterial action which benefits the community in the ecosystem. This chapter highlights the important findings to elaborate this statement at the microbial level.

The results on the effects of ilmenite and iron are presented in this chapter and are divided into two sections.

Section 6.2. : Reductive phase

Section 6.3. : Oxidative phase

6.2. Reductive phase

6.2.1. Microcosm experiments with berm sediments: Abundance and activity at bacterial and primary level

6.2.1.1. Abundance and activity at bacterial level

6.2.1.1.1. Abundance of iron reducing bacteria as determined by MPN method: Seasonal and down core variation in the berm sediments

6.2.1.1.1.1. Results

Most probable number (MPN) estimates revealed that the population of iron reducing bacteria in the sediment decreased with depth in November and ranged from 7.5 cells g^{-1} at (20-40) cm layer to 9.3 x 10^3 cells g^{-1} at (0-5) cm layer (Fig. 6.1). A sub-surface maxima was observed at (5-10) cm layer depth during August and the population ranged from 0.75 x 10^3 cells g^{-1} at (20-40) cm layer to 2 x 10^3 cells g^{-1} at (5-10) cm layer. However, there was no such definite trend during April and the population varied from 30 cells g^{-1} at (10-15) cm layer to 1.5 x 10^3 cells g^{-1}. Correlation analysis between the MPN numbers and ferrous iron [Fe(II)] concentration revealed that the Fe(II) and MPN influenced each other negatively (r = 0.42) during the monsoon season. On the other hand, during November, this relationship became positive and caused nearly 71% variation (Fig 6.2.).

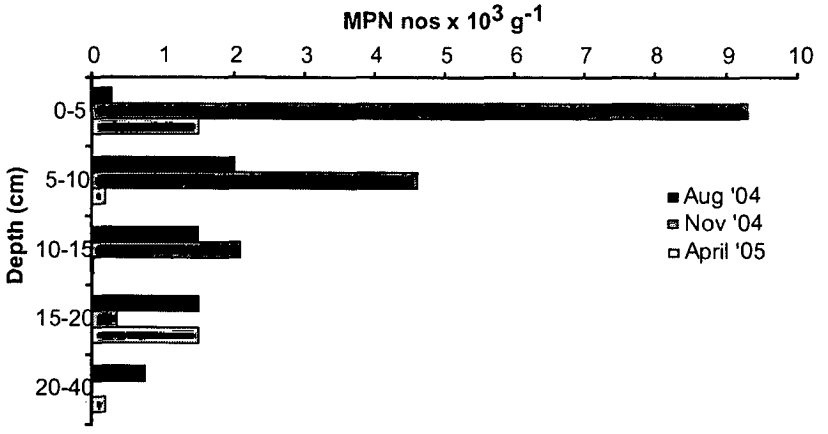


Fig 6.1: Enumeration of iron reducers using ferrozine-MPN assay

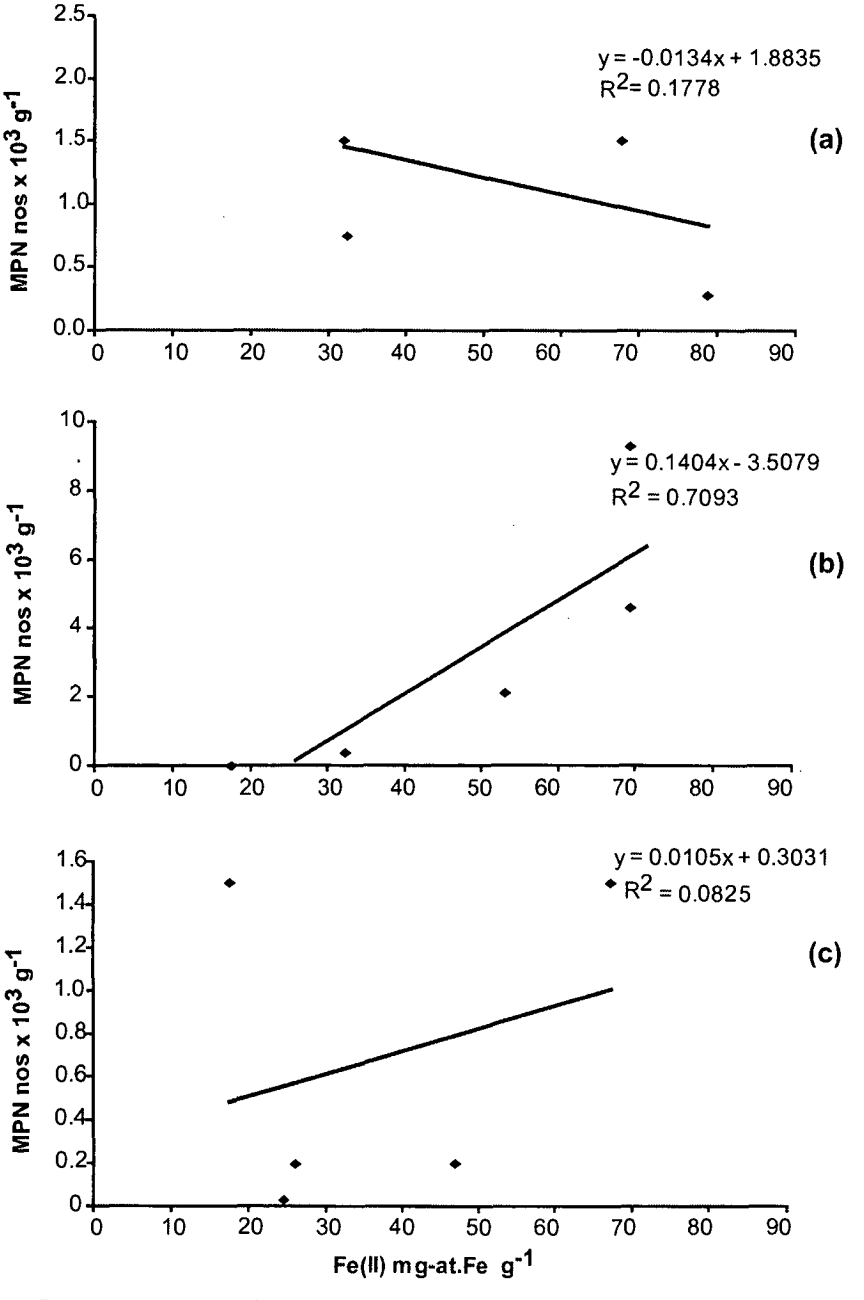


Fig 6.2: Correlation analysis between Fe(II) concentration and MPN during (a) August, (b) November and (c) April

6.2.1.1.2. Iron released from ilmenite by natural flora in microcosms: Seasonal and down core variation in the berm sediments

6.2.1.1.2.1. Results

Iron release from ilmenite occurred both in native and heat- and azide treated control at RT. Sodium molybdate at a concentration of 20mM was used to inhibit sulfate reducing activity. Fe(II) indicated as a pink colouration due to the binding to ferrozine reagent, accumulated in the native as well as in both the controls i.e heat killed and azide amended medium. However, the levels of accumulation of Fe(II) in the native experiment sediments were considerably higher than the accumulation in the controls. The biological release of iron from ilmenite started from the 3d and peaked on 7d and was generally higher than the control. In the experimental tubes, the average rate of removal of iron in the form of Fe(II) was nearly three times higher than the corresponding control. The concentration of Fe(II) released from ilmenite in the experimental tubes varied from 1.19 to 145.89 mg-at.Fe g^{-1} (Fig. 6.3., Plate 6.1). However, in the azide control it varied from non detectable levels to 97.52 mg-at.Fe g^{-1} while in the heat killed control it varied from 2.47 to 190.83 mg-at.Fe g^{-1}. In the experimental tubes, highest potential iron reduction of 146 mg-at.Fe g^{-1} was recorded during the monsoon season of August. Depth wise the maximum was the same and encountered at deeper depth of (15-20) cm layer. In the post-monsoon month of November, the highest potential iron reduction was recorded at shallower depth of (5-10) cm at 107.22 mg-at.Fe g^{-1}. Similarly, in April the highest potential iron reduction was recorded at (5-10) cm at 74.43 mg-at.Fe g^{-1}. The average potential iron reduction rates varied from 4.6 mg-at.Fe g^{-1} d^{-1} in April to 8.65 mg-at.Fe g^{-1} d^{-1} during August.

6.2.1.1.3. Discussion

Iron reduction and oxidation can be of prime importance for aerobic and anaerobic microbiological processes within sand. In the Kalbadevi beach sediments, the berm sediments usually had higher accumulation of ilmenite (Valsangkar, 2005). Hence this sediment along with the associated microflora was used in the present study to validate the hypothesis that natural consortia of microflora from this region could mobilize iron from the mineral ilmenite at

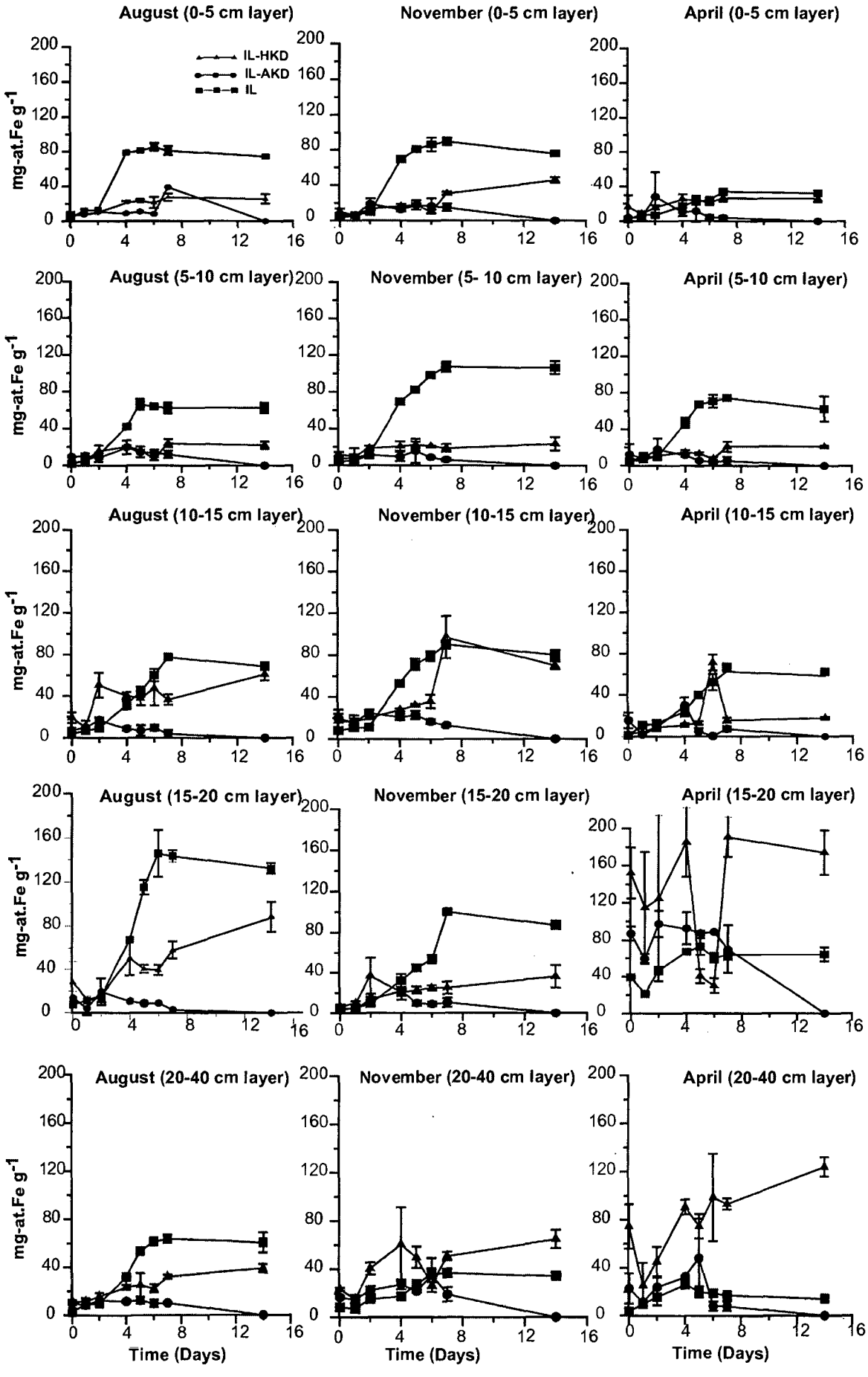


Fig 6.3: Removal of iron from ilmenite containing Kalbadevi beach sediment using native bacteria. IL- Ilmenite with native bacteria,
IL- HKD: heat killed sediment,
IL- AKD: azide killed sediment.

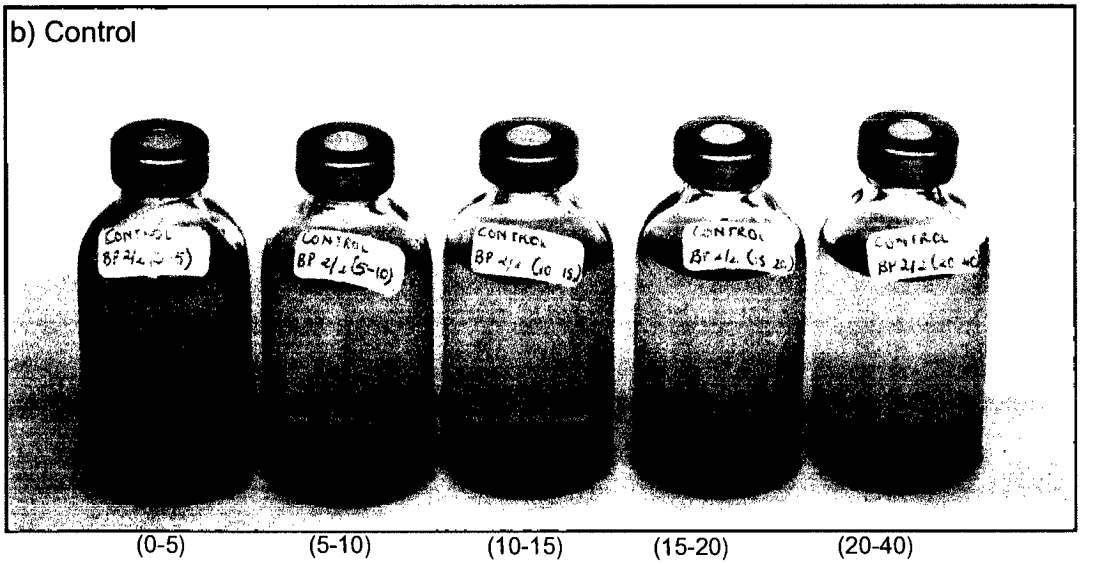


Plate 6.1: Fe(II) released from ilmenite rich Kalbadevi beach sediment by natural flora in microcosms (down core variation during post monsoon)
a) experiment using native bacteria
b) heat killed control

* Ferrozine binds with Fe(II) and gives a pink colouration

an appreciable rate. In the present study, microcosm experiments using native bacteria and ilmenite showed that these microbes were capable of releasing soluble iron from the mineral ilmenite into the medium. This was perhaps an inherent ability of the native bacteria since chemical removal of iron from mineral media containing heat and azide treated sample was relatively low. Similar trends were observed in the experiments that were replicated over three sampling months of August, November and April which define the monsoon, post-monsoon and pre-monsoon season, respectively. The average potential iron reduction rates in the Kalbadevi sediments ranged from 4 to 9 mg-at.Fe g^{-1} d^{-1}. Generally, the maximum rates of microbial iron reduction reported for natural freshwater and marine sediments varies over several orders of magnitude ranging from 2-100 nmol Fe(III) cm^{-3} h^{-1} (Thamdrup, 2000; Roden and Wetzel, 2002; Jensen et al., 2003; Canavan et al., 2006).

Concentration of Fe(II) in the experimental sediments was generally higher than the controls especially at the surface layers. This indicated that the microbial rate of release of iron from the ilmenite was higher than the controls. Reductive dissolution is known to occur in natural ilmenites which contain significant amounts of Fe(III) (White et al., 1994). However, the microbial participation acts as a catalyst and increases the reaction several fold. At deeper depths, the chemical removal of Fe(II) seemed to prevail in cell free controls especially in the heat killed controls. The heat killed deep sediments overcame the effect of heat during the non monsoon months especially during the pre-monsoon. In this beach sediment, Firmicutes occurred at a frequency of nearly 41% (Chapter 7). It is quite possible that these spore formers may be resistant to heat and proliferate at later stages to participate in the release of iron from sediment. *Bacilli* spp have been known to be involved in solubilizing large amounts of iron from limonite, goethite and hematite (de Castro and Ehrlich, 1970). Studies have also shown that spore formers such as *B. subtilis* had incredible heat resistance that prevented the complete inactivation of the enzyme with temperature control (Gardner et al., 2006; 2008). It may also imply that free enzymes adhering to sediment particles are only temporarily disabled when heat killed.

MPN analysis offers a convenient culture-based method for enumerating respiratory (dissimilatory) iron reducing bacteria (DIRB). The number of iron reducing bacteria as estimated by Ferrozine-MPN method was high during the post-monsoon season of November suggesting that the proliferation of these group of bacteria are dependent on the higher bio-availability of organic carbon which generally increases during the post-monsoon months. MPN estimates of the iron reducers also indicated that this group of bacteria decreased with increasing depth again suggesting that they were constrained by availability of the organic carbon. It is well known that iron reducers do not require strongly reducing conditions and are well distributed in surficial sediments. Their tolerance of intermediately oxidized environments is reflected in their distribution. Water acts as a barrier to oxygen penetration, allowing its depletion and the generation of suboxic conditions (Ponnamperuma, 1972). Further, biological oxygen demand generated by organic matter accumulating at the deeper layers creates oxygen-reducing conditions and consequently, lowers the E_h. As other terminal electron acceptors are reduced, the redox potential continues to drop (Cummings et al., 2002).

The activity of iron reduction was the highest during the monsoon season and occurred at deeper depths. During post-monsoons, nearly 71% of the variation in the Fe influenced the MPN estimates positively suggesting its effect on the proliferation of bacteria. On the other hand, during monsoons, nearly 18% of the variation in the Fe influenced the MPN estimates negatively suggesting that the concentration of the released iron was beyond optimal level. Perhaps the dissolution of ferrous ions is facilitated more in fresh water thus enabling iron reduction to proceed at a faster rate. The trend depicts that the biological rate of release of iron in surface sediments is more than the deeper layers. As this process follows the trend in the distribution in organic matter, microorganisms can reduce ferric iron during the metabolism of organic matter (Lovley, 2000). In the previous chapters of 4 and 5 it had been shown that organic matter in the berm sediments is high (1.3 mg g^{-1}). Previous studies have also revealed that the availability of Fe(III) for microbial reduction is also an important factor controlling the extent of organic matter

decomposition with Fe(III) serving as the terminal electron acceptor (Lovley, 2000). In turn, the in situ dissimilatory iron reduction is influenced by a variety of factors, including the microbial community structure and biomass (Dollhopf et al., 2000), the quality and quantity of the organic matter (Chen et al., 2003), and the type and abundance of Fe minerals (Bonneville et al., 2004). Also, Fe(III) respiration is coupled to a substantial portion of organic matter remineralization in the surface sediments of marine and freshwater environments (Kostka et al., 2002). Under suboxic conditions, ferric iron is a potential electron acceptor for organic matter degradation (Froelich et al., 1979; Lovley and Phillips, 1988). Thus iron reducers in the berm sediments play a significant role in bringing Fe(II) into the solution and making it bio-available for other trophic levels.

Sodium molybdate at a concentration of 20 mM prevented sulfate reducing activity and therefore binding of iron to the sulphide does not occur. Molybdate is known to inhibit sulfate reduction (Oremland and Capone, 1988). It is well known that sulphide produced can chemically reduce ferric iron, and this will affect the measured iron reduction rate. The activity of sulphate reducing bacteria (SRB) was effectively inhibited by molybdate (Ito et al., 2002, Nielsen et al., 2002). Hence all the iron was available for iron reduction and not diverted for precipitation by sulphide. It is hence tempting to interpret the lack of any inhibitory effect of molybdate on iron reduction as evidenced by reduction of Fe(III) to Fe(II). It is thus possible that the native dissimilatory iron reducers contribute to the major part of iron reduced from ilmenite in Kalbadevi sediment.

6.2.1.2. Effect of varying concentration of ilmenite on heterotrophs

6.2.1.2.1. Results

Varying concentrations of ilmenite were added to elucidate the response of culturable heterotrophic bacteria in terms of their abundance on 1% strength nutrient broth in 1.5% agar. With the addition of ilmenite to the sediment in a ratio of 1:1 to 1F, CFU retrieved on day 3, 5, and 15 increased by 63, 44, and

31% respectively more than the control. CFU retrieved from the control flask was 1.12 x 10^6, 2.28 x 10^6, and 4.56 x 10^6 CFU g^{-1} on 3rd, 5th and 15th day respectively. Similarly when the quantity of the ilmenite was doubled i.e in the ratio of 2:1 in 2F, CFU retrieved at day 3 increased by nearly 50% more than the control. However, when the quantity of ilmenite added was in the ratio of 3:1, the retrievability increased >88% by the third day. This retrievability was maintained at level which was always 50% more than the control. Highest retrievability was recorded from 3F on all the days (Fig 6.4h).

Generic diversity increased with the increasing concentration of the ilmenite addition from 5 to 6 genera (Fig 6.4a-g). When different proportions of ilmenite ranging from namely 0, 50, 66, 75, 80% were used, the retrievability of different genera changed. The most dominant genus was *Pseudomonas* spp. It was retrieved on 50% ilmenite at 10^6 CFU g^{-1}. Interestingly, the next maximum group was *Enterobacter* reaching 10^6 at 0% and 10^5 at 80%. Both *Alcaligenes, Aeromonas* and *Marinococcus* were retrieved at 10^6 CFU g^{-1} at 75%. *Aeromonas* was retrieved at 10^6 CFU g^{-1} at 75%. Least retrievable was *Acinetobacter* and was retrieved only on 80% concentration of ilmenite. Preliminary analysis of siderophore activity showed that all the isolates were actively producing siderophores at the end of the 15th day.

6.2.1.2.2. Discussion

Microbes inhabiting terrestrial or aquatic environments acquire iron directly from natural sources that are for the most part insoluble. Ferric minerals serve as a primary nutritional source of iron for a variety of microbes, including water-borne pathogens and environmental species. Their iron-scavenging activities has important geochemical and environmental repercussions, both for global iron cycling, trace metal mobilization as well as mineral instability over geological time (Cornell and Schwertmann, 1996; Hersman et al., 2001; Kraemer, 2004). Moreover, studies have shown that a number of bacterial pathogens require sufficient iron acquisition to enhance significantly their virulence capability (Expert et al., 1996; Genco and Desai, 1996; Mietzner et al., 1998; Schryvers and Stojilkovic, 1999; Vasil and Ochsner, 1999).

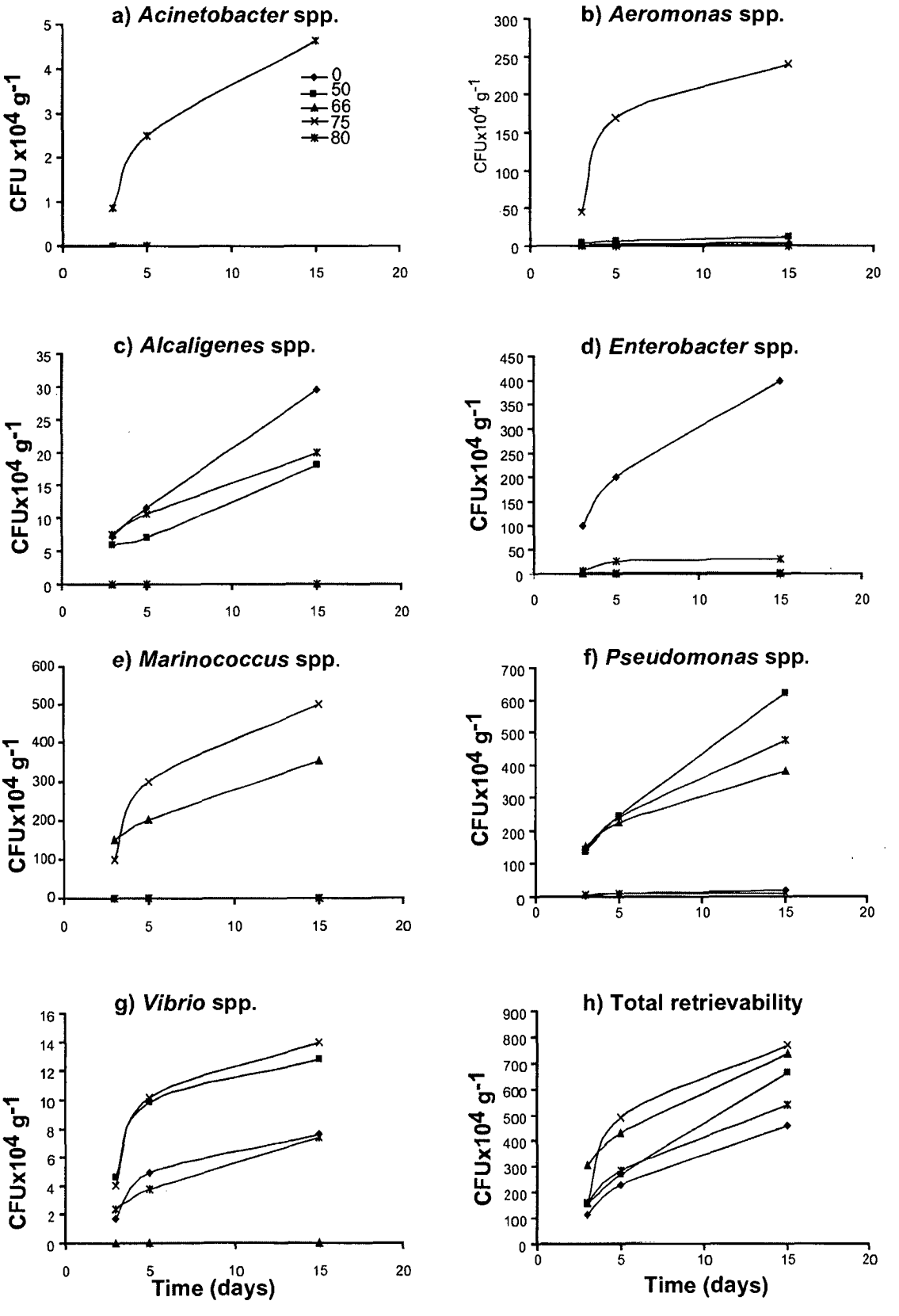


Fig 6.4: Effect of varying concentration of ilmenite on the different genera of bacteria, (a-g)- different genera, h) total retrievability

Maximum culturability is at a ratio of one part of sand and three parts of ilmenite. Siddiquie et al., (1984) reported heavy minerals to range from 7-79 % at Kalbadevi. Increases in bacterial cell abundance have been reported previously in Fe-amended bottle experiments from the equatorial Pacific (Price et al., 1994), Gerlache Strait, Antarctica (Pakulski et al., 1996), and coastal California (Hutchins and Bruland, 1998). The average abundance of bacteria in the control and experimental flasks was in the order of 10^6 CFU g^{-1}. Most of the isolates were gram-negative. Previous study by Atlas and Barther, (1981) have found that most of the marine bacteria i.e. >95% were gram negative and motile.

Pseudomonas spp. were predominant in all the experimental flasks. *Alcaligenes* and *Marinococcus* were optimally retrieved at 75% while *Pseudomonas* was optimally retrieved at 50% suggesting that high concentration of ilmenite may be detrimental. Bacterial species of the genus *Bacillus*, *Arthrobacter*, *Clostridium* and *Pseudomonas* have been reported as Fe-reducing bacteria (Ottow and Glathe, 1971; Lovley, 1993). Likewise, *Acinetobacter* are also retrievable at 80% ilmenite suggesting that they could be potential pathogens. It is known that some *Acinetobacter* spp such as *Acinetobacter haemolyticus* is an opportunistic pathogen and produces siderophores such as acinetoferrin (Okujo et al., 1994). Some of the *Acinetobacter* are known to express siderophore-mediated iron-acquisition systems (Dorsey et al., 2004). Likewise *Aeromonas* spp is known to be involved in Fe(III) reduction (Knight and Blakemore, 1998). The culturability of *Vibrio* was not affected by ilmenite suggesting that this group of organisms do not respond to varying concentration of iron or are adaptable to wide concentration of iron released from ilmenite. It is known that some of *Vibrio* possess active transport system for iron and do not seem to be affected very much by concentration of ilmenite (Mazoy et al., 1997). With *Enterobacter* it is interesting to note that there are definitely two groups - those that grow at 80% ilmenite and those that grow at lower concentration.

Thus the effect of increasing concentration of ilmenite on retrievability showed that it improved by nearly 88% especially on 75% concentration of ilmenite.

6.2.1.3. Effect of iron released from ilmenite at bacterial level on abundance and activity at primary level

In order to appreciate the effect of the reduced iron released, experiments were conducted to monitor the changes at the bacterial and primary level i.e. changes in bacterial and phytoplankton abundance. Diversity at the phytoplankton level was examined. Also, net changes in Fe(II) and Fe(III) were monitored throughout the experiment.

6.2.1.3.1. Results

6.2.1.3.1.1. Change in iron concentrations with time

Generally, the concentration of Fe(II) and Fe(III) followed an irregular pattern of alternate increase and decrease in both the controls (C1-only seawater, C2-seawater + $FeSO_4.7H_2O$), while in the presence of ilmenite, these two components of Fe increased significantly with time ($r > 0.952$, $n = 11$, $p \leq 0.001$) (Fig 6.5, Table 6AT1, Appendix III).

6.2.1.3.1.2. Effect of $FeSO_4.7H_2O$ and ilmenite on total living microbial biomass - ATP

The ATP concentration increased during the first few days of incubation, followed by a decrease. In the flasks containing seawater and ilmenite i.e. Exp-1, ATP was nearly 50 times higher than the C-2 and Exp-2. This concentration increased significantly with time ($r = 0.836$, $n = 11$, $p \leq 0.001$) (Fig 6.6a, Table 6AT1, Appendix III).

6.2.1.3.1.3. Effect of $FeSO_4.7H_2O$ and ilmenite on total bacterial counts (TC)

TC showed an increase with time. At the end of 84d the maximum cell numbers were recorded in C-2. The numbers increased from 2.9×10^7 to 16.9×10^7 cells mL^{-1} in C-1, to 17.2×10^7 cells mL^{-1} in C-2, to 3.18×10^7 cells mL^{-1} in Exp-1, and to 5.13×10^7 cells mL^{-1} in Exp-2 flask. This increase in TC was significant only in controls [($r = 0.936$(C-1), $r = 0.654$(C-2)]. TC also related positively with Fe(III) in C-1 ($r = 0.599$) (Fig 6.6b, Table 6AT1, Appendix III). However, in the experimental flasks, TC did not relate with any parameters and increase in TC was static at 10^7 cells mL^{-1}.

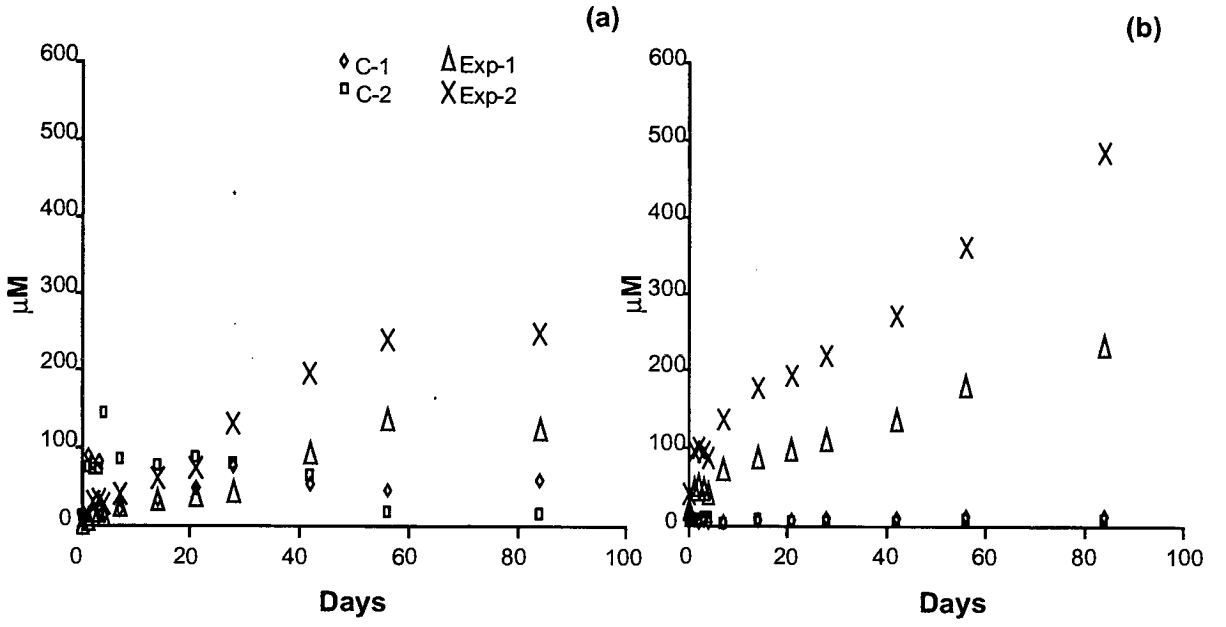


Fig 6.5: Changes in concentration of (a) Fe(II) and (b) Fe(III) with time in control (C-1 - seawater, C-2 - $FeSO_4.7H_2O$) and experimental microcosms (Exp-1.- seawater + ilmenite, Exp-2 - seawater + ilmenite + $FeSO_4.7H_2O$)

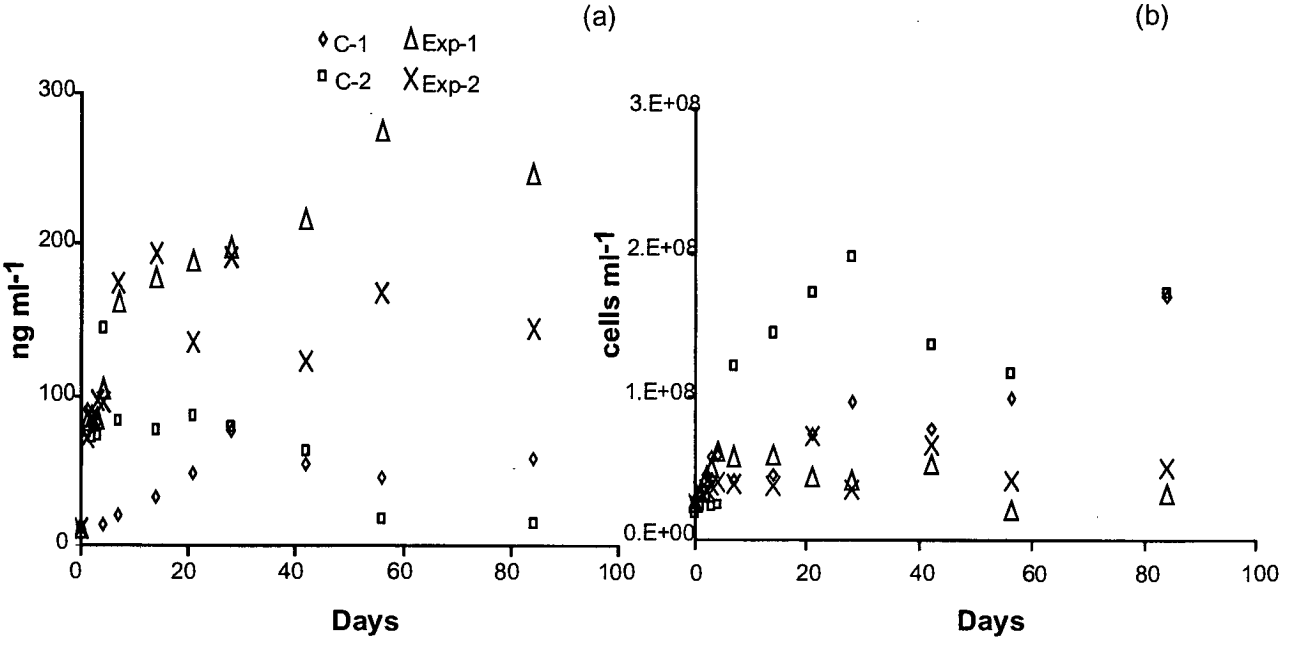


Fig 6.6: Changes in (a) ATP concentration and (b) bacterial cell counts (TC) with time in control (C-1 - seawater, C-2 - $FeSO_4.7H_2O$) and experimental microcosms (Exp-1.- seawater + ilmenite, Exp-2 - seawater + ilmenite + $FeSO_4.7H_2O$)

6.2.1.3.1.4. Effect of $FeSO_4.7H_2O$ and ilmenite on Chlorophyll *a* (Chl *a*)

Chl *a* concentration increased up to 14d and decreased thereafter. It peaked on 7d in control flasks and on 14d in Exp-1 and 2. Chl *a* related significantly to many parameters like phytoplankton abundance, ATP, algal abundance, Fe(II) and Fe(III) in Exp-1 (Fig 6.7a, Table 6AT1, Appendix III).

6.2.1.3.1.5. Effect of $FeSO_4.7H_2O$ and ilmenite on phytoplankton

Phytoplankton cell abundance followed a varied growth pattern (Fig 6.7b). Initial cell number was 8.0(±0.4) x 10^3 cells L^{-1}. Maximum cell counts of 4.5 x 10^4 cells L^{-1} was recorded on the 7d in C-1, and reduced thereafter. In C-2, the peak abundance was attained on the third day with 8.6 x 10^4 cells L^{-1}. It attained maximum counts on the 42^{nd} day with 32.8 x 10^4 cells L^{-1} in Exp-1. Here, there was a significant increase in cell abundance with time ($r = 0.853$, $n = 11$, $p \leq 0.001$) and it related positively with ATP, Fe(II) and Fe(III) (Table 6AT1, Appendix III). In the Exp-2, there was significant increase in abundance ($r = 0.768$, $n = 11$, $p \leq 0.01$), and maximum abundance was recorded on the 56^{th} day with 27.8 x 10^4 cells L^{-1} (Fig 6.7b). Surprisingly, the phytoplankton cell abundance followed the same increase in concentration of ATP. However, this relationship was not significant. In C-2, the cell abundance peaked within 3d at lower number of 8.61 x 10^4 cells L^{-1}. In Exp 1 and 2, the abundance peaked much later i.e. on 42d, which was >20times higher than control.

6.2.1.3.1.6. Effect of $FeSO_4.7H_2O$ and ilmenite on distribution of different genera of phytoplankton

Diatoms dominated in terms of the number of genera and abundance. Initially, the phytoplankton assemblage comprised of 14 genera namely *Asterionella* spp., *Biddulphia* spp., *Coscinodiscus* spp., *Ditylum* spp., *Grammatophora* spp., *Navicula* spp., *Nitzschia* spp., *Phaeocystis* spp., *Prorocentrum* spp., *Rhizosolenia* spp., *Synedra* spp., *Thalassionema* spp., *Thallassiothrix* spp., and *Triceratium* spp. (Fig 6.8). During the course of the experiment, 29 genera were identified. Highest number of genera were recorded on 3d in C-1 (16 genera), C-2 (17 genera) and Exp-2 (28 genera) and 14d in Exp-1 (27 genera). *Nitzschia* spp. dominated followed by *Navicula* spp. Though *Coscinodiscus* spp. was present in large numbers in the controls, their

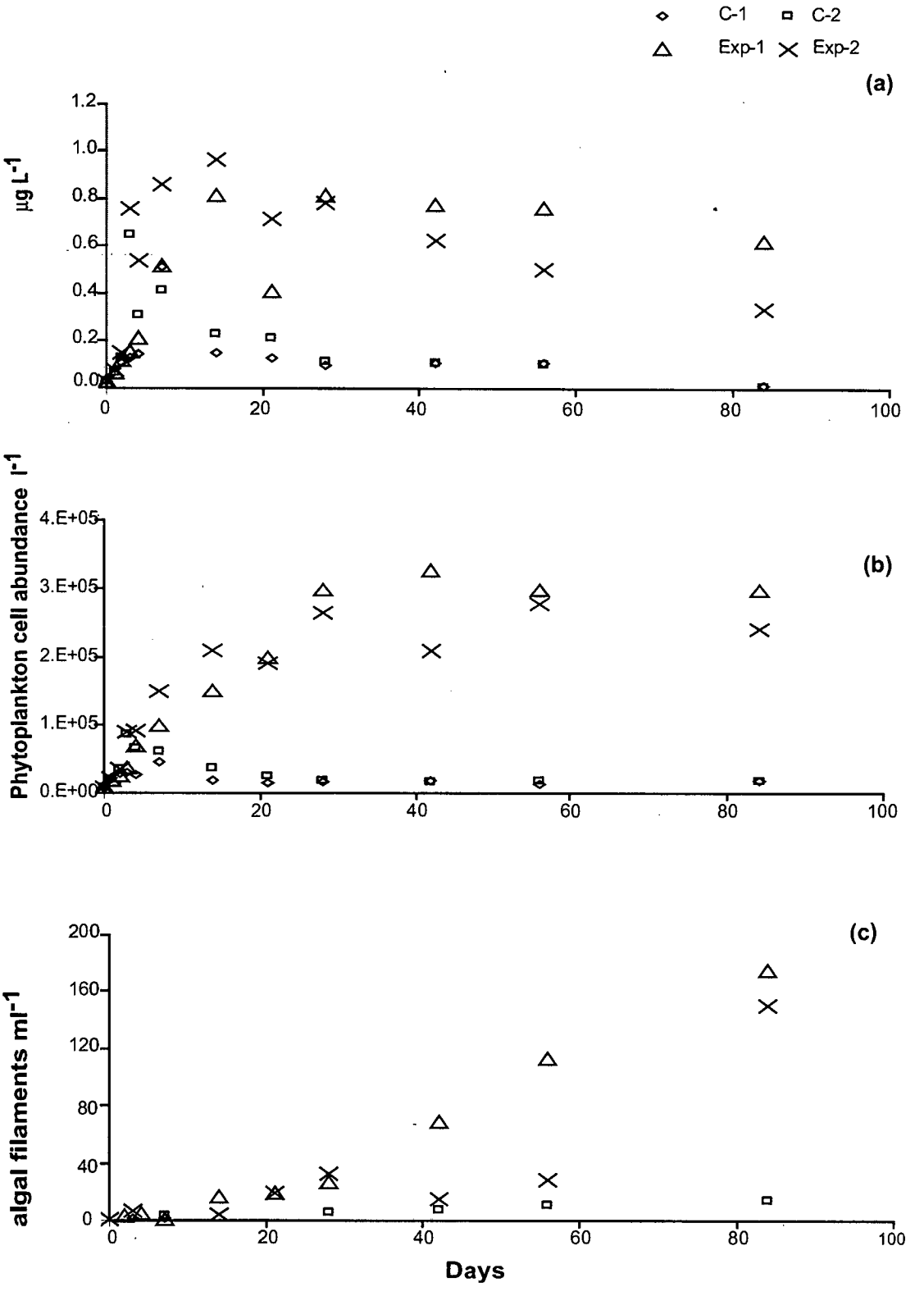


Fig 6.7: Changes in (a) Chl a concentration, (b) phytoplankton cell abundance, (c) algal abundance with time in control (C-1 - seawater, C-2 - $FeSO_4.7H_2O$) and experimental (Exp-1 - seawater + ilmenite, Exp-2 - seawater + ilmenite + $FeSO_4.7H_2O$) microcosms

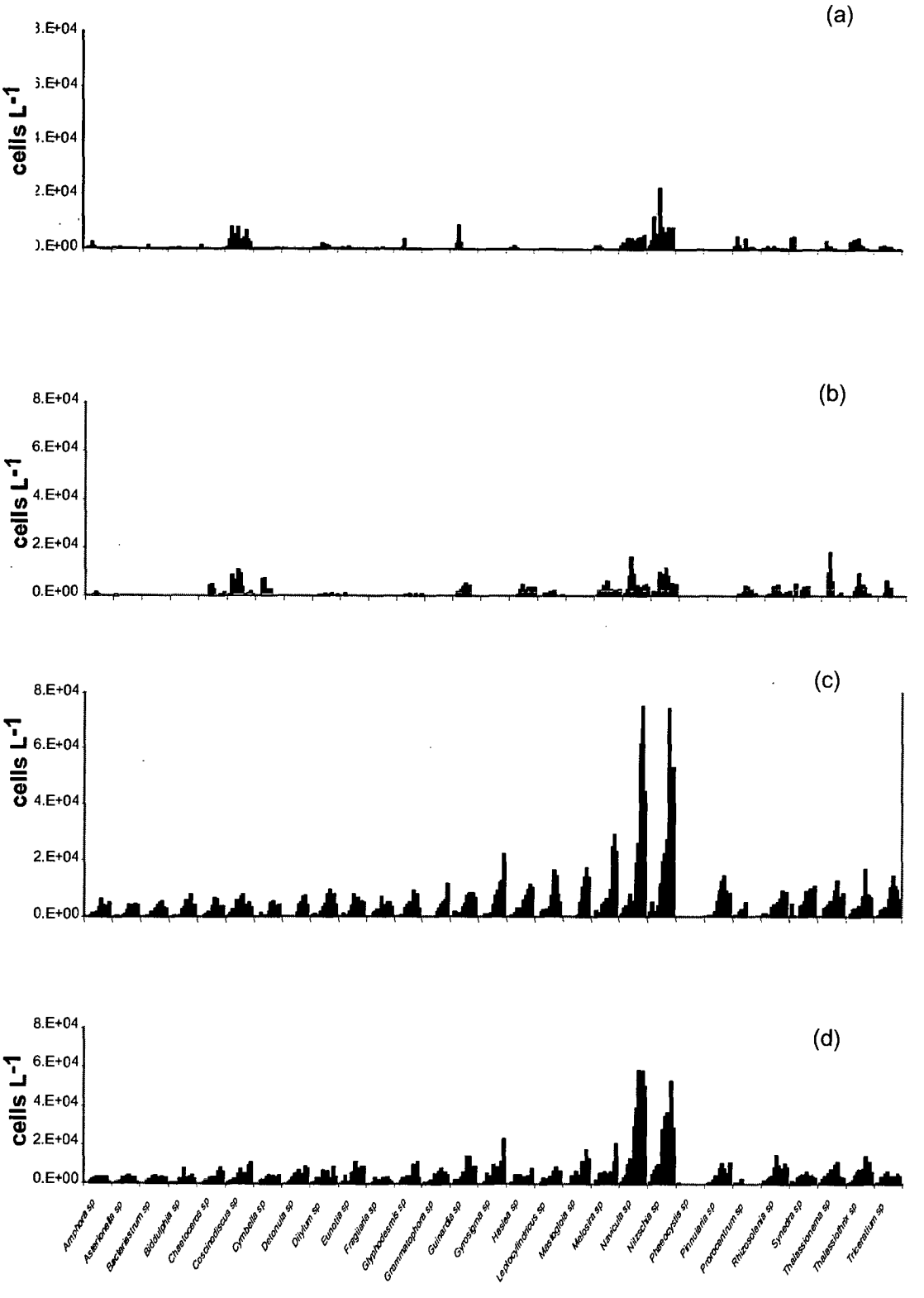


Fig 6.8: Ilmenite alone in microcosm (c) Exp-1 promotes higher abundance and diversity than (d) Exp-2, (a) C-1 and (b) C-2.

numbers were reduced sharply in Exp-1 and 2. *Eunotia* and *Pinnularia* spp. were found to increase in Exp-1 and 2. These species were conspicuously absent in control flask during the later stages of incubation. While Exp-2 showed highest species richness (d= 4.647), Exp-1 showed highest diversity index (H'= 3.156) and evenness (J'= 0.9576) (Table 6.1)

Table 6.1: Maximum abundance and genera in different microcosms, their species richness, evenness and diversity index

Microcosms	Days	Total individuals (N= cells x 10^4 L^{-1})	Total Genera (G)	Species richness (D)	Species evenness (J')	Diversity index (H')
C-1	3	3.03	16	3.049	0.8522	2.363
	4	2.65	15	3.008	0.8225	2.227
	7	4.50	8	1.341	0.7611	1.583
C-2	3	8.61	17	2.701	0.8453	2.395
	4	6.59	17	2.885	0.9001	2.55
Exp-1	3	3.69	22	4.394	0.9273	2.866
	14	14.95	27	4.168	0.9373	3.089
	21	19.95	27	3.905	0.9576	3.156
	42	32.81	27	3.608	0.8479	2.795
Exp-2	2	3.58	25	4.647	0.8924	2.873
	3	8.93	28	4.641	0.9305	3.101
	56	27.79	27	3.703	0.8494	2.799

There was massive development of green algae in Exp-1 and 2 (Fig 6.7c, Plate 6.2). Bubbles were seen attached to the mat at the end of 84d. However in C-2, the mats were translucent and distributed throughout the flasks. SEM images showed that diatoms as well as algal filaments apparently covered the ilmenite grains (Plate 6.3). Preliminary counts showed that ciliates numbered 12 x 10^2 cells L^{-1} in C-1, 8.8 x 10^2 cells L^{-1} in C-2, 14 x 10^2 cells L^{-1} in Exp-1 and 12.8 x 10^2 cells L^{-1} in Exp-2.

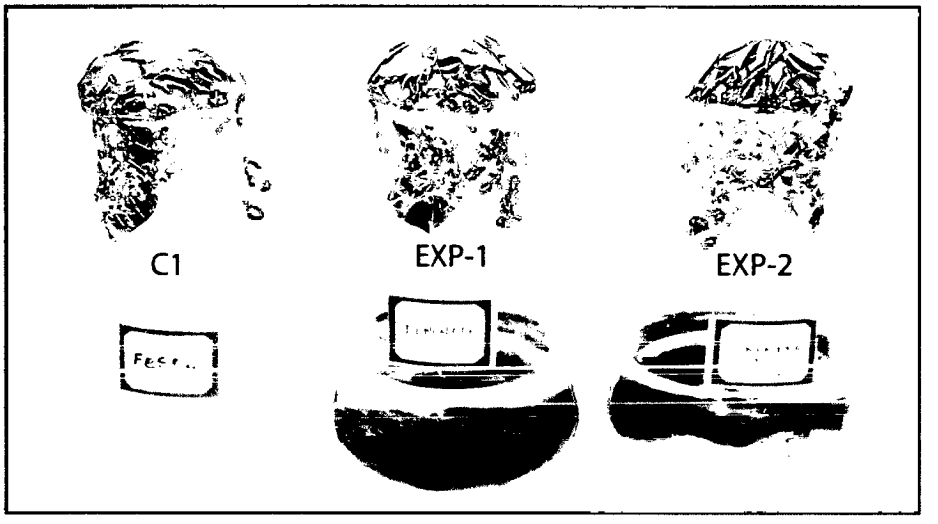


Plate 6.2: Effect of iron released from ilmenite on abundance and activity at primary level

a) C2 flask containing $FeSO_4.7H_2O$

b) Exp-1 flask containing ilmenite grains

c) Exp-2 flask containing ilmenite and $FeSO_4.7H_2O$

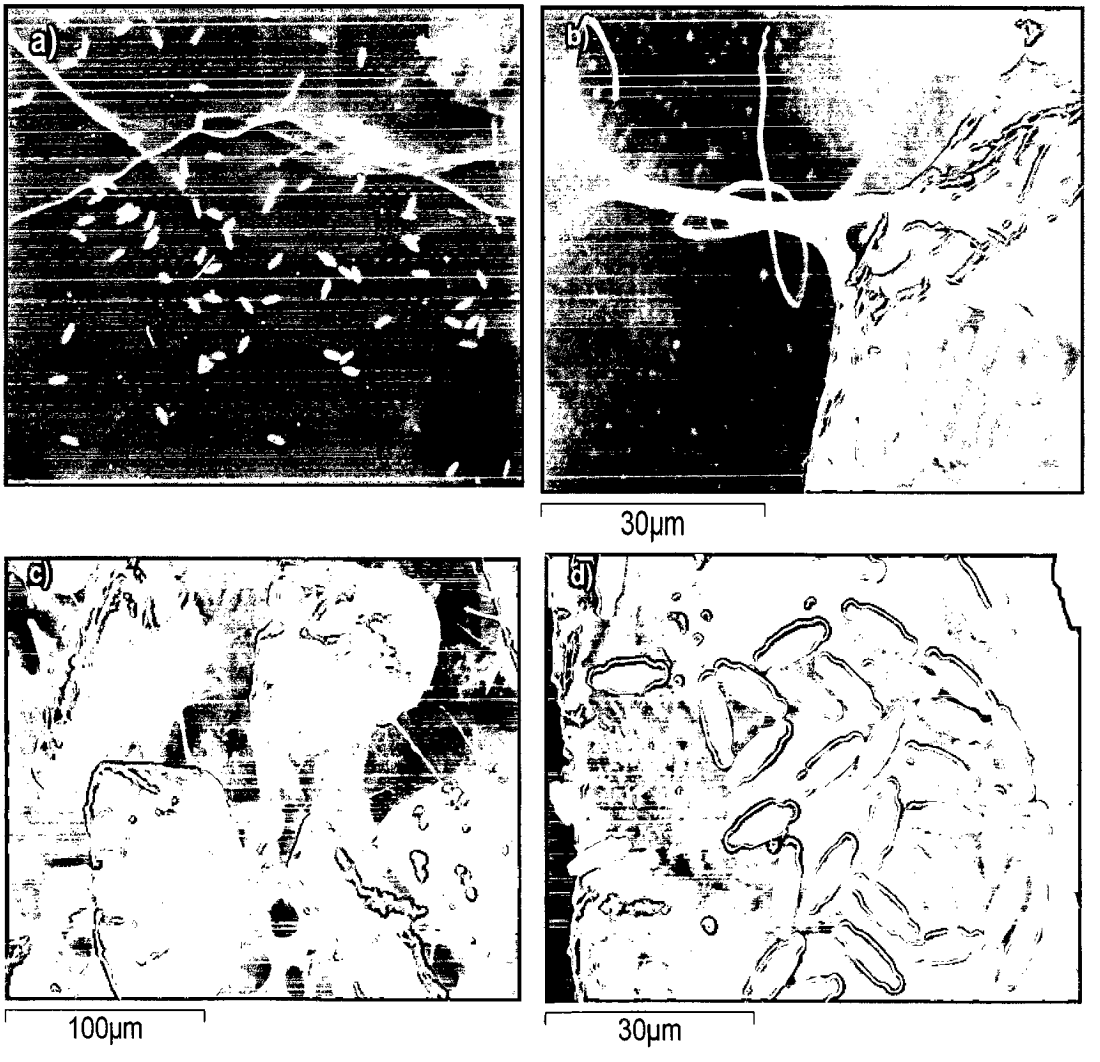


Plate 6.3: Colonization of ilmenite grains by diatoms and green algae in Exp-1 flask containing ilmenite grains

a) Image using light microscopy

b-d) SEM images

6.2.1.3.2. Discussion

It is well known that in marine environment, iron is an important micronutrient controlling primary production, the availability of which depends on the speciation of iron. Consequently, there are ecosystems that are limited by this micro-nutrient. The transportation of iron to the marine environment is usually through three pathways i.e. via fluvial input, atmospheric deposition and processes on seafloor such as diagenesis, sediment re-suspension and hydrothermal venting (Ussher et al., 2004). Thus iron containing placer minerals like magnetite, ilmenite released from terrestrial rocks and transported by rivers and streams from the hinterland into the adjoining seas could be a major source of iron especially in the near-shore environments.

The experiments conducted to simulate natural systems in microcosms show that iron released from ilmenite could be stimulatory. In Exp-1 flask, there was a significant accumulation of iron in the form of Fe(II) and Fe(III) though there was no added $FeSO_4.7H_2O$ suggesting microbial participation. Interestingly, the r values which highlight the release of iron with time were close to the values in Exp-2 where $FeSO_4.7H_2O$ was added (Table 6AT1, Appendix III). This release of iron by indigenous bacteria has been dealt in section 6.2.1.1. The release of iron not only supports bacteria but other living organisms. This influence on living organisms could be measured in terms of ATP. It is well known that iron plays a major role in the reduction of oxygen for synthesis of ATP, photosynthesis as well as respiratory electron transport (Geider and La Roche, 1994; Neilands, 1995). This experiment clearly demonstrates that iron released from the ilmenite could contribute to the high biomass which in turn leads to high concentration of ATP. This is corroborated by high phytoplankton and benthic algal biomass in Exp-1 but not by bacterial counts.

Total bacterial counts were generally in the range of 10^{7-8} cells mL^{-1}. Surprisingly, the controls recorded bacterial numbers that were one order more than the experiment. Moreover, in the presence of ilmenite, this numbers seem to be more or less static at 10^7 cells mL^{-1}. This lack of increase in bacterial biomass could be due to the grazing by the protozoans at the next

trophic level. It is quite possible that the presence of grazers in the size range of <200μm could contribute to these low bacterial numbers. Ciliates were generally encountered in the samples and they are also known to predate on the bacteria. Therefore the bacterial abundance remaining apparently at the same level and one order below control could be due to these grazers. The bacterial abundance was thus kept in a dynamically static state by the secondary grazers, that were more abundant in experimental than the control flasks. This lack of difference in Exp-1 and 2 suggests that bacteria are not inhibited by higher concentration of iron in Exp-2. These observations suggest that bacteria can tolerate higher concentrations than primary producers. Study by van Wambeke et al., (2008) has shown that after stimulation of heterotrophic bacteria with Fe, the number of heterotrophic flagellates also increased. However, this leads to enhanced bacterial predation. The stronger relationship noticed between bacterial numbers and Fe(III) concentration in the control C-1 flask could be due to the use of Fe(III) as an electron acceptor in respiratory cycles for maintenance. This relationship is uncoupled in experimental flasks especially Exp-2 where iron is non-limiting.

Iron is not only required in the respiratory but also in the photosynthetic cycles. Chl *a* was also used as a proxy for live phytoplankton biomass because of the difficulty in differentiating between live and dead phytoplankton. During the course of the experiment, both phytoplankton density and Chl *a* increased in the experimental flasks. It was much higher than C-1 and C-2 and peaked on 14d. In C-1 and C-2, the Chl *a* concentrations peaked by 3d, and there was a gradual decrease thereafter. The delayed peak in the Exp-1 and 2 is attributable to the slower release of iron from ilmenite. The earlier attainment of the peak in the controls could be due to more readily available iron but at much lower concentration. The concentration of Chl *a* is highest in Exp-2 due to increased iron released from ilmenite as well as due to the added $FeSO_4.7H_2O$. Thus the experiment clearly demonstrates that the iron released from the mineral ilmenite is used in photosynthesis and thus stimulate the growth of phytoplankton. In mesoscale experiments conducted elsewhere, phytoplankton biomass and productivity,

especially in large-celled phytoplankton such as diatoms, increased several fold after Fe enrichment in the surface mixed layer (Martin et al., 1994; Coale et al., 1996, 2004; Boyd et al., 2000; Tsuda et al., 2003). The relationship between iron and Chl *a* is interesting in the present set of experiments. A strong relationship between Chl *a* and iron is encountered only in Exp-1. It is low and insignificant in C-1, C-2 and Exp-2 for different reasons, iron being highly limiting in C-1 and C-2 and in excess in Exp-2. In Exp-1, the concentration of iron governs the growth and sustenance of phytoplankton for a longer period. Besides concentrations of Chl *a* related significantly to phytoplankton abundance suggesting high viability.

The average abundance of phytoplankton in Exp-1 and Exp-2 increased significantly with time ($p \leq 0.01$) and recorded a high abundance which was >20 times more than C-1. SEM images clearly revealed the colonization of the ilmenite grains not only by the filaments of algae but also by diatoms (Plate 6.2 an 6.3). As mentioned earlier, cell abundance related positively with Chl *a*, ATP, Fe(II) and Fe(III) in Exp-1. Both Exp-1 and 2 supported 27 and 28 genera as compared to 16 and 17 in C-1 and C-2. The total number of phytoplankton count, diversity index and species evenness index was highest in Exp-1. Diatoms were dominant in the control as well as the experimental microcosms. Generally, in the natural marine ecosystems, the Bacillariophyta (diatoms) are found to be the most dominant phytoplankton group. Bottom dwelling genera such as *Nitzschia* spp and *Navicula* spp. proliferated at the later stages of the experiment in all the microcosms suggesting they had tolerance to a wider range in iron concentration. However, in the presence of ilmenite i.e. Exp-1 and 2, percentage occurrence of two genera namely *Eunotia* and *Pinnularia* spp. increased during the later stages of the experiment. Their percentage increased from non-detectable levels to >4% of the total genera at 85d. Both these species have been known to occur in iron enriched waters of moderately high conductivity (Czarnecki and Cawley, 1997), thus suggesting their preference for higher concentrations of iron. Site specific studies have shown that *Pinnularia* occurs as brown mucilaginous mats on wet soils and along the border of streams and pools commonly in

thermal and non-thermal sulfuric acid habitats having high concentrations of metals at pH 1.0-3.0 (Albertino, 1995). Strands of filamentous algae and bubbles were also predominantly present especially in Exp-1 indicating active algal and plankton growth (Plate 6.2 and Plate 6.3). In contrast, C-1 showed no visible growth throughout the experiment. Growth in C-2 was marked by white floccules of diatomite indicating early loss of viability. Such observations have been made by Sutherland et al., (1998). "Blister" or "bubble" mats have been usually attributed to oxygen trapped in the microalgal mucilage (Jørgensen et al., 1983; Yallop et al., 1994).

The present experiments with microcosms clearly highlight the influence of the bio-available iron released from ilmenite by microbial action on the abundance and diversity of phytoplankton. It also shows that in the presence of ilmenite alone, there is both increase in diversity and evenness that is better than in the presence of combination of $FeSO_4.7H_2O$ and ilmenite. The health of the ecosystem is thus maintained by the diversity it promotes. The stimulatory effect of iron released from this mineral could be beneficial to the biocoenosis in Kalbadevi Bay.

6.2.2. Iron released from ilmenite by selected isolates

In order to discern the contribution from morphologically and numerically different types of bacteria for iron release, experiments were carried out to screen them for ability to release iron from ilmenite.

6.2.2.1. Results

Nearly 60% of the 100 isolates obtained on different media had the ability to release Fe from mineral ilmenite. The data presented here pertains to four of the best isolates which could effectively release iron from ilmenite.

6.2.2.1.1. Characterization of the studied bacterial isolates

Microbial dissolution of ilmenite was followed in the laboratory using bacterial isolates. Preliminary analyses revealed that the experiments on four bacterial isolates showed that the culture when grown in media amended with mineral ilmenite was capable of leaching out iron in the form of Fe(II).

6.2.2.1.2. Cell and colony morphology

The biochemical characterization of these isolates is given in the table 6.2. All the strains were gram negative and non motile. S3 and S11 were cocobacilli while S7 and S8 were coccoid. All the strains were oxidase positive.

6.2.2.1.3. Phylogeny of studied isolates

Potential isolates were subjected to 16S rDNA analyses for identifying them upto species level (Table 6.2).

Table 6.2: Biochemical and 16S rDNA characterization of the bacterial isolates

Isolates number	S3	S7	S8	S11
Gram character	Gm –ve coccoid	Gm –ve coccobacilli	Gm –ve coccobacilli	Gm –ve coccoid
Motility	Non motile	Non motile	Non motile	Non motile
Oxidase	+	+	-	+
Catalase	-	-	-	-
OF test	Oxidative	Oxidative	Oxidative	Oxidative
Amylase	+	-	-	-
Protease	+	+	+	-
Lipase	-	-	-	-
DNase	+	-	+	+
Nearest homology	uncultured alpha proteobacterium B23; (GenBank entry: EU360292) (Homology: 99.1%)	bacterium CWISO21; (NCBI Accession no: DQ334359) (Homology: 99.5%)	Flavobacteriaceae bacterium GB058; (Accession No: AB433333) (Homology: 99.4%)	*Yangia pacifica* (T); type strain: DX5-10; AJ877265 (Homology: 99%)
Hierarchy view according to 16S rDNA analyses				
Domain	Bacteria	Bacteria	Bacteria	Bacteria
Phylum	Proteobacteria	Proteobacteria	Bacteroidetes	Proteobacteria
Class	α-Proteobacteria	γ-Proteobacteria	Flavobacteria	α-Proteobacteria
Order	Rhizobiales	Alteromonadales	Flavobacteriales	Rhodobacterales
Family	Hyphomicrobiaceae	Alteromonadaceae	Flavobacteriaceae	Rhodobacteraceae
Genus	*Filomicrobium*	*Alteromonas*	*Gaetbulimicrobium*	*Citricella*

Phylogenetic analysis of the 16S rDNA sequence of S3 isolate indicated that it belonged to class Alphaproteobacteria. The nearest known relative of S3 isolate is an uncultured Alphaproteobacterium B23 (GenBank entry: EU360292). S3 isolate has 99.1% sequence identity to uncultured Alphaproteobacterium B23 over 1200 unambiguously aligned positions (Fig 6AF1, Appendix IV). Isolate S7 was grouped within the gamma class of the

Proteobacteria and had close relationship of 99.5% with bacterium CWISO21, (NCBI Accession no: DQ334359) (Table 6.3, Fig 6.9).

Table 6.3: Alignment View and Distance Matrix Table (With S7 sequence taken as reference sequence)

S_ab score*	Organism Name		NCBI Accession No
0.979	*Alteromonas* sp.	AS-30	AJ391191
0.984	*Alteromonas* sp.	AS-31	AJ391192
0.983	biphenyl-degrading bacterium	BP-PH	AB086226
0.987	*Pseudoalteromonas* sp.	KT0812A	AF239705
0.989	uncultured bacterium;	PDA-OTU9	AY700607
0.995	bacterium	CWISO21	DQ334359
0.980	uncultured Alteromonas sp.	IG-1-14	AB262377
0.980	uncultured bacterium	S25_1053	EF574709
0.995	*Pseudoalteromonas* sp.	c7	EU420060
0.978	uncultured Alteromonas sp.	C50-021	AM941188
0.981	uncultured bacterium	Red2	EU627901
0.982	*Alteromonas macleodii* 'Deep ecotype'		CP001103
0.982	*Alteromonas* sp.	UST981101-023	EU982328
0.980	*Alteromonas alvinellae*	C73	FJ040190
0.980	uncultured bacterium	AV19F21b	FJ905627
0.982	*Alteromonas* sp.	H86	FJ903192
0.981	*Alteromonas* sp. unclassified_Alteromonadaceae (1)	H91	FJ903194
0.982	*Alteromonas* sp.	WH063	FJ847830

*S_ab: Sequence Match Score

S7 culture Aligned Sequence Data: (1411bp)

>S7

ACCATGCAAGTCGAACGGTAACATTTTTAGCTTGCTAATCGATGACGAGTGGCGGACG
GGTGAGTAATGCTTGGGAACTTGCCTTTGGGAGGGGGATAACAGTTGGAAACGACTGC
TAATACCGCATAACGTCTACGGACCAAAGGGGGCTTAGGCTCCTACCTGAAGAGAGGC
CCAAGTGAGATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAACGATCTCTAGCT
GTTCTGAGAGGAAGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGT
GTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTTGTGAGGAAAGGTTGGTAGT
TAATACCTGCCAGCTGTGACGTTAACAACAGAAGAAGCACCGGCTAACTCCGTGCCAG
CAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ACGCAGGCGGTTTGTTAAGCTAGATGTGAAAGCCCCGGGCTCAACCTGGGATGGTCAT
TTAAAAATGGCAGACTAGAGTCTTGGAGAGGGGAGTGGAATTCCAGGTGTAGCGGTGA
AATGCGTAGATATCTGGAGGAACATCAGTGGCGAAGGCGGCTCCCTGGGCAAAGACT
GACGCTCATGTGCGAAAGTGTGGGTAGCGAACAGGATTAAATACCCTGGTAGTCCACA
CCGTAAACGCTGTCTACTAGCTGTTTGTGGATTTAATCCCGTGAGTAGCGAAGCTAACG
CGCTAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACG
GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTT
ACCTACACTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCT
GACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGAGA
CTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTAC
GTGTAGGGCTACACACGTGCTACAATGGCATATACAGAGGGAAGCGAGACAGTGATGT
GGAGCGGACCCCTTAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCAT
GAAGTCGGAATCGCTAGTAATCGCAGGTCAGCATACTGCGGTGAATACGTTCCCGGGC
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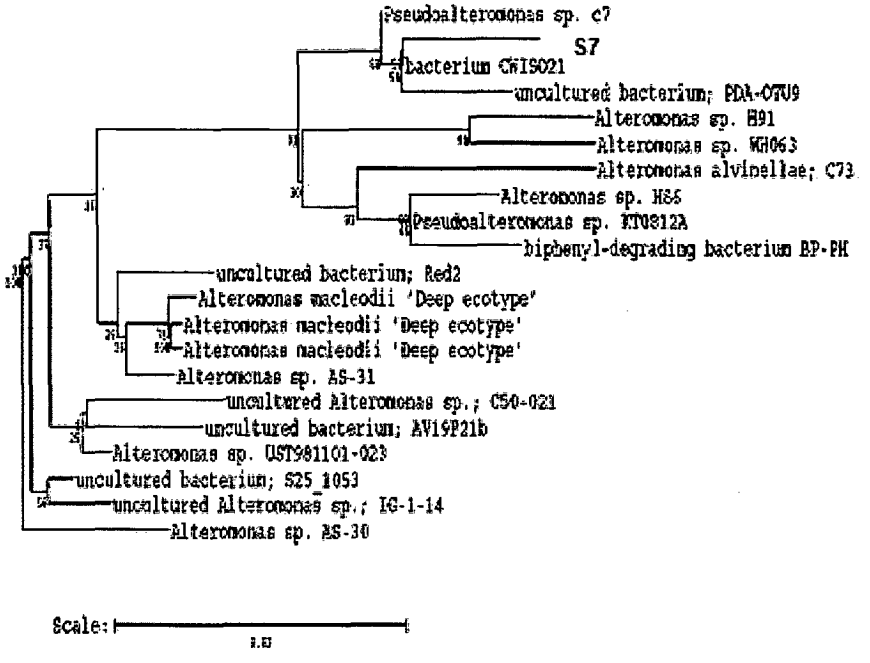


Fig 6.9: Phylogenetic affinity of S8 isolate

16S rDNA analysis revealed that the S8 isolate was most closely related (99.4%; 1,214 nucleotide positions considered) to the Flavobacteriaceae bacterium GB058; (Accession No: AB433333) a member of the family Flavobacteriaceae within the Flavobacteria class of Bacteroidetes (Fig 6AF2, Appendix IV). The closest known relative of S11 isolate was *Yangia pacifica* (T), type strain: DX5-10 with 99% similarity in 1325 nucleotides considered (Accession No. AJ877265) (Fig 6AF3, Appendix IV).

6.2.2.1.4. Cell growth and reduction of ilmenite

The pH in the cell free control and the experiment flasks fluctuated between 4.5-7.0 during the entire experimental period for nearly all the tested isolates (Table 6.4, Fig. 6.10). Concentration of Fe(II) in the media of the test isolate, S7, was double that of the control. Cell numbers of S7 isolate showed an increasing trend in presence of increasing concentration of Fe(II) (Plate 6.4). The S8 and S11 isolates displayed a sinusoidal growth curve. It initially increased by day 13 than showed a decreasing trend. These numbers again increased by day 62. TEM analysis showed the presence of intracellular metal accumulation in S8 and S11 isolate (Plate 6.5).

Table 6.4. Microcosm studies with isolates using ilmenite as a substrate

Isolates tested	Control	S3	S7	S8	S11
pH change during experiment	6.46 -6.69	6.47- 6.78	5.11 -6.64	6.48 -6.69	6.18 -6.82
Maximum cell yield (x 10^6 cells mL^{-1}) * initial in parentheses		8.72 (1.45)	382 (101)	20.48 (14.32)	60.48 (1.74)
Maximum amount of Fe(II) estimated in the solution (μM)	0-91	0-17	0-184	0-21	0-19
Rate of Fe(II) removal (μM d^{-1})	-12.12 to 15.62	-2.52 to 15.77	-19.90 to 35.48	-2.53 to 18.06	-1.94 to 13.29

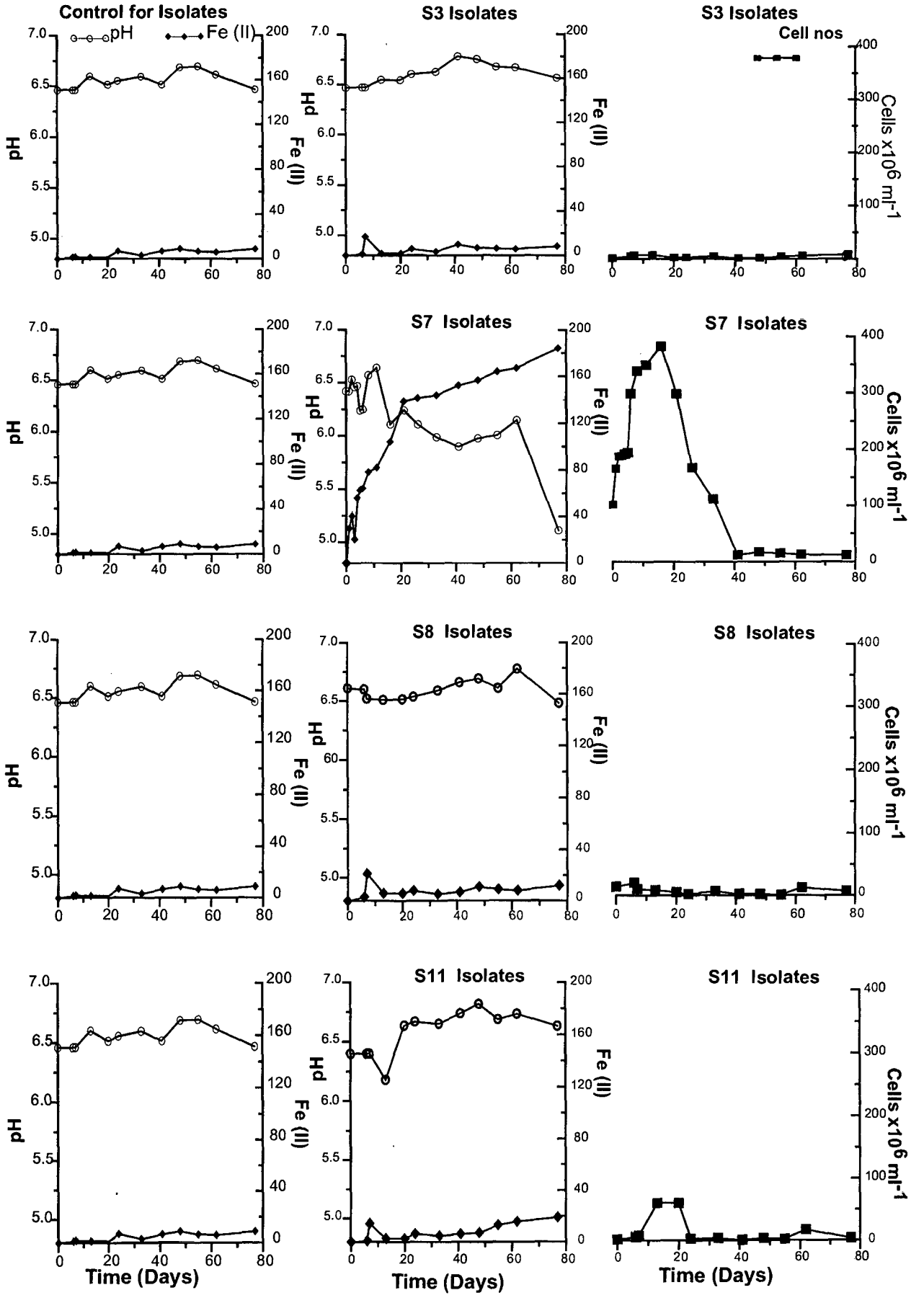


Fig 6.10: Change in pH and cell nos during removal of Fe (II) from ilmenite by the selected isolates

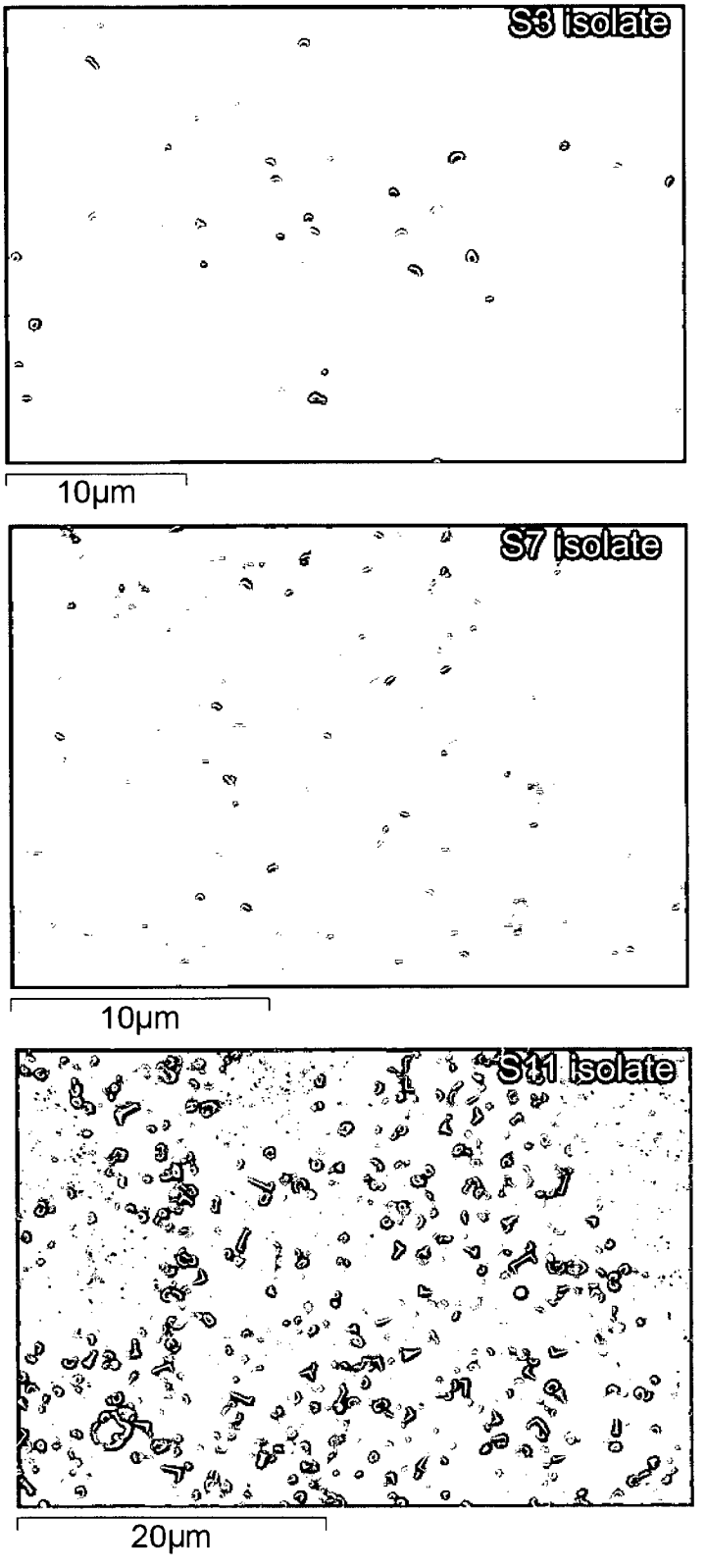


Plate 6.4: SEM images of isolates involved in the release of iron from ilmenite

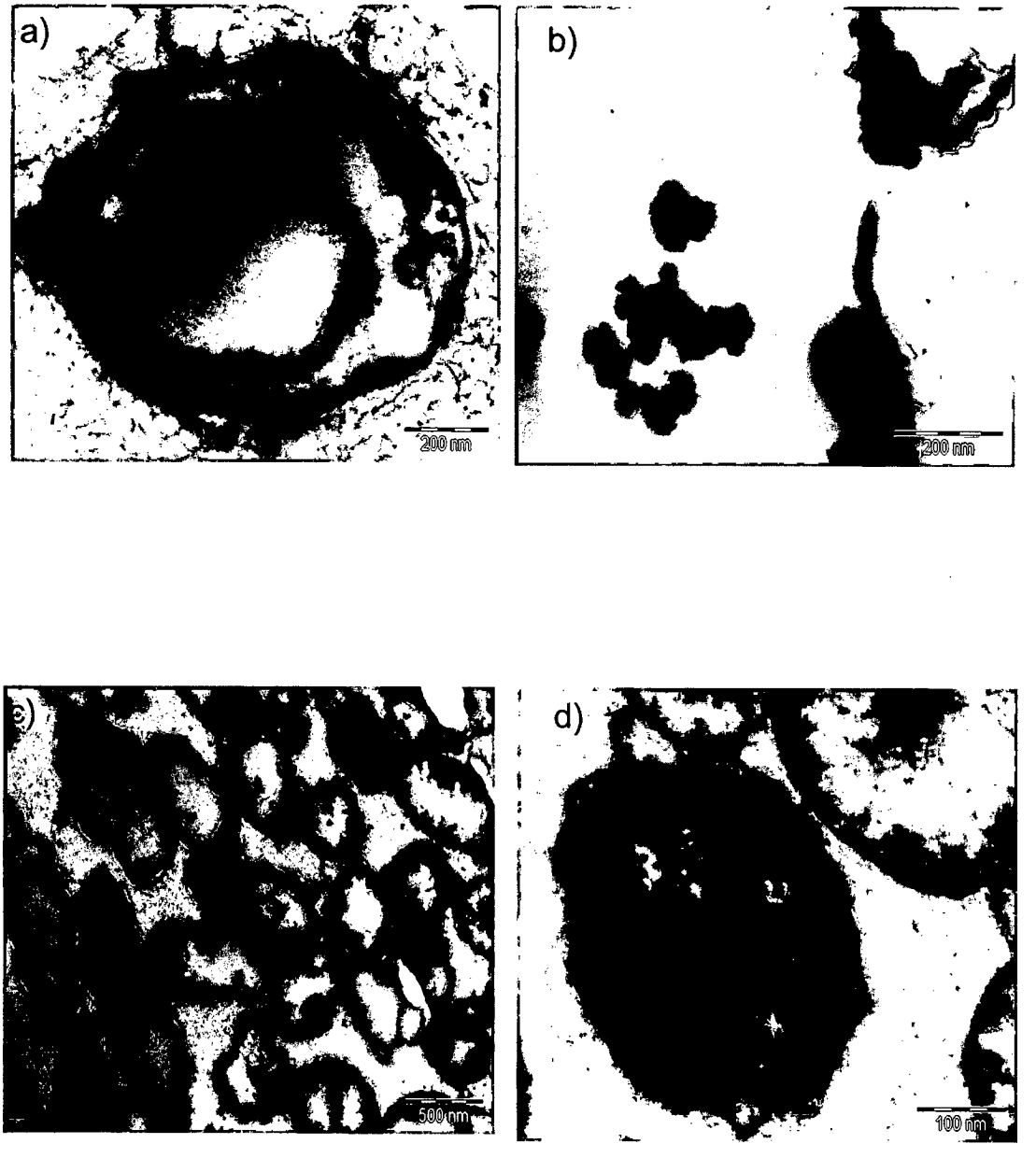


Plate 6.5: TEM images of S8 isolate (a-b) and S11 isolate (c-d)

Though the trends in the relationship with Fe(II) and cell numbers were visible, there was no significant correlation between the two parameters. Chemical rate of removal of Fe(II) in the solution fluctuated between -12.12 to 15.62 μM d^{-1}. However, the biological rate of removal of Fe(II) from ilmenite varied between -19.90 to 35.48 μM d^{-1} and the S7 isolate displayed the highest rates of removal.

6.2.2.2. Discussion

Iron in sediments often exists as poorly crystalline Fe(III) oxides (Lovley, 1993; Nealson and Saffarini, 1994). Iron oxides and oxyhydroxides which may occur in marine environments are goethite (α-FeOOH), akaganéite (β-FeOOH), lepidocrocite (γ-FeOOH), feroxyhite (δ'-FeOOH), ilmenite ($FeTiO_3$), hematite (α-Fe_2O_3), magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) (Burns and Burns, 1981). The extent of diversity among dissimilatory metal reducers has been reviewed by Lovley (1993) and Nealson and Saffarini (1994). It is well known that microorganisms can reduce Fe(III) (hydr)oxide minerals, hematite, and magnetite (Roden and Zachara, 1996; Fredrickson et al., 1998; Zachara et al., 1998). The four tested isolates belonged to Proteobacteria and Bacteroidetes. The experiment with the isolates is clearly suggestive of the diversity of the organisms in the Kalbadevi beach. Lonergan et al., (1996) in his study of phylogenetic analysis of dissimilatory Fe(III)-reducing bacteria indicated that the metal reduction capacity is a characteristic that is widely spread in the domain *Bacteria*.

All the isolates were capable of releasing Fe(II) from the mineral ilmenite. This rate of removal was the highest to S7 isolate which had a taxonomic affinity to Gammaproteobacteria. The negative rate of removal of Fe(II) in the solution suggested that the Fe(II) brought into the solution was re-oxidized to Fe(III). Growth rate and cell numbers can be caused by minor differences in the innoculum size, the viability of the cells in the innoculum, temperature, pH, etc and may have an implication in the variation in the rate of Fe(II) removal. This type of variability in reduction experiments with microoganisms have previously been noted (Fredrickson et al., 1998). It is probable that the use of

higher innocula of S7 isolate and perhaps more rapidly metabolizing cells may have established relatively more favourable redox conditions. This may also probably explain the higher rate of Fe(II) removal in the solution as well as the equally faster re-oxidation. Low accumulation of ferrous iron in medium containing other isolates i.e S3, S8 and S11 could indicate possibly that these isolates were capable of extracting the Fe(II) from ilmenite at a net rate that is slow.

Thus it may be inferred that ilmenite which was previously known as recalcitrant mineral (Page and Huyer, 1984; Page and Grant, 1987) and resistant to bacterial attack is possibly more accessible to bacterial reduction than hitherto believed.

6.3. Oxidative phase (Oxidation of iron released from ilmenite)

6.3.1. Effect of varying concentration of chemical form of iron, $FeSO_4.7H_2O$ on a bacterial isolate.

6.3.1.1. Results

6.3.1.1.1. Ecological background

The total bacterial abundance in the Kalbadevi berm sediment ranged between 10^{5-6} cells g^{-1}. The morphotypes representing the isolate Fe13 were frequently encountered on the isolation medium and made up to 10% of the total iron utilizing bacteria retrieved on ferric ammonium citrate plates

6.3.1.1.2. Characterization of the bacterial isolate

The biochemical characterization of this isolate, Fe13 showed that it is a gram-negative short rod belonging to Gammaproteobacteria member *Pseudomonas* sp. Results of 16S rDNA analysis confirmed its generic identity. The isolate was closely related to *Pseudomonas agarici* at a similarity index of 98%.

6.3.1.1.3. Bacterial cell growth in presence of increasing concentration of iron

Cell growth in terms of cell numbers was monitored to assess the response of the cells to increasing iron concentration. The initial inoculum of cells was 0.084(±0.022) × 10^9 cells mL^{-1}. Maximum growth was attained in 9d. In EM-E1 medium containing only seawater and glucose, the cell abundance increased to 0.78(±0.27) × 10^9 cells mL^{-1} (Fig 6.11i). However, the cell numbers in EM-E2, EM-E3, EM-E4 and EM-E5 increased 25%, 30%, 32% and 6% more than the Fe-unamended EM-E1 medium. In EM-E2, the cell numbers reached a maximum of 0.97(±0.048) × 10^9 cells mL^{-1} in 9d. Similarly, in EM-E3, the maximum cell numbers recorded was 1.02(±0.014) × 10^9 cells mL^{-1} in 9d. In EM-E5 medium, the peak cell abundance was lower at 0.82(±0.09) × 10^9 cells mL^{-1}. The calculated instantaneous growth rate constant "μ_g" increased with the increasing concentration of iron. Consequently, the corresponding mean generation time for cells was lower at 100 μM-Fe concentration (Table 6.5). The time required for the cells to double in numbers was low when the medium was amended with Fe concentration ranging between 1 to 100 μm $FeSO_4.7H_2O$.

Table 6.5: Growth rate constant (μ_g), mean generation time (g), cell specific activities at varying concentration of iron at the end of 9 days.

Concentration of $FeSO_4.7H_2O$ (μM)	μ_g	g	cell specific activity (M $cell^{-1}$ d^{-1}) Fe(II)→ Fe(III)
Control	0.354	2.811d	2.61
1	0.508	2.551d	8.73
10	0.758	2.503d	-10.3
100	0.992	2.503d	119.96
1000	1.133	2.737d	-221.9

Like the cell numbers, ATP concentrations and cellular proteins increased with time. Biomass yield measured in terms of cellular ATP concentration also increased with time. However the average concentration of cellular ATP did

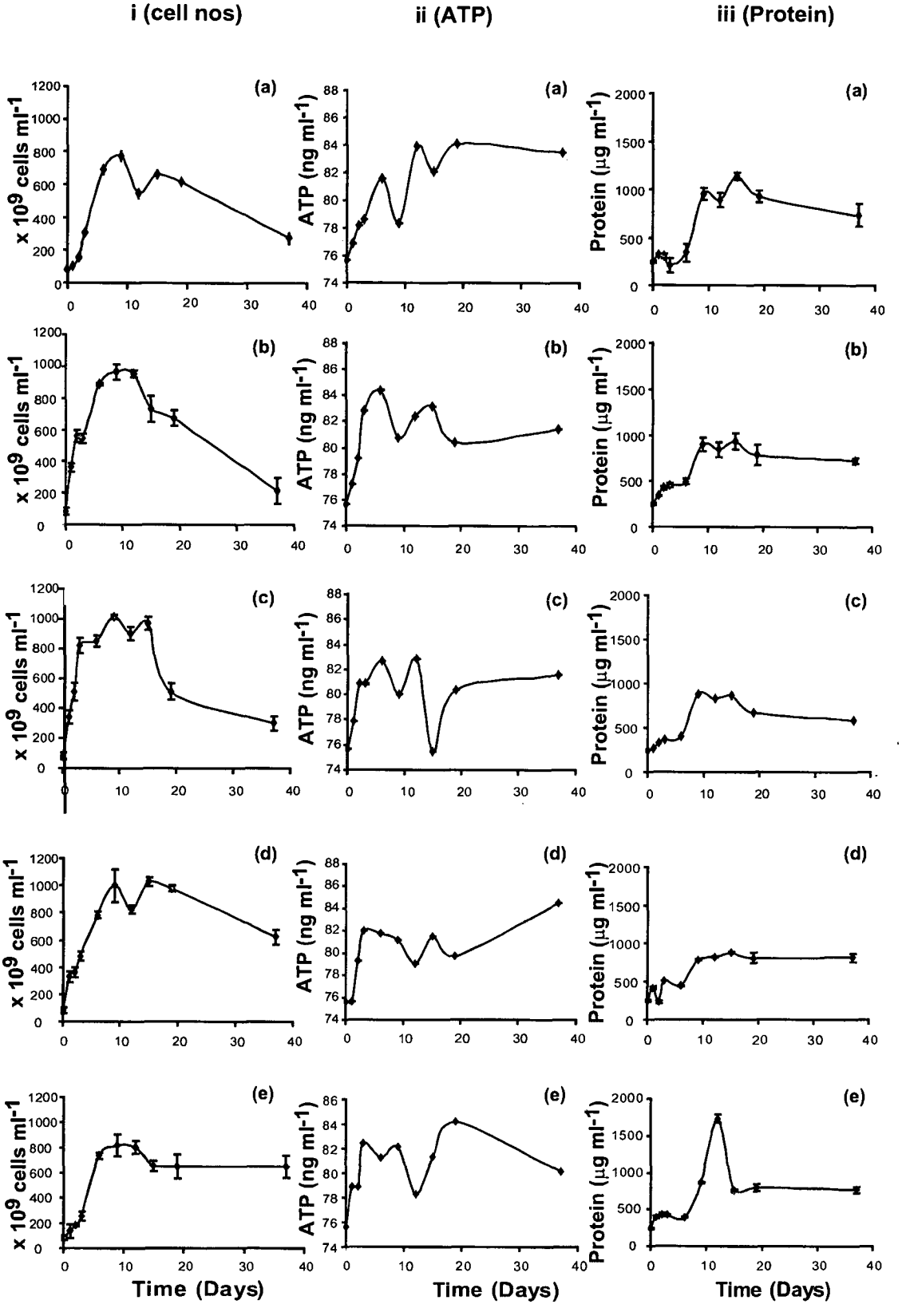


Fig 6.11: Change in (i) cell nos. (ii) ATP content and (iii) protein concentration of isolate Fe 13 in presence of varying concentration of iron (a) EM medium alone, (b) EM medium + 1 µM $FeSO_4.7H_2O$, (c) EM medium + 10 µM $FeSO_4.7H_2O$, (d) EM medium + 100 µM $FeSO_4.7H_2O$ and (e) EM medium + 1000 µM $FeSO_4.7H_2O$

not differ between the Fe amended media and Fe unamended medium (Fig 6.11ii). Cell protein concentrations increased from 245.31(±13.61) to 1131.68(±36.58) μg mL^{-1} in the EM-E1 medium (Fig 6.11iii). Amendments of Fe in EM-E2, EM-E3, EM-E4 and EM-E5 medium resulted in maximal values of 930.47(±82.97) μg mL^{-1} in 15d, 878.22(±11.71) μg mL^{-1} in 9d, 877.11(±11.71) μg mL^{-1} in 15d, and 1735.31(±50.06) μg mL^{-1} in 15d respectively. pH measurements showed a decrease of the initial pH but no difference at the end of the incubation period (Fig 6.12).

6.3.1.1.4. Bacterial cell morphology in presence of iron amendment

The above biochemical changes were accompanied by morphological changes. Changes in cell size and aggregation behavior, biofilm and precipitate formation was evident in SEM under 8000x magnification (Plate 6.6). Cells from unamended medium were short rod-shaped bacteria measuring 1.0x0.4μm (Plate 6.6b). Aggregation, biofilm and cluster formation was generally visible in amended experimental flasks and rarely in control. When the medium was amended with 1 μM-Fe (Plate 6.6c) the cell size decreased to 0.8 x 0.3 μm and was accompanied by the formation of a biofilm. At 10 μM-Fe amendment, the cell size further decreased to 0.7 x 0.2 μm and the cells aggregated in clusters (Plate 6.6d). The size of the biofilms formed was larger than those formed at 10 μM-Fe amendment. At 100 μM-Fe concentration, the cells seemed to increase marginally in size to 0.8 x 0.3 μm and were accompanied by precipitates and thicker biofilm formations (Plate 6.6e). However, cells from 1000 μM-Fe amendment were examined under lower magnification of 3000x using SEM. At 1000 μM, the cells became very large measuring 2.5 x 0.6μm, and were associated with biofilm and precipitates (Plate 6.6f).

6.3.1.1.5. Chemical nature of accumulated iron

EDS analysis further confirmed that the metal precipitate was iron oxide (Fig 6.13). The point x-ray analysis carried out on cells as well as on Fe precipitate in the microscope field revealed that the intensity of peaks for Fe on bacterial cell was 20% higher than the background precipitate. XRD analysis was

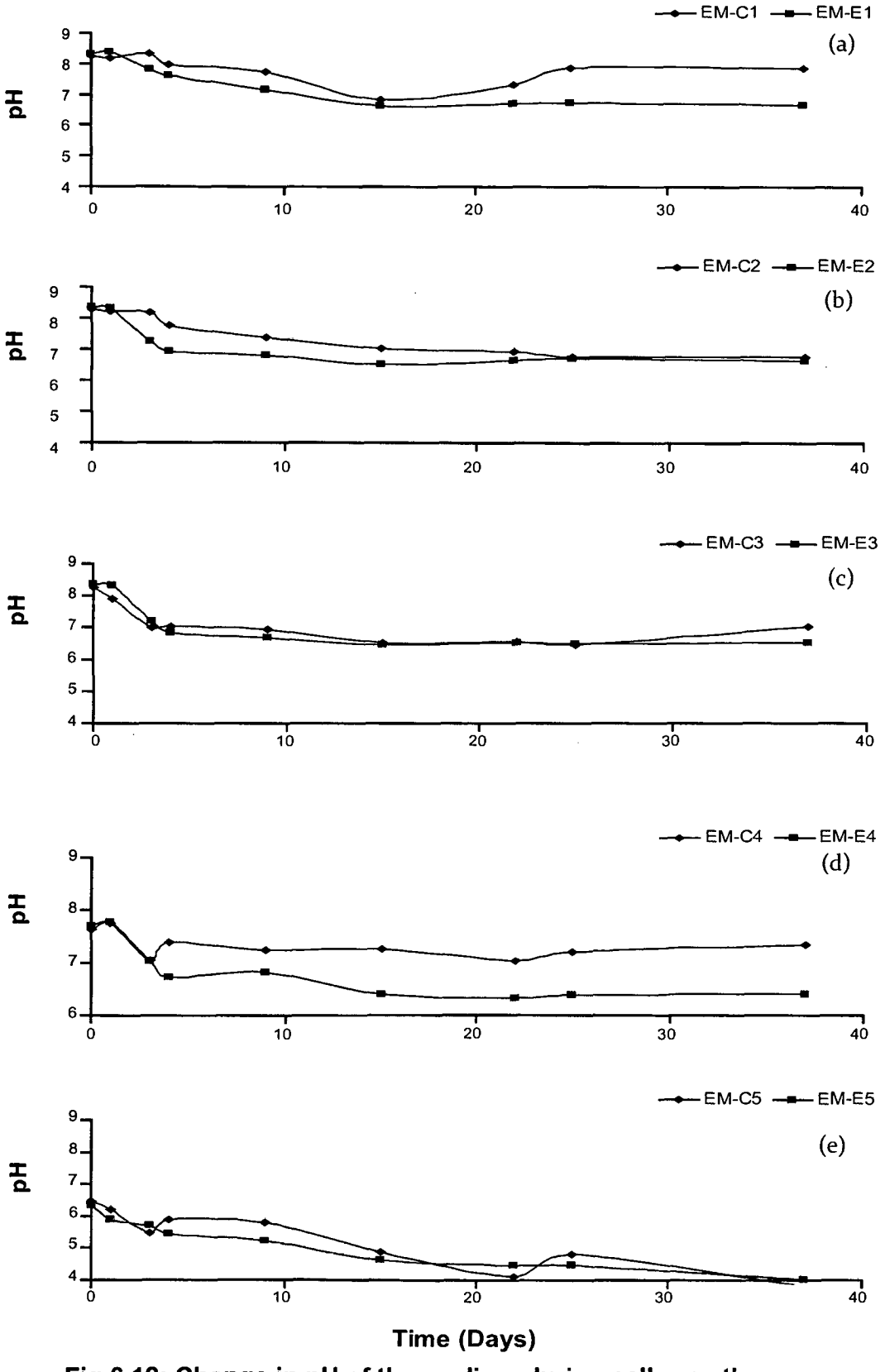


Fig 6.12: Change in pH of the medium during cell growth
(a) EM medium alone, (b) EM medium + 1 μM $FeSO_4.7H_2O$,
(c) EM medium + 10 μM $FeSO_4.7H_2O$,
(d) EM medium + 100 μM $FeSO_4.7H_2O$
and (e) EM medium + 1000 μM $FeSO_4.7H_2O$

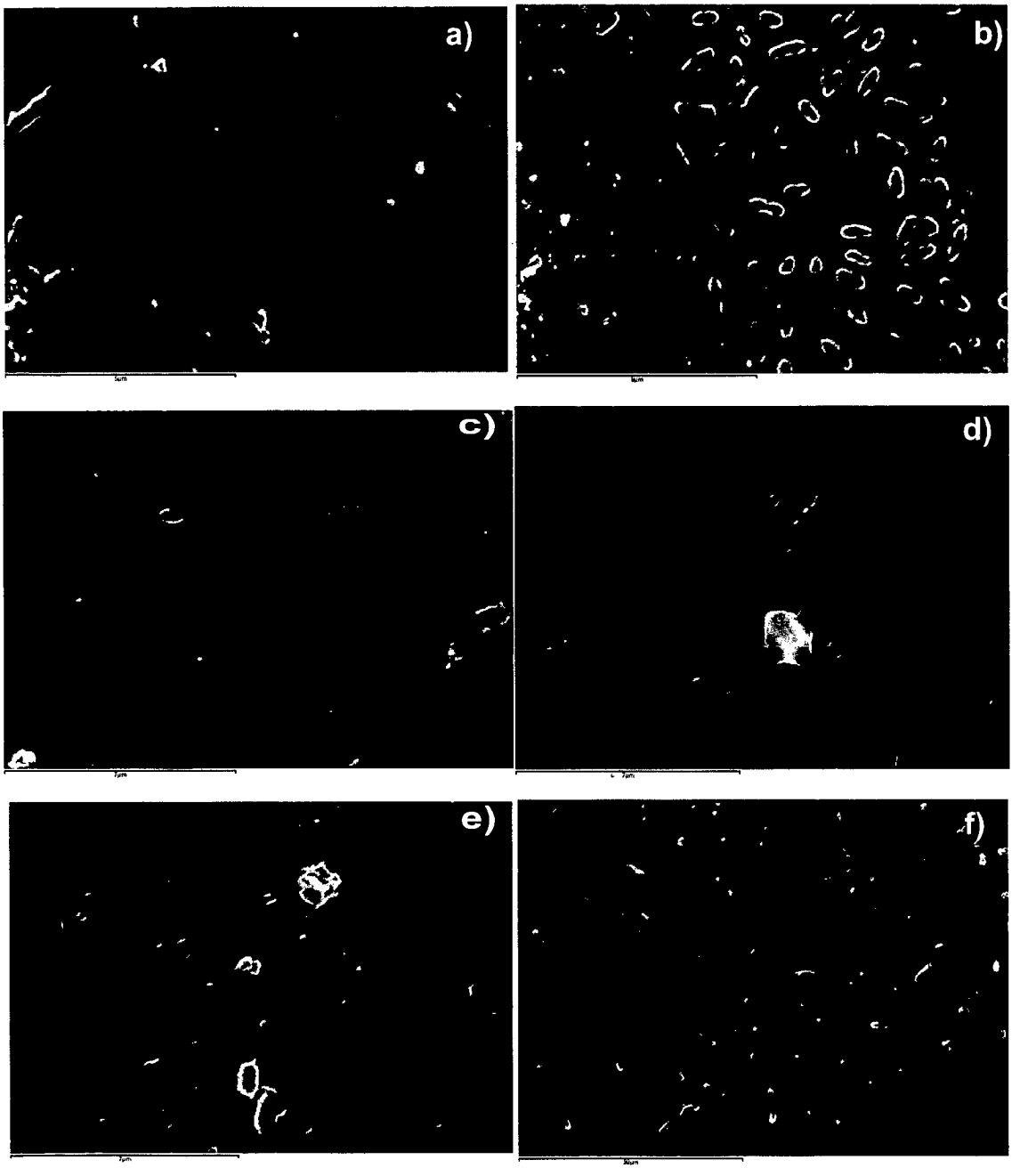


Plate 6.6: SEM images of bacterial cells and matrix formation by Fe13 strain (a) represents 0-d culture, (b) EM medium alone, (c) EM medium + 1 μM $FeSO_4.7H_2O$, (d) EM medium + 10 μM $FeSO_4.7H_2O$, (e) EM medium + 100 μM $FeSO_4.7H_2O$ and (f) EM medium + 1000 μM $FeSO_4.7H_2O$
(Magnification for A to E-x8000, F- x3000)

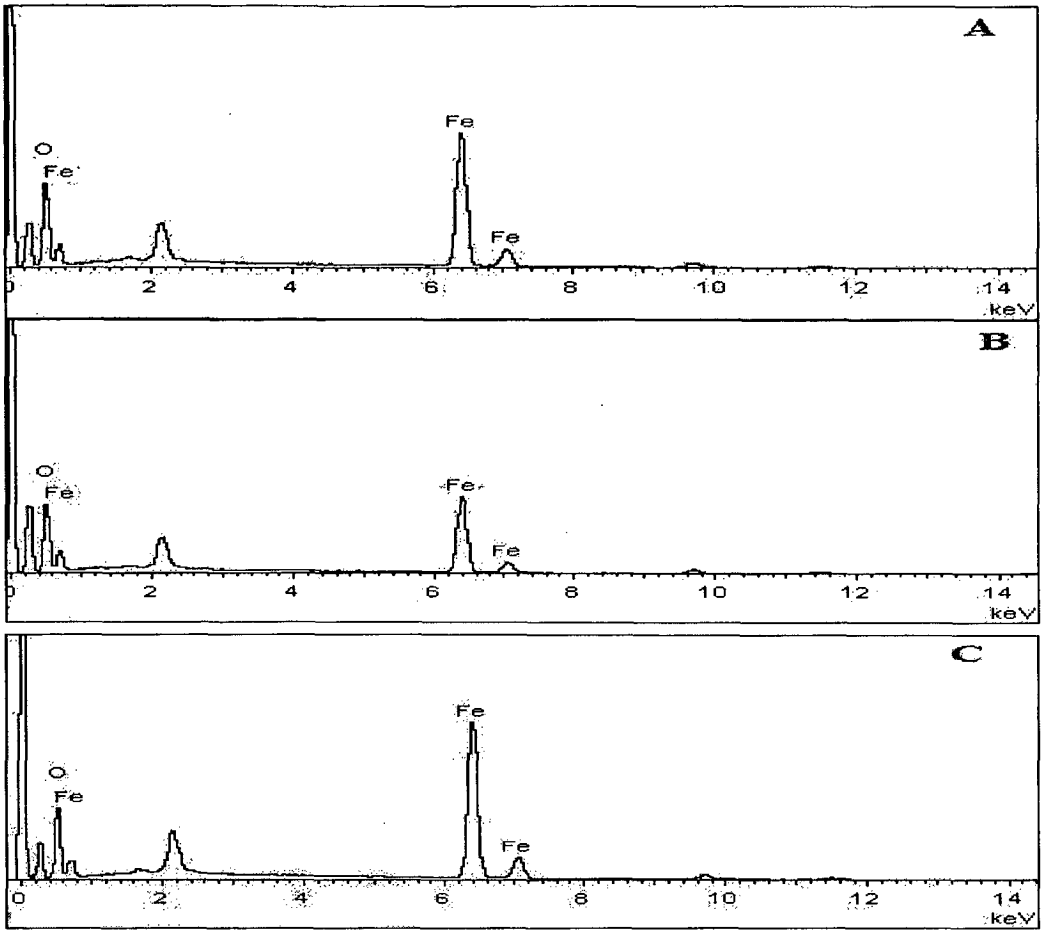


Fig 6.13: SEM-EDS spectrum showing typical elemental composition in (A) precipitate in control flask, (B) precipitate in experimental flask, (C) cells in experimental flask

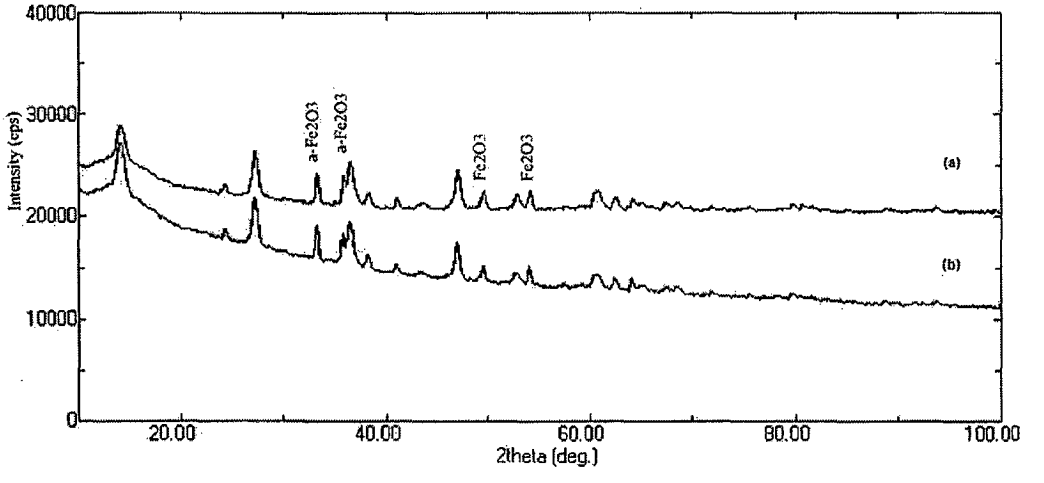


Fig 6.14: XRD analysis on precipitate in a) control b) experimental flask

performed to understand the mineral type in bacterial cells. The peaks for biogenic and abiogenic mineral phases were found to be overlapping each other. However, the intensity of the peaks in experiment (Fig 6.14b) was slightly lower than in control (Fig 6.14a). The diffractograms of the iron precipitate confirmed the oxidized form of iron to be Fe_2O_3 and α-Fe_2O3. Based on the "d" values, the mineral form of Fe was identified as hematite (Fe_2O_3) and goethite (α-Fe_2O_3). The d values for corresponding mineral hematite was 1.692, 1.838 and for goethite was 2.692 and 2.51 (Fig 6.14).

6.3.1.1.6. Change in Fe(II) and Fe(III) rate constant by Fe13

The optimal growth concentration of iron was at 100 μM where increase in soluble iron was accompanied by maximum increase in cell numbers. The rate constants for converting soluble Fe to insoluble increased with increasing Fe concentration (Table 6.6). On 9d when the cell numbers peaked, the rate constant "$\mu_{Fe(II)}$" for soluble iron was negative up to the 10 μM-Fe concentration and became positive at higher concentrations of 100 and 1000 μM (Table 6.6). The result showed that the cell specific activity for the Fe(II)→Fe(III) reaction generally increased with increasing iron-amendment except for some cases which did not follow the trend. The maximum cell specific activity for soluble Fe was recorded at 100μM $FeSO_4.7H_2O$ concentration. It ranged from -221 x 10^{-20} M $cell^{-1}$ d^{-1}in EM-E5 to 120 x 10^{-20} M $cell^{-1}$ d^{-1} in EM-E4 medium.

Table 6.6: Change in rate constant (μ) in control and experiment at varying concentration of iron at the end of 9days.

Concentration of $FeSO_4.7H_2O$ (μM)	$\mu_{Fe(II)}$	
	control	experimental
Control	-0.326	-0.556
1	-0.290	-0.193
10	-0.254	-0.006
100	0.254	0.737
1000	0.000	4.372

6.3.1.1.7. Change in Fe(II)/Fe(III) ratio by Fe13

The average Fe(II)/Fe(III) ratio in the control series ranged from 0.11±0.06 in EM-C1 to 2.96±0.71 in EM-C4. On the other hand, the average ratio in the experimental series during the course of the experiment was 0.64±0.89 in EM-E1, 0.1±0.03 in EM-E2, 0.12±0.04 in EM-E3, 2.72±1.67 in EM-E4 and 2.22±1.14 in EM-E5 (Fig 6.15). The trend in the change over time in ratio of Fe(II)/Fe(III) using voltammetry were comparable to the change in ratio using ferrozine assay at lower concentration of ≤100uM (Fig 6.16).

6.3.1.2. Discussion

The influence of increasing concentration of iron on an isolate from placer rich sediments was studied in order to monitor the increase in cell number and to determine its participation in the changes in Fe(II)/Fe(III) ratio.

Total bacterial cell abundance in the Kalbadevi beach sediments varies from 10^{5-6} cells g^{-1} and is generally low compared to other sandy intertidal beaches. The natural abundance of bacteria in the present study area is generally an order of magnitude less than reported elsewhere (Schmidt et al., 1998; Luna et al., 2002; Moreno et al., 2006). Bacteria are found commonly attached to the grains in sandy beach sediment and their abundance is controlled by various factors having complex relationships (Meadows and Anderson, 1968; Yamamoto and Lopez, 1985). The isolate was able to grow in the presence of the inorganic iron ($FeSO_4.7H_2O$) at circumneutral pH and can bring about a net fall in the Fe(II) concentration alternatively thus demonstrating the metabolic ability of this dominant group of *Pseudomonas* from ilmenite rich placer sediments. *Pseudomonas* spp. are generally known for their metabolic versatility

The growth of the isolate was accompanied by increase in ATP and protein concentrations confirming active participation (Riemer et al., 2004). ATP concentrations which are similar to that of control without iron could be due to the effect of glucose. The scanning electron micrographs demonstrate that the

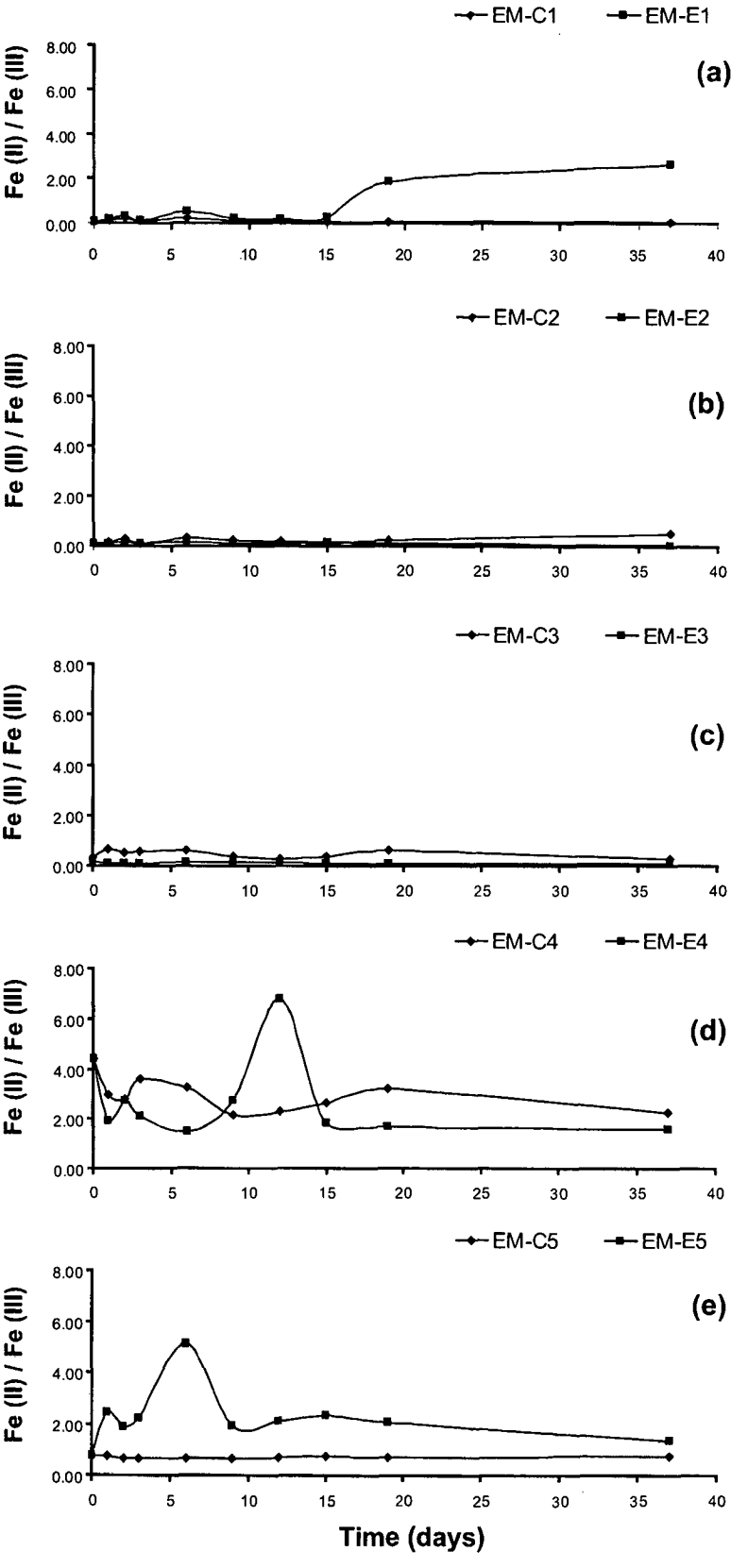


Fig 6.15 Change in Fe (II)/Fe(III) ratio during cell growth
(a) EM medium alone, (b) EM medium + 1μM $FeSO_4.7H_2O$,
(c) EM medium + 10μM $FeSO_4.7H_2O$,
(d) EM medium + 100μM $FeSO_4.7H_2O$
and (e) EM medium + 1000μM $FeSO_4.7H_2O$

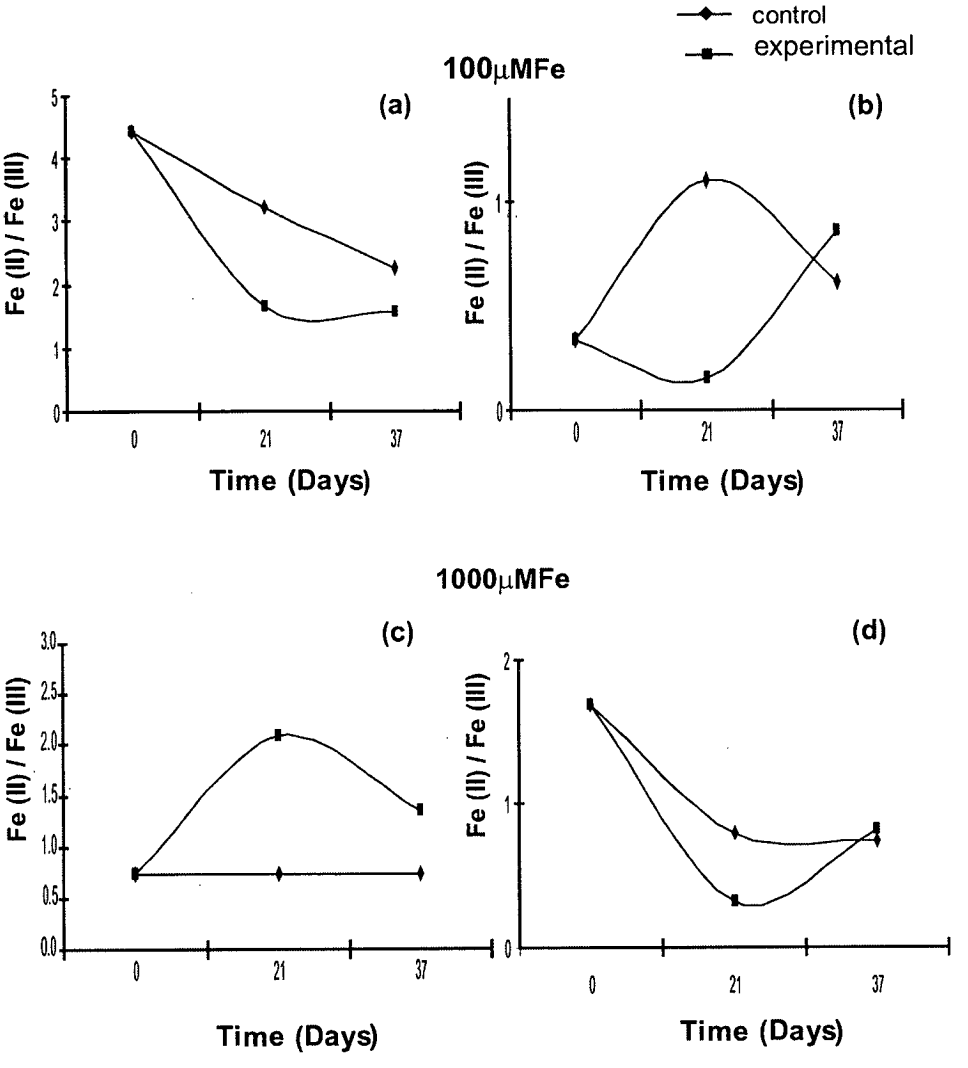


Fig 6.16: Comparative study of Fe(II)/Fe(III) ratio using ferrozine assay by spectrophotometery (a & c) and modified Metrohm method by voltammetry (b & d)

bacterial cell size decreases with the increasing iron concentration. The cells in the unamended medium appear to be dividing and hence appear large. However, at the highest concentration of iron tested, the cells appear very elongated (2.5x0.6μ, Plate 6.6f). At 1 and 10 μM amendment, the aggregation of the cells and the formation of biofilm suggest that this physiological adaptation could be towards improvement in iron scavenging ability. At a still higher concentration of 100 μM the matrices enlarge perhaps bypassing the necessity for aggregation. The findings complement the previous work, which, show that iron-deficient cells were smaller than iron-sufficient cells (Page and Grant 1987). This study highlights that when the cells are actively scavenging (in this case ambient 1 to 100 μM) they tend to decrease in size till a threshold is reached.

Generally, the quantity of iron recovered in various fractions do not account for the total iron added initially at the start of the experiment especially at higher concentrations. This is because some of the iron gets converted into hematite and goethite which may not be amenable to reduction by hydroxylamine. Moreover, at higher concentrations some of the iron gets lost as a film on the walls of the vessels. Hence for measuring ferrous iron production, some researchers have used ratio of Fe(II):total Fe, in order to better account for potential error which may inherently be introduced by sampling of a suspended solid phase (Langley et al., 2009). Yet others have used some other ratios such Fe(II)/Fe(III), [Fe(II)+ Fe(III)]/Fe(II) ratio (Sand et al., 1992; Propp and Propp, 2001). Studies on the cell interiors have revealed that the higher the concentration of iron, the lower the Fe(II)/Fe(III) ratio. Further, when the oxygen was less in the medium, the ratio was higher. (Kot and Bezkorovainy, 1993; Kot et al., 1994). In this study, the Fe(II)/Fe(III) ratio was used to monitor changes. Comparison between live experimental and abiotic controls showed definite trends (Fig 6.15).

The Fe(II)/Fe(III) ratios were counterchecked using the voltammeter. Samples containing initial concentration of 100 and 1000 μM of Fe were re-analyzed using voltammetry since the ferrozine method is said to have its limitations of

detecting all the iron in the solution (Anastacio et al., 2008). Figure 6.16 elaborates the results obtained by ferrozine assay and compares it with that obtained by voltammetry using pyrophosphate method. Though the behavior of controls is different, the trend in the experimental curves is comparable. From 0 to 21d, there is a fall in the ratio suggesting oxidation. Subsequently, the trend is either maintained or there is slight increase in the ratio suggesting relative increase in respiration and relative decrease in use of iron as an energy source. Higher average ratios were recorded for concentrations of 100 and 1000 μM indicating that the pool of soluble iron in the medium was more at these concentrations. Also, these ratios were higher in the control medium indicating that most of the iron was in the soluble phase. Similarly, higher ratios of Fe(II)/Fe(III) were recorded using the ferrozine method. This was due to hydroxylamine based extraction method which extracted only the hydroxylamine extractable iron. This is the inherent drawback of the method. However, in this experiment the amount of iron that the bacteria could oxidise to Fe(III) was assessed.

Measurement of release of reduced iron showed that the change is rarely steady suggesting of net oxidation and reduction phases both in control and experiment. However, the changes in the experiment are always more than in the control with the final trend being more oxidative. Like total activity, specific activity (activity normalized for cell growth) followed the same pattern. It is perhaps at this concentration the bacterium used the iron optimally for growth. At higher concentration, the precipitation is higher but growth rate decreases.

Studies have shown that microorganisms have the capability to couple between Fe(II) oxidation and Fe(III) reduction in a localized iron cycle and this can influence the biogeochemistry of other elements including carbon, phosphorus and sulfur (Neubauer et al., 2002). Some researchers opine that this oxidation is more passive than active. They argue that the probability in many cases of the so-called "biological iron oxidation" by these bacteria is confined to absorption of chemically oxidized iron by the sheath or slime layer surrounding the sheaths (Bridge and Johnson, 1998). However, these studies

clearly demonstrate that the oxidation is not passive but active. In this study, the bacterial isolate did not form a sheath, but a biofilm, which probably entrapped the oxidized form of iron. EDS images analyzed revealed that the precipitated form as well the cells were rich in Fe and O. Further, XRD analysis indicated the presence of haematite and goethite. The XRD diffractogram clearly show the similarity in iron oxidation productions between both the control and experiment. However, semi quantitative analysis suggests that these products could be relatively higher in the experiment than in the control. Mean generation time (g) calculated from the increase in cell numbers was higher for concentrations between 1 and 100 µM. At these concentrations, the bacterial isolate oxidizes the soluble iron for mixotrophic growth, thus attaining maximum cell abundance at 100 µM.

The foregoing experiments show that this isolate, Fe13, from ilmenite rich sediment is capable of scavenging the reduced iron and use it as an energy source. It generally expresses optimal mixotrophic growth when the ambient concentration is 100 µM. It is therefore suggested that the ambient concentration of iron in the environment governs the contribution of bacteria like Fe13 to iron oxidation in microaerophic to anaerobic conditions in beach sediments. At very high concentration it accelerates oxidation with reduced cell yield. The experiment elucidates the metabolic capability of the isolate. It could act as an efficient geochemical agent that could mitigate iron concentrations, by oxidizing or precipitating it when the concentrations are high. Thus this bacterial strain may be conveniently harnessed for bioremediation under iron-replete conditions.

Chapter 7

Chapter 7: Bacterial Diversity using 454 technology

7.1. Introduction

In order to understand the biodiversity of bacteria involved in iron cycling holistically, total DNA from sediments was extracted and subjected to 454 high throughput screening especially of bacterial forms under the aegis of International co-operative run of International Census of Marine Microbes (ICoMM).

Bacteria are ubiquitous in nature and account for most of the benthic biomass. They play a significant ecological and biogeochemical role in marine ecosystems by regulating the transformation of major elements such as carbon, nitrogen, phosphorus, oxygen, sulphur as well as in the mobility of metals (Guezennec and Fiala-Medioni, 1996; Ringelberg et al., 1997; Danovaro et al., 2000). Further, they are involved in the degradation of organic matter (Aller and Aller, 1998). The identification of these bacteria has traditionally been achieved by cultivation techniques such as plate counting. However, the cultured bacteria appear to constitute only a small fraction of the total cell counts in each sample (D'Hondt et al., 2004) and thus nearly 99% of naturally occurring microorganisms are not cultivated by standard techniques (Amann et al., 1995). Hence alternate methods are used to describe community constituents.

One such method is the identification of rRNA genes (rDNA) in DNA extracted directly from the environment (Pace et al., 1985; Amann et al., 1995; Pace, 1997). In general, the spectrum of cultured isolates is smaller than the diverse lineages of bacteria that are detected using DNA-based molecular methods. Environmental rDNA sequences, in principle, are also used to infer some properties of the organisms that they represent. For example, if a sequence is related to those of a group of cultivated organisms with common properties, then the environmental organism represented only by the sequence also is expected to have those properties (Dojka et al., 1998). Sequences greater than 97% identity are typically assigned to the same species while those with

>95% identity to the same genus, and those with >80% identity to the same phylum (Stackebrandt and Goebel, 1994; Bond et al., 1995; Borneman and Triplett, 1997; Hugenholtz et al., 1998; Everett et al., 1999; McCaig et al., 1999; Sait et al., 2002).

A number of molecular microbial diversity studies have reported diversity of the bacteria in various environments using conventional sequencing of 16S rRNA genes, (Liesack and Stackebrandt, 1992; Stackebrandt et al., 1993; Borneman et al., 1996; Borneman and Triplett, 1997; Kuske et al., 1997; Ludwig et al., 1997; Hansen et al., 2007; Fuhrman and Hagström, 2008). Recently, massively parallel pyrosequencing of amplicons from the V6 hypervariable regions of small-subunit (SSU) ribosomal RNA (rRNA) genes is commonly used to assess microbial diversity and richness (Cristea-Fernstrom et al., 2007; Huse et al., 2007; Liu et al., 2007; Roesch et al., 2007; Sundquist et al., 2007; Dowd et al., 2008)

The high-throughput technology of pyrosequencing (Margulies et al., 2005) is a cost-effective alternative to traditional sequencing methods especially for 16S rRNA-based microbial diversity studies as well as metagenomic studies (Sogin et al., 2006; Huber et al., 2007; Roesch et al., 2007). The 454 pyrosequencing has not only revealed bacterial community structures in the environment but has also provided a platform for researchers to examine the rare phylotypes (Margulies et al., 2005; Sogin et al., 2006; Huber et al., 2007; Roesch et al., 2007). Pyrosequencing is a DNA based sequencing technique. In this technique, the release of pyrophosphate (PPi) during DNA synthesis is detected. The PPi which is released is converted to ATP by ATP sulfurylase. This conversion provides energy to luciferase to oxidize luciferin. Light is generated in this reaction that is proportional to the number of incorporated nucleotides. Thus the sequence of the template can be determined by the number of added nucleotides known by performing hundreds of thousands of these reactions in parallel (Ronaghi, 2001).

7.2. Results

7.2.1. Preliminary analysis of the data

16S rRNA gene fragments (tags) from microbial communities in Kalbadevi beach and bay sediments were examined. Sequences occurring only once in the whole dataset i.e. singletons were removed. Consequently only the remaining tags were classified. There was an average of 15,121 tags after the elimination of incorrectly identified sequences or sequences of poor quality. Thus the number of different bacterial sequences ("ribotypes") analyzed here averaged at 2593 per sample (Table 7.1). The average read length of the sequences was 60.7 (±3.88).

7.2.2. Bacterial diversity in Berm-Beach and Bay sediments

Diversity of bacterial community in berm sediments was compared with that in the bay sediments. Rarefaction curve for the S2 bay sediments was higher than the S1 berm sediment (Fig 7.1). The non-parametric richness indices such as Chao1 and ACE, calculated at 3% dissimilarity, showed higher Ace values in predicting number of OTUs for each system. Using two estimators of diversity, the maximum number of unique sequences of operational taxonomic units (OTU, roughly corresponding to the species level) did not exceed 2200 in the Kalbadevi sediments at the 3% dissimilarity level (Table 7.2). Use of most common estimators showed that the bacterial diversity in the bay sediments was greater than the berm.

Table 7.1: Data summary of the pyrosequenced sediment from Kalbadevi region.

Sample ID	**S1**	**S2**
Sample location	**Berm, Beach**	**Bay**
DNA concentration recovered (ng/µL)	6.0	4.3
Total reads	18899	19898
Trimmed tags	14893	15349
Ribotypes	2052	3134

* Trimmed tags are the numbers of reads remaining after the removal of singletons. Unique tags are the numbers of distinct sequences within a set of trimmed tags Ribotypes ~ Phylotypes

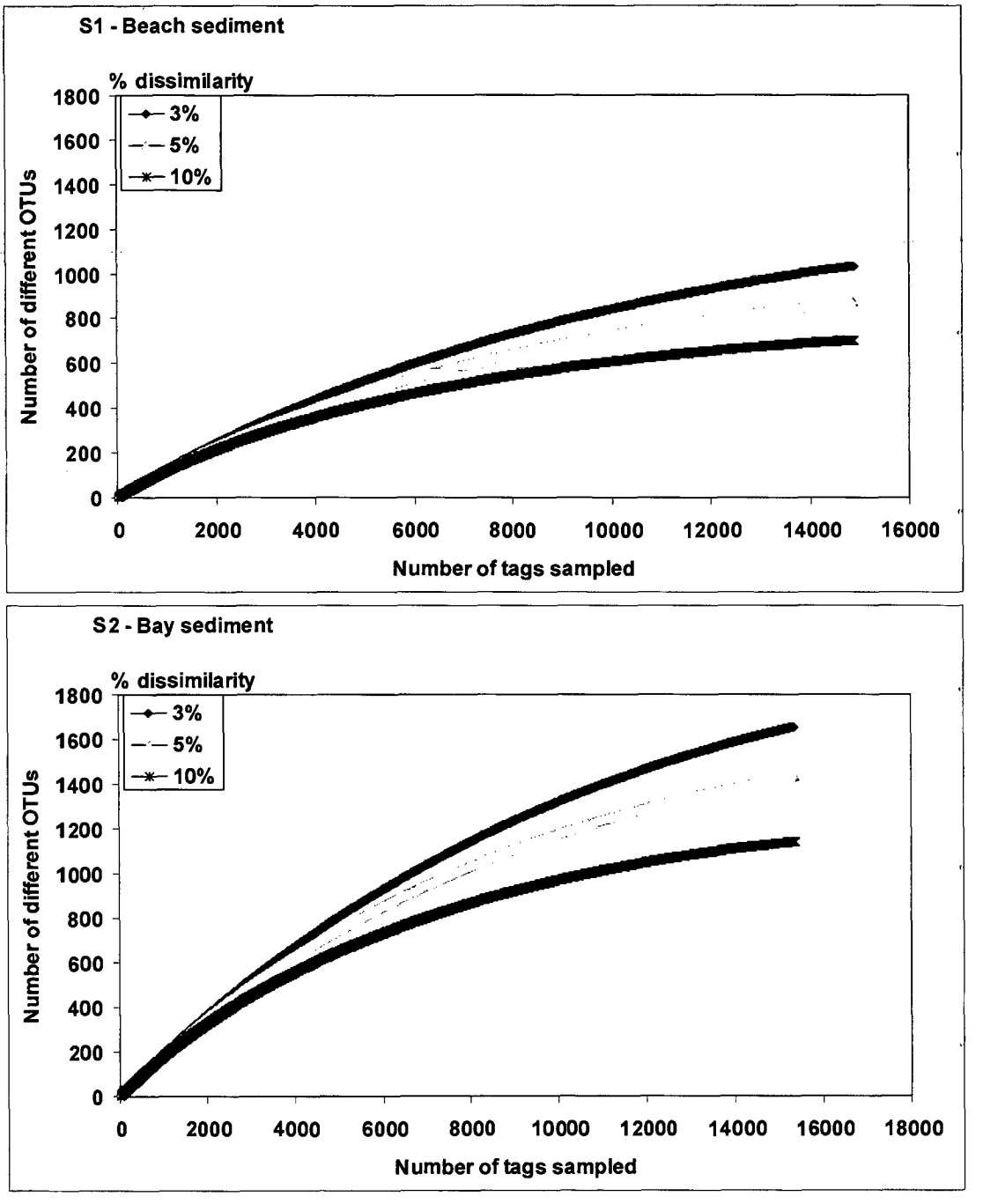


Fig 7.1: Rarefaction curves for the two bacterial communities.

Table 7.2: Similarity-based OTUs and species richness estimates.

Sample ID	Reads	Cluster distance based on dissimilarly								
		3%			5%			10%		
		OTU	ACE	Chao1	OTU	ACE	Chao1	OTU	ACE	Chao1
Berm	14893	1035	1410	1283	872	1101	1018	704	839	774
Bay	15349	1655	2199	2014	1434	1801	1646	1142	1360	1246

*The species richness estimates were determined by using the program DOTUR

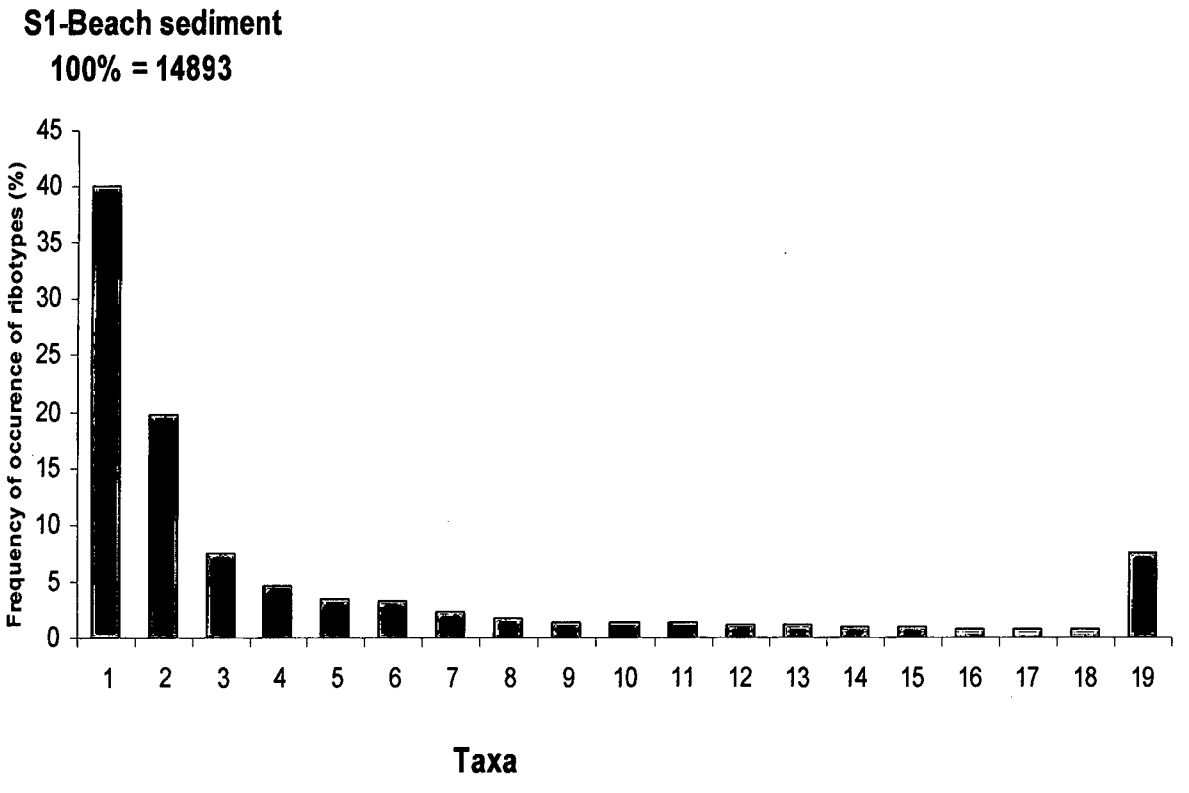
OTU – Operational taxonomic unit

ACE – Abundance based coverage estimator

7.2.3. Variation based on total tag abundance

Ribotypes in the beach sediments were identified and affiliated to 22 phyla and 63 orders. Predominant phyla were Firmicutes (41%), Actinobacteria (31%), Proteobacteria (13%) and Acidobacteria (6%). An unknown phylum formed nearly 3%. Orders which comprised of tags occurring more than 100 times were placed under major order (Fig 7.2) while those that occurred less than 100 times were placed under minor orders (Table 7AT1 and 7AT2, Appendix III). The minor order comprised nearly 7% of the 14,893 tags found in beach sediment (Table 7AT1, Appendix III). Within Firmicutes which occurred at a frequency of 41% of the total tag abundance, nearly 98% of these tags were affiliated to the order Bacillales. Other orders in the phylum Firmicutes were <1.5%. Among the tag abundance of Actinobacteria, Actinomycetales comprised nearly 63% followed by Rubrobacterales (23.8%) and an unknown order (11%) (Fig 7.2).

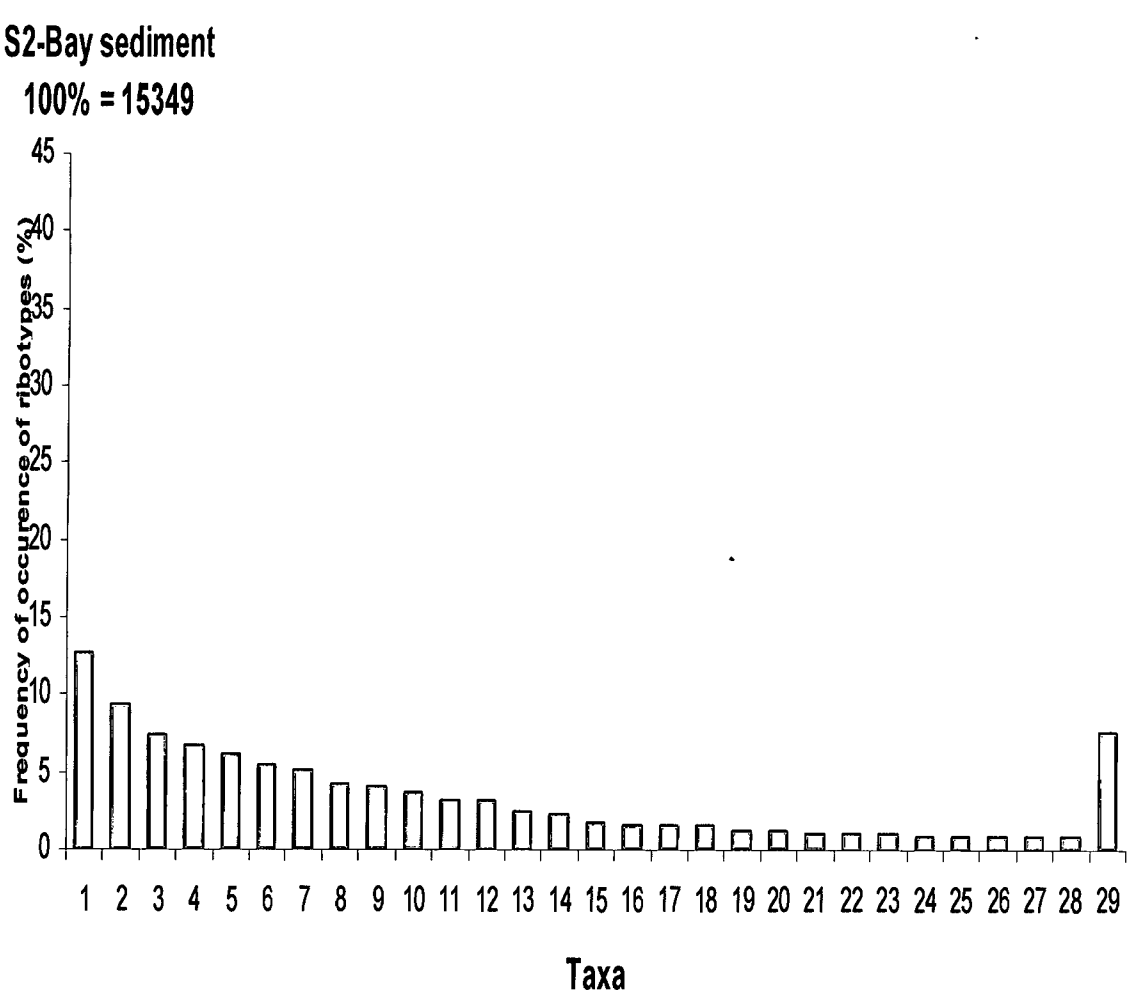
On the other hand, the ribotypes in the bay sediments mainly comprised of 30 phyla belonging to 87 orders. In the bay sediments, Proteobacteria sequences represented 44%, Actinobacteria 14%, Acidobacteria 11% and an unknown phyla 7%. Firmicutes comprised only 0.5% in these sediments. Similar to the beach sediments, distinction was made between major and minor orders (Fig 7.3 and Table 7AT2, Appendix III). Like the beach sediments, only 7% of the 15,349 tags occurred < 100times. α-, β-, γ-, δ-, and ε-Proteobacteria class



Key

1 Firmicutes Bacilli Bacillales
2 Actinobacteria Actinobacteria Actinomycetales
3 Actinobacteria Actinobacteria Rubrobacterales
4 Acidobacteria Acidobacteria Acidobacteriales
5 Actinobacteria Actinobacteria unk
6 Unknown unk unk
7 Proteobacteria Alphaproteobacteria Rhizobiales
Proteobacteria Betaproteobacteria
8 Burkholderiales
Proteobacteria Deltaproteobacteria
9 Myxococcales
10 Proteobacteria Betaproteobacteria unk
11 Proteobacteria unk unk
Gemmatimonadetes Gemmatimonadetes
12 Gemmatimonadales
13 Proteobacteria Deltaproteobacteria unk
Planctomycetes Planctomycetacia
14 Planctomycetales
15 Chloroflexi Anaerolineae unk
Proteobacteria Alphaproteobacteria
16 Rhodospirillales
Bacteroidetes Sphingobacteria
17 Sphingobacteriales
18 Acidobacteria unk unk
19 Others

Fig 7.2. Taxonomic breakdown of bacterial V6 from the two locations. S1- berm-beach sediments. Columns show the Phylum Class Order distribution for taxonomically assigned tags that occurred more than 100 times, the remaining tag sequences are grouped into "Other" and presented in table 7AT1 (appendix III). unk - unknown



Key

1 Proteobacteria Gammaproteobacteria unk
2 Acidobacteria Acidobacteria Acidobacteriales
3 Actinobacteria Actinobacteria unk
4 Unknown unk unk
5 Proteobacteria Deltaproteobacteria Desulfobacterales
6 Proteobacteria unk unk
7 Actinobacteria Actinobacteria Actinomycetales
8 Proteobacteria Gammaproteobacteria Chromatiales
9 Proteobacteria Deltaproteobacteria unk
10 Proteobacteria Alphaproteobacteria Rhizobiales
11 Planctomycetes Planctomycetacia Planctomycetales
12 Firmicutes Clostridia Clostridiales
13 Proteobacteria Alphaproteobacteria Rhodobacterales
14 Gemmatimonadetes unk unk
15 Acidobacteria unk unk
16 Verrucomicrobia Verrucomicrobiae Verrucomicrobiales
17 Actinobacteria Actinobacteria Rubrobacterales
18 Bacteroidetes Flavobacteria Flavobacteriales
19 Proteobacteria Deltaproteobacteria Myxococcales
20 Nitrospira Nitrospira Nitrospirales
21 WS3 unk unk
22 Deferribacteres Deferribacteres Deferribacterales
23 Proteobacteria Deltaproteobacteria Desulfuromonadales
24 Chloroflexi Anaerolineae unk
25 Proteobacteria Alphaproteobacteria unk
26 Bacteroidetes Sphingobacteria Sphingobacteriales
27 Proteobacteria Deltaproteobacteria Syntrophobacterales
28 Chloroflexi unk unk
29 Others

Fig 7.3. Taxonomic breakdown of bacterial V6 from the two locations. S2- bay sediments. Columns show the Phylum Class Order distribution for taxonomically assigned tags that occurred more than 100 times, the remaining tag sequences are grouped into "Other" and presented in table 7AT2 (appendix III)

unk - unknown

represented 17, 0.7, 40, 30 and 0.3% Proteobacteria phylum respectively. An unknown order comprised nearly 29% of γ-Proteobacteria tag abundance while 14% tag abundance of δ-Proteobacteria was made up of Desulfobacterales. The orders Rhizobiales and Rhodobacterales comprised nearly 6 and 8% respectively of the α-Proteobacteria tag abundance. Among the 14% Actinobacteria, nearly half of this tag abundance was affiliated to unknown order, followed by Actinomycetales (36%) and Rubrobacterales (11%) (Fig 7.2). With the exception of WS1, the ribotypes such as BRC1, WS1, WS3, OP3, OP5, OP8, OD1, OD10, OD11, TM6, TM7, having no phylogenetic association with known taxonomic divisions occurred <1% of the total tag abundance at both the sites.

7.2.4. Variation based on ribotype abundance

The number of clusters at ≥97% similarity for the beach sample was 1035 while the bay sediments recorded 1655. The 20 most abundant clusters in the beach and bay sediments are given in table 7.3 and 7.4.

7.3. Discussion

Comparing the diversity of the bacteria in bay and beach sediments using the rarefaction curve indicated that there was a systematic difference in diversity between the two areas.

Firmicutes dominated the beach sediments while Proteobacteria dominated the bay sediments of Kalbadevi. Study by de Castro and Ehrlich (1970) has suggested that *Bacilli* spp are involved in the solubilizing large amounts of iron from limonite, goethite and hematite. Nishio and Ishida (1989) while studying the culturable diversity of bacteria from sediments of a lagoon found that the predominant iron solubilizing bacteria were affiliated to *Bacilli*. The presence of Actinobacteria and Firmicutes in the beach sediments suggests that the bacteria are involved in the decomposition and humus formation.

Table 7.3: Phylogenetic classification of the twenty most abundant >97% clusters in the Kalbadevi beach sediments.

Sr. No.	Domain	Phylum	Class	Order	Family	Genus	Frequency
1	Bacteria						2.32
2	Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Rhodothermaceae	*Salinibacter*	1.45
3	Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	*Gemmata*	1.26
4	Bacteria						0.97
5	Bacteria						0.97
6	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		0.87
7	Bacteria	Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae	*Gp4*	0.87
8	Bacteria						0.77
9	Bacteria						0.77
10	Bacteria	Acidobacteria	Acidobacteria				0.77
11	Bacteria						0.68
12	Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Sphingobacteriaceae	*Pedobacter*	0.68
13	Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	*Gemmata*	0.68
14	Bacteria						0.68
15	Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	*Gemmata*	0.58
16	Bacteria						0.58
17	Bacteria	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	*Gemmata*	0.58
18	Bacteria	Chloroflexi	Anaerolineae				0.58
19	Bacteria	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	*Sorangium*	0.58
20	Bacteria	Chloroflexi	Anaerolineae				0.58

Table 7.4: Phylogenetic classification of the twenty most abundant >97% clusters in the Kalbadevi bay sediments.

Sr. No.	Domain	Phylum	Class	Order	Family	Genus	Frequency
1	Bacteria	Proteobacteria					0.60
2	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales			0.60
3	Bacteria	Proteobacteria					0.60
4	Bacteria	Actinobacteria	Actinobacteria				0.60
5	Bacteria	Proteobacteria					0.54
6	Bacteria	Proteobacteria					0.48
7	Bacteria	Proteobacteria					0.48
8	Bacteria	Actinobacteria	Actinobacteria				0.42
9	Bacteria	Proteobacteria					0.42
10	Bacteria	Actinobacteria	Actinobacteria				0.42
11	Bacteria	Proteobacteria					0.36
12	Bacteria	Actinobacteria	Actinobacteria				0.36
13	Bacteria	Proteobacteria					0.36
14	Bacteria	Actinobacteria	Actinobacteria				0.36
15	Bacteria	Proteobacteria	Deltaproteobacteria				0.36
16	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	*Hyphomicrobium*	0.36
17	Bacteria	Proteobacteria	Deltaproteobacteria				0.36
18	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	*Hyphomicrobium*	0.36
19	Bacteria	Proteobacteria	Deltaproteobacteria				0.36
20	Bacteria	Actinobacteria	Actinobacteria				0.36

The frequency of occurrence of the order Actinomycetales of the phylum Actinobacteria is 20% of the total ribotypes in the beach and 5% in bay sediments. Presence of this group clearly indicates that these organisms may be involved in the transformation of the organic matter as well as in the dissolution of minerals present. The key role of Actinomycetes in degrading complex substrates is well known. It has been suggested that the higher abundance of Actinobacteria indicates that these bacteria are involved in the decomposition of organic materials, such as cellulose and chitin, thereby playing a vital part in organic matter turnover and carbon cycling (Acosta-Martínez et al., 2008). The phylum Actinobacteria is comprised of Gram-positive bacteria with an overall high mol% G+C content. Other than the soil habitat, Actinobacteria also inhabit aquatic systems, extreme environments such as acidic thermal springs (Mohagheghi et al., 1986), Antarctic regolith (Meys et al., 2000), gamma (Phillips et al., 2002) and UV irradiated biotopes (Warnecke et al., 2005). This ability to inhabit various environments probably defines the capability to sense and adapt to a wide range of metals. To survive in these environments the ferric uptake regulator (Fur) may be important (Santos et al., 2008). According to Gremion et al. (2003), Rubrobacter of the Actinobacteria phylum were predominant in heavy metal contaminated bulk and rhizosphere soil. They further demonstrated that this group formed the dominant part of metabolically active bacteria. Further, the order Rubrobacterales which accounts for 7% of the total ribotypes in the beach sediments may also be involved in the iron oxidation. Study by Bryan and Johnson (2008) showed that an isolate Pa33, a novel iron-oxidizing acidophilic actinobacterium that was isolated from spoil material of an abandoned copper mine was involved in ferrous iron oxidation. Presence of the Actinobacteria and Firmicutes also indicates the possibility of nitrophenol, nitroaromatic compound, pesticide and herbicide degradation (Chaudhuri and Thakur, 2006). The Kalbadevi beach is near to agricultural fields where the use of such pesticides and herbicides could be common.

The Kalbadevi bay sediments had high abundance of Proteobacteria ribotypes which accounted for nearly 44% of the total tag abundance. Proteobacteria are usually thought to be involved in bioremediation of heavy metals from solid as well as liquid sources, degradation and recycling of woody tissue of plants and biodegradation of oil-contaminated soil and toxic compounds as well as in nitrogen fixation along with the Cyanobacteria (Chaudhuri and Thakur, 2006).

The Kalbadevi beach sediments have pH 6.5-7.0 while the bay sediments had pH 7.5-8.0. It was surprising to note that Acidobacteria ribotypes formed 5% and 11% of all ribotypes in the beach and bay sediments respectively. Acidobacteria are usually found to dominate low pH (<5.5) soils, while Proteobacteria dominated high pH soils (Mannisto et al., 2007). Generally, Acidobacteria phylum members are usually encountered in soils and sediments. However, this phylum is associated with the ability to withstand metal-contaminated, acidic and other extreme environments (Barns et al., 1999; Barns et al., 2007).

Thus the 454 pyrosequencing technique gave a holistic understanding of bacterial diversity in these iron rich systems.

Chapter 8

Chapter 8: Delineation of the influence of sand mining on the bacterial responses in placer rich Kalbadevi sediments (Beach and Bay)

8.1. Introduction

Bacteria are known to respond instantaneously to changes in environmental conditions. Their abundance, ubiquity, small size and rapid turnover time play an important role in environmental monitoring (Danovaro et al., 1993; Fabiano and Danovaro, 1994). This chapter elaborates the response of bacteria and biochemical parameters to simulated sand mining. It monitors the impact of mining on intertidal beach sediment, coastal sediments and bay water.

8.2. Result

8.2.1. Simulated disturbance on the beach

The results of the present studies are restricted to three phases, pre-disturbance (phase I, PI), immediately after disturbance (phase II, PII) and 24 hours after disturbance (phase III, PIII). Results presented here are averaged for the 40 cm core at each tidal level.

8.2.1.1. Direct total bacterial counts (TC)

The distribution of TC along the beach transect is shown in figure 8.1a. The abundance within the sediment ranged over an order of magnitude (10^5 to 10^6 cells g^{-1}) during the baseline observations. On an average, Stn B sediment recorded the highest abundance ($2.90 \pm 1.83 \times 10^6$ cells g^{-1}) while Stn M recorded an order less. The average population declined immediately after disturbance by nearly an order from 1.99×10^6 cells g^{-1} to 0.45×10^6 cells g^{-1}. This decreasing trend in abundance did not seem to revert to original numbers even after 24 hours and continued to be 85% lower than the baseline values.

8.2.1.2. Direct total viable counts (TVC)

Both types of viability yielded 10^5 cells g^{-1} and followed the trend of total bacterial abundance (Fig. 8.1 b and c). Overall, TVCa ranged from $0.18 \pm 0.21 \times 10^6$ cells g^{-1} at Stn H to $0.99 \pm 1.03 \times 10^6$ cells g^{-1} at Stn L. Likewise,

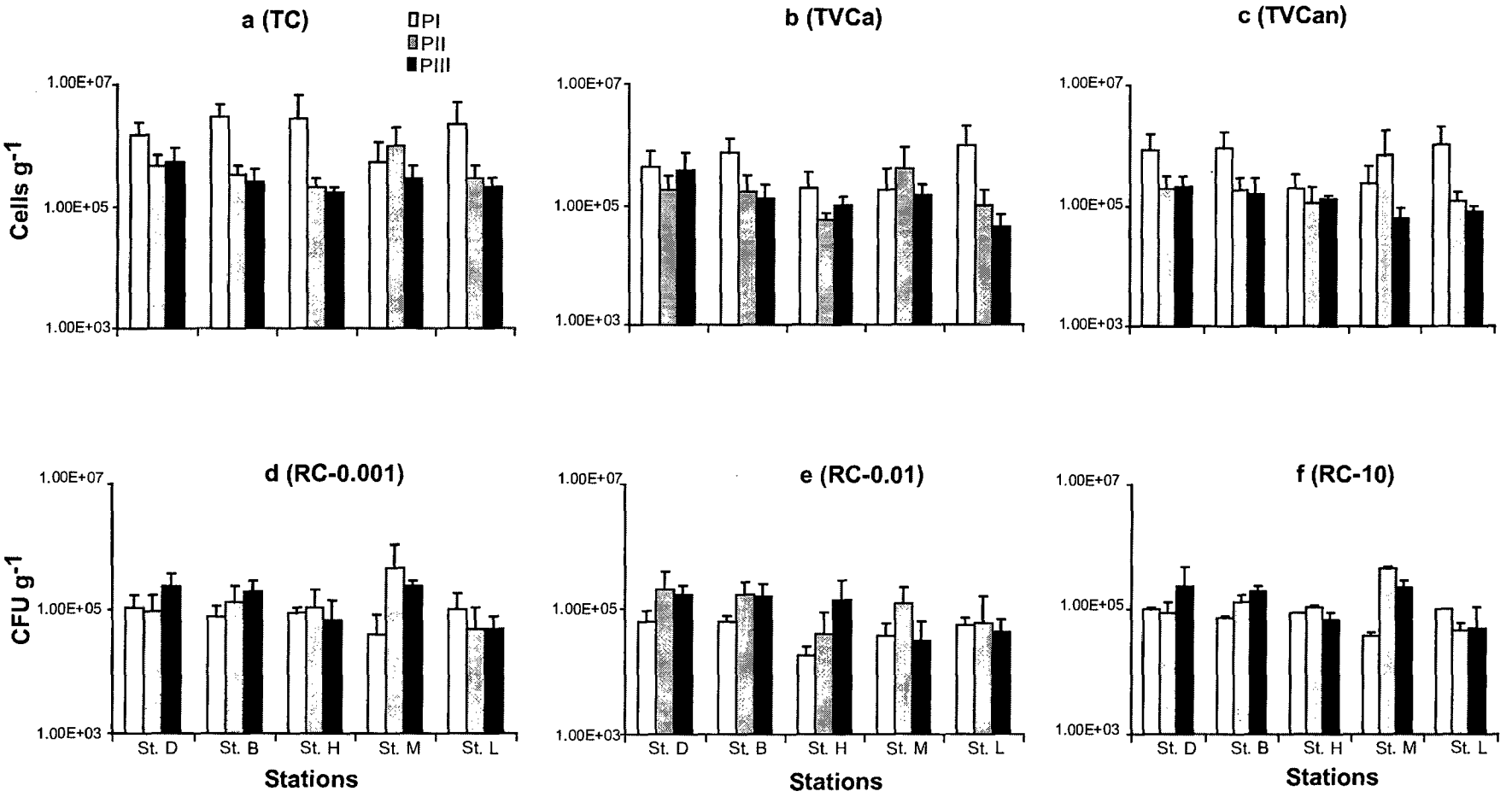


Fig 8.1: Variation in (a) TC, (b) TVCa, (c) TVCan, (d) RC-0.001, (e) RC-0.01 and (f) RC-10 (f) during Phase

PI - Pre-disturbance

PII - Immediately after disturbance

PIII - 24 hours after disturbance

higher TVCan population of 1.00 ± 1.02 x 10^6 cells g^{-1} was recorded at Stn L. With disturbance, the average viabilities of the 40 cm cores decreased by 60%. However, the anaerobic viability decreased by 79% after 24 h.

8.2.1.3. Retrievable counts of heterotrophs (RC)

Maximum retrievability of 10^5 CFU g^{-1} was recorded at Stn D. Generally, bacteria forming the culturable fraction retrieved on the three nutrient broth concentrations (NB) of 10, 0.01 and 0.001% also followed the trend of TC (Fig. 8.1 d, e, f). Prior to disturbance, the average retrievability of RC-10 population was 0.42 ± 0.28 x 10^4 CFU g^{-1}, RC-0.01 was 4.73 ± 1.87 x 10^4 CFU g^{-1}, and RC-0.001 was 7.99 ± 4.60 x 10^4 CFU g^{-1}. This fraction of retrievability accounted for 0.2 to 4% of TC. However, with disturbance there was an improvement in culturability. The RC fraction increased by 66, 34 and 47% on 10, 0.01 and 0.001% NB respectively (Table 8.1).

8.2.1.4. Biochemical parameters of the sediment

In the present study, the ATP values were generally elevated at Stn D and Stn B, with still higher values at Stn H (222 ± 60 ng g^{-1}). Immediately after disturbance, these ATP values tended to increase by about 45% at Stn D, Stn B and Stn M region followed by a drastic decrease by 90% after 24 h (Fig. 8.2a). Carbohydrate generally decreased towards the surf zone and its concentration in the sediment varied from 230 ± 164 μg g^{-1} at Stn H to 917 ± 660 μg g^{-1} at Stn D (Fig. 8.2b). The immediate response to disturbance was an increase by 123 % at Stn H and a decrease of 66% at Stn L. The 24 h response was mixed, with the concentration increasing by 37% at Stn B and decreasing by 42% at the Stn M suggesting variable rates of replenishment and degradation. Unlike the distribution in carbohydrates, the lipid concentration varied irregularly. The Stn M had the lowest sediment lipid concentration of 44 ± 15 μg g^{-1} while the highest of 93 ± 43 μg g^{-1} was found at Stn D (Fig 8.2c). Like the other constituents of LOM, lipids also exhibited a varied response during phase III. It decreased by 8% at Stn D while at Stn L it increased by 139%. Like carbohydrates, the protein content ranged between 39 ± 26 μg g^{-1} at Stn M to 438 ± 352 μg g^{1} at Stn B. The concentration at Stn D and Stn B sediments were higher than at the other tidal levels (Fig. 8.2d).

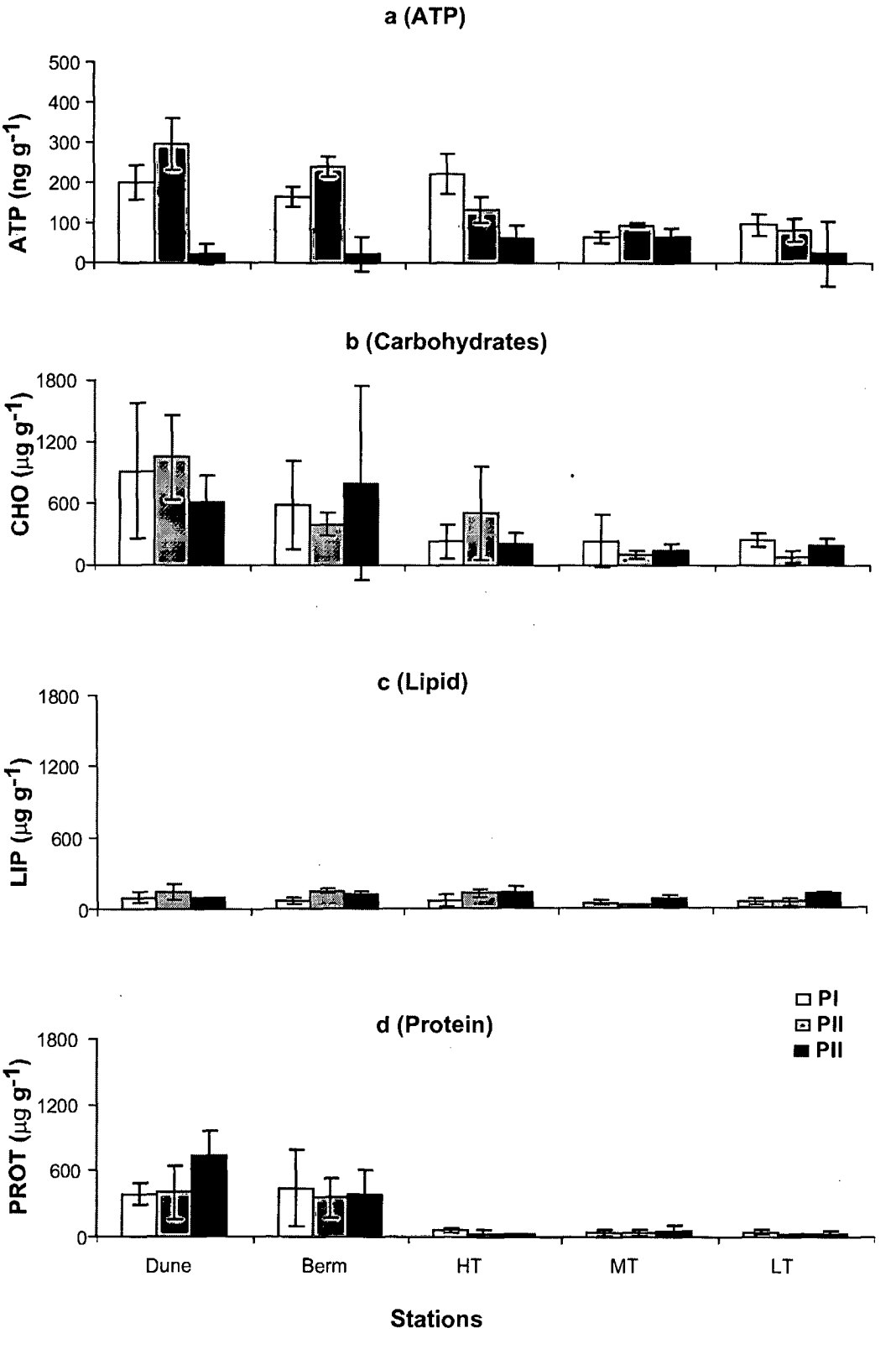


Fig 8.2: Variation of (a) ATP, (b) Carbohydrates, (c) Lipid and (d) Protein during phase PI - Pre-disturbance
PII - Immediately after disturbance
PIII - 24 hours after disturbance

The protein concentration decreased by 60% at Stn H and increased by 90% at Stn D after 24 h. During phase I, carbohydrates contributed 62%, protein 24% and lipid 14% to sedimentary LOM. Major changes in LOM are not discernible during phase II. However, after 24 h, protein and lipid contribution increased to 25 and 24% while carbohydrates decreased to 52%.

Table 8.1: Change in the percentage contribution of different bacterial parameters to total bacterial abundance during different phases of simulated mining at Kalbadevi beach.

Phase	TC	Contribution to TC (%)				
	(x 10^5 cells g^{-1})	TVCa	TVCan	RC-0.001	RC-0.01	RC-10
PI	19.9	25	32	4.0	2.4	0.2
PII	4.53	40	57	36	27	14
PIII	2.97	52	44	51	36	66

*Phase I – Pre-disturbance
II – Immediately after disturbance
III – 24 hours after disturbance

8.2.1.5. Evolution of interrelationship during different phases of disturbance

Examination of interrelationships between bacterial and biochemical parameters suggest the extent of interdependence between them. Though these relationships were few and weak before disturbance, they were more significant after 24 h when the transect was examined as a whole (Table 8AT1, Appendix III)

8.2.2. Simulated disturbance in the bay

The result of the present study is restricted to three phases, 3 hours pre-disturbance (PD), during disturbance (DD) and monitoring 2 hours after disturbance (MM).

8.2.2.1. Direct total bacterial counts (TC)

The average TC in the sediment was 1.71(±0.37) x 10^7 cells g^{-1} during PD. The TC increased by 70 and 21% at input and output point respectively during MM phase (Table 8.2). Unlike the sediment, the TC in the water column

ranged over an order from 10^6 to 10^7 cells mL^{-1} during PD. However, the disturbance had variable influence on the water column. It increased at the input point by 14% while at the output point it decreased by 54% (Table 8.3).

Table 8.2: Overview of the change in bacterial and biochemical parameters in the offshore Kalbadevi bay sediment after 2h of simulated mining.

Parameter	Suction (S)	Discharge (D)
TC (x10^7 cells g^{-1})	↑70% (1.41)	↑21% (2.07)
TVCa (x10^7 cells g^{-1})	↑24% (0.8)	↑389% (0.18)
TVCan (x10^7 cells g^{-1})	↓ 9% (0.42)	↓0.8% (0.17)
RC-10 (x10^7 CFU g^{-1})	↓98% (0.07)	↑286% (0.09)
CHO (μg g^{-1})	↑12% (48)	↑44% (78)
LIP (μg g^{-1})	↓17% (127)	↑72% (26)
PROT (μg g^{-1})	↑17% (108)	↓5% (205)

* Numbers in parentheses are initial values.

Table 8.3: Overview of the change in bacterial parameters in the water column after 2h of offshore simulated mining in Kalbadevi bay.

Parameter	Surface (1mbsf)		Sub surface (3mbsf)	
	Suction (S)	Discharge (D)	Suction (S)	Discharge (D)
TC	↑26%	↓23%	↑1%	↓86%
(x10^7 cells mL^{-1})	(0.85)	(0.96)	(1.41)	(1.40)
TVCa	↓53%	↓91%	↓69%	↓75%
(x10^7 cells mL^{-1})	(0.18)	(0.22)	(0.01)	(0.02)
TVCan	↑12%	↓57%	↓79%	↓92%
(x10^7 cells mL^{-1})	(0.03)	(0.03)	(0.01)	(0.02)
RC	↓71%	↓94%	↓56%	↓99%
(x10^7 CFU mL^{-1})	(0.007)	(0.003)	(0.06)	(0.03)

* Numbers in parentheses are initial values.

8.2.2.2. Direct total viable counts (TVC)

During PD, the average TVCan in the sediment was 3.24(±1.32) x 10^6 cells g^{-1} and decreased marginally at input and output point during MM phase (Table 8.2). Generally the TVCa population in the water column was 10^6 cells mL^{-1}

during PD. With disturbance, the TVCa population decreased over 50% at all the sampling points (Table 8.3). The average TVCa in the sediment was 7.29(±5.16) $x10^6$ cells g^{-1} before disturbance and increased by 24 and 389% at input and output point respectively during MM (Table 8.3). The TVCan population in the water column varied from 10^{5-6} cells mL^{-1} during baseline observations. However, during phase MM, the TVCan population behaved like the aerobic population and reduced over 50% with the exception of input point at 1 m where TVCan increased by 12% (Table 8.3).

8.2.2.3. Retrievable counts of heterotrophs (RC)

The RC population in the sediment was 5.82(±4.17) $x10^5$ cells g^{-1} during phase PD. The population decreased by 98% at input point while it increased nearly 4 times at output point during phase MM (Table 8.2). During PD phase, the RC population in the water column ranged from 10^{4-5} cells mL^{-1}. Like the other bacterial parameters, RC population also decreased during phase MM (Table 8.3).

8.2.2.4. Biochemical parameters of the sediment

Generally, sedimentary carbohydrates recorded an increase after the disturbance experiment. The discharge point was more affected than the others and recorded an increase by 44% from initial 77 $\mu g\ g^{-1}$. Lipids in the sediment decreased by 17% from 127 $\mu g\ g^{-1}$ at suction point and increased by 72% from 26 $\mu g\ g^{-1}$ at discharge point. At PD, the sedimentary protein at suction point was 108 $\mu g\ g^{-1}$ and at discharge point it was 205 $\mu g\ g^{-1}$. After MM, the concentration increased at suction point but decreased by 5% at discharge point (Table 8.2).

8.3. Discussion

The study of benthic communities is useful and sensitive tool for identifying sediment-related stress (Alongi, 1990). The analysis of changes in benthic community structure has now become one of the main methods of detecting and monitoring the biological effects of marine disturbance (Chou et al., 2004). Bacteria could perhaps act as one of the component to identify this stress as these organisms are ubiquitous in their distribution and have short

generation times. In addition, they are sensitive to changes in the physical, chemical and other biological parameters of the environment.

Biochemical parameters like sedimentary protein concentration in the berm region as well as the carbohydrate concentration in the dune were generally high. Naturally, higher sedimentary protein concentration in the berm region could not only be due to the greater abundance of bacteria but also due to the proteinaceous exudates that could emanate from the roots of the existing vegetation (Hertenberger et al., 2002). A similar reason could also be attributed to the maximum carbohydrate concentration at the dune. Vascular plants are known to contribute to sedimentary carbohydrates (Bhosle and Dhople, 1988). Following disturbance, the sedimentary protein, lipid and carbohydrate patterns are clearly disturbed. Change in protein, carbohydrates and lipid concentration at the dune and the berm may be due to the redistribution from the deeper layers to the surface sediments. The increased sedimentary protein concentration during phase III suggests increased bacterial activity due to death or decay of other organisms. The increase at high tide is attributed to higher concentrations of carbohydrates, which surface due to mechanical disturbance. Disturbance improved the sediment lipid concentration. However, the homogenous distribution even after disturbance in the intertidal area may be due to the masking effect by the tidal and wave action. It is well known that strong hydrodynamism permits deposition of coarse sediments and this allows water to run easily thus preventing the accumulation of organic matter (Langezaal et al. 2003). In this study, there is a general decrease in labile organic matter with depth and from the dune to the low tide level. Labile organic matter (LOM) which is derived from the sum of protein, carbohydrates and lipids gives the holistic status of the nutrient of the sediment. During phase I, carbohydrates, proteins and lipids contributed significantly to LOM. Percentage contribution was 62% CHO ($r = 0.969$, $p \leq 0.001$) > 24% PROT ($r = 0.864$, $p \leq 0.001$) > 14% LIP ($r = 0.667$, $p \leq 0.001$). Thus, most of the LOM was dominated by carbohydrates through the whole transect. Studies on preferential utilization of carbohydrate, protein and lipid - has shown that the latter compounds are more rapidly recycled (Pusceddu et

al., 1999). However in the present study area, the high background carbohydrate contribution of 62% ($r = 0.969$, $p \leq 0.001$) to LOM could be attributed to the sand dune vegetation and it is also suggested that the input rate of this substrate is greater than its degradation rate. During phase III, protein contribution increased to 25% while carbohydrates decreased to 51% indicating an increase in the newly generated organic matter. This was corroborated by the increase in protein/carbohydrate ratio (PROT/CHO). PROT/CHO ratio also increased from 0.39 to 0.48 within 24 h of disturbance suggesting availability of newly generated detritus (Dell'Anno et al., 2000). As proteins are more readily utilized by bacteria than carbohydrates, a high PROT/CHO ratio indicated the generation of recently produced organic matter. Further, the background ratio of 0.39 also indicated that the ecosystem continuously generated detritus. Biochemical characteristic like ATP act as a proxy for total living biomass and is a central compound in the energy metabolism of all living organisms. It is generated by energy-yielding reactions and is subsequently consumed in energy-requiring reactions in the cell. Upon cell death, production of ATP is arrested and is rapidly degraded (Vosjan et al. 1987). Hence, ATP measurements have been used in various marine environments (Holm-Hansen and Booth, 1966), including sediments (Karl and LaRock, 1975; Stoeck and Duineveld, 2000) to understand its distribution and dynamics. In the present study, the ATP values were generally elevated at the dune and berm, with still higher values in the high tide sediments (222 ± 60 ng g^{-1}). This could be because of the entrapment of small meiobenthic/zooplankton organisms at this level. Reichgott and Stevenson (1978) have shown that copepods are major contributors to sediment ATP. Immediately after disturbance, these values tended to increase by about 45% at dune, berm and mid tide region and fall drastically by 75% after 24 h. Immediate rise in ATP could be due to instantaneous microbial stimulation by factors such as LOM only to be followed by drastic inhibition in 24 h. In the present study, influence of LOM on ATP variability evolved from non significant to a significant 63% ($p \leq 0.01$, $n=60$) variation during phase II.

The natural abundance of sedimentary bacteria of Kalbadevi beach was generally 10^6 cells g^{-1} and decreased with depth ($p \leq 0.05$). This abundance is generally an order less than reported elsewhere (Luna et al., 2002). High concentration of placer minerals and the associated vegetation comprising of *Ipomoea pes-caparae* shrubs in the berm and *Casuarina* tree plantation in the dune may influence high numbers at certain locations on the beach. However, the total bacterial load did not show any relationship to the biochemical variables and LOM, thus suggesting that these were not the only influencing factors. Similar results were also obtained in deep-sea sediments of Central Indian Ocean Basin (Nair et al., 2000). Interactions with the environment as well as the distribution of resident bacteria could be affected by any mechanical disturbance. However, microorganisms are known to respond quickly especially to the changes in the biotic and abiotic factors in the sediment. Simulated disturbance at the study site generally decreased the bacterial population immediately after disturbance. In addition, the depth wise distribution patterns were also disturbed ($p > 0.05$). This decrease could perhaps be due to the mechanical displacement of sediments where manual sieving and mixing of the sediment mixed regions harboring lower and higher bacterial abundance. Kaneko et al., (1995) also observed a decrease in the Pacific Ocean sediments while conducting such artificial disturbance experiment. The results of the Indian Ocean Deep-sea Experiment (INDEX) are also in accordance with the above findings (Nair et al., 2000; Fernandes et al., 2005; Loka Bharathi and Nair, 2005). Earlier, laboratory scale experiments conducted by Findlay et al, (1990) with intertidal sediments of Florida also strengthen our observations. However, in this study the depthwise distribution perhaps restored to original trend within 24 h ($p \leq 0.05$). Though the above findings relate to all the tidal levels, the mid tide region behaved differently. This difference in behavior could be attributed to the concentration of both meio and macrofauna at this level (Anon, 2005). Bacterial population increased and then decreased thus varying over an order within a period of 24 h after disturbance. This rhythmic rise and fall in numbers could be due to bioturbation and feeding by benthic fauna (Boucher and Chamroux, 1976; Moreno et al., 2006). The low bacterial abundance

during pre-disturbance perhaps synchronizes with such a feeding and therefore the low numbers in this phase. With disturbance, there is a likelihood of mechanical displacement of the benthic animals disrupting the trophic chain. Such a decrease from an initially higher level in the meiofaunal population to a lower level following disturbance has been observed (Anon, 2005). It is perhaps for this reason that the bacterial population responded differently with an increase at the mid tide.

It is known that bacteria have rapid growth rates and individual species within functional groups often have differing responses to changing environments (Torsvick et al., 1996). Thus any change in viability or culturability could be an indicator of this response. High viability under both aerobic and anaerobic conditions was encountered at the berm, dune and the low tide region. This high numbers could be attributed to the influence of the plant exudates while that recorded at low tide may be due to continuous nutrient replenishment from the surf waters. In addition, there was a strong relationship ($r = 0.72$, $p \leq 0.001$) between TVCa and TVCan. This suggested that there is higher abundance of facultative anaerobes in the bacterial community. However, with disturbance this interrelationship reduced marginally ($r = 0.54$, $p \leq 0.001$). In addition, there was a general reduction in viable forms encountered. The viable aerobic forms decreased by 69% after disturbance. Further, the interrelationship between viable aerobic forms and total bacterial abundance increased from 0.32 ($p \leq 0.05$) during phase I to 0.59 ($p \leq 0.01$) during phase III. This increase suggested that the viable aerobic forms could have increased their role in controlling the total abundance of bacteria. The effect of disturbance was more prominent after 24 h on direct viability under anaerobic conditions, which decreased by 79%. This effect could possibly be attributed to higher stress on the viability of anaerobic population brought about by aeration due to mixing of sediment. Thus this instantaneous response by decrease in viability could be related to stress on the system by disturbance and the incubations lasting up to 7 h would not take into account the recuperation that is required by the organisms. Stevenson (1978) has stated that the bacterial cells respond to stress by decrease in size and activity. It is

possible that in the present study, that the decrease in viability could be a response to stress imposed by mechanical disturbance. However, in the absence of disturbance, bacteria could resume their normal development over time. In the dormant state, the bacteria function at very low metabolic rates and do not undergo cell division. When the stress is released, they tend to become more viable and resume cell division (Roszak and Colwell 1987). This perhaps could be the reason why bacteria respond by higher culturability. Plating and incubation gives sufficient time for bacteria to recuperate, thus becoming more viable to form colonies. In environmental samples, culturable counts usually represent only a small fraction (0.1% ± 10%) of the active microbial community (White et al., 1998) as most organisms are in the viable but not cultivable state (Xu et al., 1982; McCarthy and Murray, 1996). However, with disturbance there is an improvement in culturability. The culturable fraction increased by 127 and 91% on 0.01% and 0.001% NB respectively. Improved culturability (RC) after disturbance could be either due to increased availability of nutrient brought to the surface due to mixing and aeration of sediment. Thus, the effect is more positive on the culturability of the microbes. This may be because this parameter would able to measure the state of the bacteria after the effect of mechanical stress decreases. Under the culture conditions, there is enough time for bacterial revival and growth. Similar results were obtained with deep-sea bacteria in the INDEX experiment. There was an increase by two orders of magnitude of culturability after the simulated disturbance (Loka Bharathi and Nair, 2005). Positive correlation between RC and TC increased from non-significant levels to significant levels at 0.44 ($p \leq 0.001$) for RC-0.001 forms, 0.398 ($p \leq 0.001$) for RC-0.01 forms and 0.58 ($p \leq 0.001$) for RC-10 forms. Thus the variation of RC-10 population had a higher influence on the variation of TC after disturbance. This increased retrievability could also be related to biochemical characteristics of the sediments.

Interrelationships between bacterial and biochemical parameters clearly implied interdependence after 24 h (Table 8AT1, Appendix III). The RC-10 fraction of bacteria retrieved on higher nutrients was also capable of bringing

a significant variation of about 28% ($p \leq 0.001$) in proteins. On the other hand, RC-0.001 population which caused a significant 8% variation in TC during phase I was capable of bringing about 20% variation in TC and 21% variation in the aerobic viable forms during phase III. These forms also contributed to 14 and 16% variation in carbohydrates and protein respectively. The variation in aerobic viable forms accounted for 31% of the variation in protein during phase III. It is suggested that relatively more oligotrophic forms were able to proliferate and improve the viability of the community after disturbance. This was further corroborated by LOM which had a greater influence on RC-0.001 forms (19%, $p \leq 0.001$) than on culturability at other concentrations perhaps suggesting the prevalence of substrate at these concentrations in this system. In addition, during phase I, ATP was also markedly influenced (0.44, $p \leq 0.001$) by culturable bacteria retrieved on RC-0.001 forms. The influence was nearly 19% at $p \leq 0.001$ before disturbance and diminished after disturbance. During phase II, ATP brought about 37 and 7% variation on RC-0.01 and RC-10 forms respectively. This increased relationship could be partly due to higher number of retrievable bacteria during phase II of the experiment. There could be other factors involved because this relationship diminishes at the end of 24 h when total ATP values decrease. In fact, after 24 hours, all variables related negatively to ATP. This could be mainly due to the destruction of the living biomass (i.e. microbial biomass other than bacteria) as evidenced by low values of ATP.

Thus, when the whole beach was examined, the interrelationship between the variables was most prominent in phase three. The relationship of culturability and viability with biochemical parameters were more significant during this phase. Relationship between ATP and biochemical parameters suggest that the living microbial biomass was more dependent on LOM, which possibly could be plant derived. Alternatively both ATP and LOM values could be from a common source namely dune vegetation.

On the other hand in sediments, the TC and viable aerobic counts increased at both the input and output points during the simulated mining in the bay

sediments. There was a marginal decrease in the anaerobic forms (Table 8.2). Though the RC decreased at the input suction point, it improved by 4 times at the output ejection point. Thus aeration induced by mechanical displacement of the sediments, increased both aerobic viability and retrievability. However, at the end of two hours after the disturbance in the offshore Kalbadevi bay, the TC at the surface input site in the water column increased marginally along with the anaerobic viable counts. It may due to churning movement of the deeper sediments. However both the aerobic and retrievable counts decreased by 53 and 71% (Table 8.3). At the subsurface depths at the same site, all the bacterial parameters drastically decreased except TC which showed a marginal increase. This was in complete contrast with the water column at the output point where all the parameters also decreased drastically except TC which showed a marginal decrease of 23%. Thus output ejection point in the water column is more impacted by bacterial decrease than the input suction point after 2 hours.

Thus, the study on simulated sand mining in a beach ecosystem assesses the short term effect on bacterial and biochemical parameters over a span of 24 h. It showed that bacterial responses could be varied. While total counts and viability decreased, culturability increased and improved the lability of the available substrates. Bacteria are instantaneous indicators of disturbance and they are also highly resilient and therefore capable of restoring ecosystem to the original state. ATP could be a much more sensitive factor that could act as a proxy of impact on not only bacteria but also other microbes instantaneously. Thus, any mechanical stress in the ecosystem is reflected in the distributory pattern of this parameter and its relationship with other physico-chemical and biological parameters. Further studies on sedimentary microbes other than bacteria like the protozoans on a longer time scale could throw more light on the influence of simulated sand mining on a larger microbial community and its long-term impact.

In conclusion, though the disturbance had a negative impact on the total bacterial counts, total viable counts, and ATP, the simulated mining experiment improved the retrievability of bacteria by an order. It is perhaps

this fraction that would contribute to restoration through self regulation. In the bay system, the effect of simulated disturbance had a contrasting effect on bacterial parameters from water and sediments by decreasing the load in water and improving their distribution in sediments most probably due to mechanical exchange of sediments.

Chapter 9

Chapter 9: Summary and Conclusion

The thesis on "the bacterial ecology of coastal and near-shore placer sediments" is a pioneering attempt to understand bacterial interactions with environmental parameters with special reference to iron released from ilmenite of beach sediments of Kalbadevi. As these beaches are likely sites for sand mining the work examines the effect of short term disturbance on the bacterial response in both on onshore sediments and offshore waters.

9.1. Summary

Placer mineral deposits are segregated group of clastic/unconsolidated sediments, sedimentary rock or its metamorphosed equivalent with economic-grade concentration of one or more valuable dense resistant minerals. Beach sediments containing placer deposits harbor considerable mineral wealth and are thus evolving as important sites for sand mining. One of the potential area for mining is Kalbadevi situated in Ratnagiri, Maharashtra. The beach and bay sediments harbor rich concentrations of placer mineral ilmenite ($FeTiO_3$). This mineral makes upto 60% of the total heavy minerals.

The main objective of this thesis was to elucidate the role of sediment microbe interaction in placer rich sediments and understand their ecology. This area has been rarely explored. This study involved an extensive sampling scheme followed by bacterial and biochemical analyses. Bacterial abundance, viability, retrievability, phylogenetic diversity both non-culturable and culturable as well as metabolic diversity were analyzed along with sedimentary labile organic matter and geochemistry of the sediments. Pyrosequencing by 454 technology under the aegis of the international cooperative run of ICoMM has been used to delineate the microbial diversity of beach and bay sediments. Laboratory experiments were carried out to complement field observations.

Both field and laboratory experiments reveal that ilmenite is stimulatory. It increases bacterial activity and diversity. This is particularly evident at the berm where the concentration of the ilmenite is relatively higher than the rest of the beach. Interestingly, the viability and retrievability encountered in these sediments is much higher than generally reported in other sediments especially in the berm and the dune region perhaps due to the release of reactive/bioavailable iron in continuous pulses from ilmenite. This effect of ilmenite was also seen under laboratory conditions. The effect of increasing concentration of ilmenite on retrievability showed that retrievability improved by nearly 88% especially on 75% concentration of ilmenite. Maximum culturability is at a ratio of one part of sand and three parts of ilmenite which is the reported range of heavy minerals at Kalbadevi i.e. 7-79% (Siddiquie et al., 1984). Increased bio-availability of this iron could perhaps select genera capable of utilizing iron for metabolic purposes.

This could perhaps get reflected in the higher trophic levels improving the general functioning of the system. The bio-available iron released from ilmenite by microbial action stimulates phytoplankton abundance and diversity. In the presence of ilmenite alone, there is both increase in diversity and evenness that is better than in the presence of combination of $FeSO_4.7H_2O$ and ilmenite. The health of the ecosystem is thus maintained by the diversity it promotes. The stimulatory effect of iron released from this mineral could be beneficial to the biocoenosis in Kalbadevi Bay.

The average potential iron reduction rates in the Kalbadevi sediments ranged from 4 to 9 mg-at.Fe g^{-1} day^{-1}. The native dissimilatory iron reducers contribute to the major part of iron reduced from ilmenite in Kalbadevi sediment. During post-monsoons, nearly 84% of the variation in the Fe influenced the MPN estimates positively suggesting its effect on the proliferation of bacteria. The number of iron reducing bacteria as estimated by Ferrozine-MPN method was high during the post-monsoon season of November suggesting that the proliferation of these bacteria are dependent on the higher bio-availability of organic carbon which generally increases during the post-monsoon months. Release of iron from ilmenite was not only

encountered with natural flora associated with whole sediments but also with strains isolated from these sediments such as S7 isolate. This rate of removal of 35 mg-at Fe g^{-1} day^{-1} was the highest with S7 isolate which had a taxonomic affinity to Gammaproteobacteria.

To answer the question whether there was a relationship between the microflora, the biochemistry and geochemistry of the sediments, three contrasting sites namely dune, berm and mid tide were analyzed in depth. Significant correlations were plotted using the software "Cytoscape". This software gave a holistic picture thus helping in illustrating and bringing into focus the complexity in relationships at mid tide level and dune. Berm which had the highest accumulation of ilmenite had the least number of significant interrelationships. Most importantly, the components interrelated with each other directly. It was evident through these analyses that different communities of microorganisms played a role in ilmenite dissolution at the three sites. Anaerobically viable forms played a significant role in the removal of iron from the ilmenite mineral while the culturable forms were important in the berm environment.

PCA analyses revealed that most of the variations could be explained by just three principal factors. In the dune region, both the soluble fraction and acid soluble fraction of iron may influence heterotrophic culturable forms. However, in the berm region anaerobic mixotrophs may be stimulated in the presence of ilmenite. At the mid tide region, the phytoplankton may perhaps utilize the iron released from ilmenite by bacterial action. The complexity in abiotic influence was clearly indicated using BIO ENV analyses wherein a combination of parameters such as depth, lipids, proteins and microbially extractable iron was required to influence the bacterial community. These analyses showed that the environmental components influencing distribution of bacterial parameters at the mid tide region were ilmenite, depth, total carbon content and total organic carbon. At the berm station, the influence of total carbon and organic carbon was relatively lower. The influence of depth was evident only in combination with protein and ilmenite.

Paradoxically though the berm system was simple showing fewer significant relationships, the culturable phylogenetic diversity in these sediments was the highest. This was in contrast to the mid tide sediments where the ilmenite concentration was the lowest and also only few number of genera were retrieved. Berm sediments also harbored resident microbial community which could utilize nearly all the substrates tested using the BioLog Ecoplates. However, bacterial isolates capable of degrading polymers by expressing extracellular enzymes was lower than the mid tide sediments.

Microcosm experiments show that natural or indigenous flora are capable of releasing iron which could account for the improved activity. Further, experiments with bacterial isolates also support these observations. Besides, laboratory experiments demonstrated that there were a second group of isolates which could modulate their oxidizing activity depending upon the ambient iron concentration. It is therefore inferred that both these groups of bacteria could synergistically control the iron released from ilmenite at levels beneficial to the microbial community and that at other trophic levels. The study highlights that ilmenite which was hitherto believed to be non reactive has now shown to be accessible to bacterial reduction rendering the recalcitrant mineral – ilmenite reactive. Thus, they play an important role as biogeochemical agents

The 454 pyrosequencing technique gives a holistic understanding of bacterial diversity in these iron rich systems. It revealed that the berm sediments harbored only 63 orders belonging to 22 phyla while the bay sediments harbored 87 orders belonging to 30 phyla. Firmicutes ribotype constituted the predominant phyla in the beach sediments while the Proteobacteria ribotypes dominated the bay sediments reflecting their ecological role. The former are known as mineralizers of Fe and perhaps even Si and the latter as degraders of organic matter. The order *Rubrobacterales* which accounted for 7% of the total ribotypes in the beach sediments may be involved in the iron oxidation.

Short term observation over 2 hours after simulated sand mining for this economically important mineral show that the bacterial community in the sand gets stimulated but those in water column get reduced perhaps due to physical displacement. However, it is possible that the stimulatory onshore effect would also hasten the offshore restoration as sediment systems are more stable than those of the water column. Of all the parameters tested, ATP was the most responsive as it increased immediately after disturbance.

9.2. Conclusion

1) Short term simulated mining stimulate bacterial culturability. Bacteria are instantaneous indicators of disturbance and they are also highly resilient and therefore capable of restoring ecosystem to the original state. ATP could be a sensitive factor that could act as a proxy for instantaneous impact of not only bacteria but also other microbes.

2) For the first time, ilmenite which was hitherto believed to be a recalcitrant mineral has now shown to be bacterially reactive. Consequently, there is continuous bioavailability of iron from this mineral to the ecosystem where it is present, as in Kalbadevi. The positive response in terms of bacterial viability and culturability cascades through the primary trophic levels and could positively affect the other trophic levels as well.

Chapter 10

Chapter 10: References

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Chapter 11

Chapter 11: Appendix

Appendix I : Biological parameters

1.1) Direct Total Counts

1.1.1) Buffered formalin

Formaldehyde (38%)
Hexamine
Saturate formalin with hexamine.
Filter sterilize and store at RT

1.1.2) Acridine orange stain (0.1%)

Acridine orange	0.1 g
Formaldehyde (5%)	100 mL

Filter through 0.22 µm polycarbonate paper
Store in amber colored bottle at 4(±2)°C

1.1.3) Antibiotic cocktail

DW	30 mL
Nalixidic acid	0.024 g
Piromedic acid	0.012 g
Pipemedic acid	0.012 g
saturated NaOH solution	150 µL

The antibiotics are dissolved in saturated NaOH solution and DW
The solution is filter sterilized and stored in vials at 4(±2) °C

1.1.4) Yeast extract (0.3%)

DW	30 mL
Yeast extract	0.3 g

The yeast extract is dissolved in DW.
The solution is autoclaved, filter sterilized and stored in vials at 4(±2) °C

1.1.5) Sulfide solution (5%)

DW	100 mL
$Na_2S.9H_2O$	5 g

The compound is mixed in autoclaved DW, filter sterilized and used immediately

1.1.6) Additions for incubation and fixing for direct total counts

	TC	TVA	TVAn
	µL	µL	µL
Sample	5000	5000	5000
Yeast extract	-	50	50
Antibiotic cocktail	-	50	50
Sulfide solution	-	-	20
Buffered formalin (0h)	250	-	-
Buffered formalin (after 7h)	-	250	250

1.2) Retrievable Counts

1.2.1) Iron Medium (IB) (Rodina, 1972)

50% SW	1000 mL
$(NH_4)_2SO_4$	1.5 g
KCl	0.05 g
$MgSO_4.7H_2O$	0.5 g
K_2HPO_4	0.05 g
$Ca(NO_3)_2.4H_2O$	0.01 g
$NaHCO_3$	0.03 g
Solution of trace elements	1 mL
Agar	1.5%

Solution of trace elements

DW	1000 mL
$ZnSO_4.7H_2O$	4.4 mg
$CaCl_2.6H_2O$	1 mg
MoO_3	3 mg
$BaCO_3$	15 mg
$Co(NO_3)_2.6H_2O$	2.5 mg
H_3BO_3	56 mg
KI	1.3 mg
$Al_2(SO_4)_3.6H_2O$	250 mg
Silicic acid	Trace

1.2.2) Modified Winogradsky Medium (IR) (Rodina, 1972)

50% SW	1000 mL
NH_4NO_3	0.5 g
$NaNO_3$	0.5 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.5 g
$CaCl_2.6H_2O$	0.2 g
Ferric ammonium citrate	10.0 g
Agar	0.8%

1.3) Phylogenetic diversity of bacterial isolates

1.3.1. Cultural Characteristics

The following characteristics were noted:
Size, Shape, Color, Margin, Elevation, Opacity

1.3.2. Morphological and biochemical tests

1.3.2.1. Gram staining

a) Crystal violet

Crystal violet	2 g
Ethyl alcohol (95%)	20 mL

a) Gram's iodine

Iodine	1 g
Potassium iodide	2 g
Distilled water	300 mL

c) Ethyl alcohol 70%

d) Safranine

Smear of the isolates was prepared on slides, air-dried and heat fixed.
The slides were treated with crystal violet (1 min) followed by Gram's iodine (1 min).
Slides were then washed with decolorizing solution (ethyl alcohol) till the blue color disappears and counter stained with safranine for 30s.
Slides were washed with water, dried and observed under oil- immersion
Gram positive bacteria are stained purple while gram negative bacteria are stained pink

1.3.2.2. Motility

Motility was observed using hanging drop method.

1.3.2.3. Oxidase test

The enzyme oxidase is part of the electron transfer system used by some organisms that use molecular O_2 as a terminal electron acceptor. Oxidase interacts with the membrane bound cytochromes and delivers cytochromes to O_2. As a result H_2O_2 or H_2O is generated. Strict anaerobes do not use oxygen and hence do not possess the oxidase enzyme. Most gram positive bacteria are oxidase negative as well as the members of the family Enterobacteriaceae. Oxidase positive bacteria possess cytochrome oxidase or indophenol oxidase (an iron containing haemoprotein). These both catalyze the transport of electrons from donor compounds (NADH) to electron acceptors (usually oxygen). The test reagent, N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride acts as an artificial electron acceptor for the enzyme oxidase. The oxidised reagent forms the coloured compound indophenol blue. The cytochrome system is usually only present in aerobic organisms which are capable of utilizing oxygen as the final hydrogen receptor. The end product of this metabolism is either H_2O or H_2O_2 (broken down by catalase).

DW	100 mL
N, N, N', N'-tetra-methyl-p-phenylenediamine dihydrochloride	1 g

A drop of oxidase reagent was placed on Whatman filter paper no.1
Isolates were picked using sterile toothpicks and smeared on treated filter paper to check for the presence of cytochrome oxidase in the isolates.
The observations were inferred from the following table

	Observation	Report
1.	Deep violet color developed immediately after smearing	Oxidase positive
2.	Deep violet color developed after 30s	delayed positiveness
3.	No color change.	Oxidase negative

1.3.2.4. Catalase test

The catalase test is used to detect the presence of catalase enzymes by the decomposition of hydrogen peroxide to release oxygen and water. Hydrogen peroxide is formed by some bacteria as an oxidative end product of the aerobic breakdown of sugars and if allowed to accumulate, is highly toxic. Catalase either decomposes hydrogen peroxide or oxidizes secondary substrates.

Hydrogen peroxide (3%)
Scrape the growth from a slant or plate with a non- metallic instrument.
Suspend it in 3 % hydrogen peroxide on a slide.
Examine for effervescence, presence of effervescence denotes catalase positive and absence denotes negative reaction.

1.3.2.4. Hugh and Leifson's medium (OF test)

Oxidative organisms can only metabolize glucose or other carbohydrates under aerobic conditions i.e. using O_2 as hydrogen acceptor. Other organisms ferment glucose and hydrogen acceptor is then another substance e.g. sulphur. This fermentative process is independent of oxygen and cultures of organisms may be aerobic or anaerobic. The end product of metabolizing a carbohydrate is acid. Oxidizing organisms produce an acid reaction towards the top of the tube. Fermenting organisms produce an acid reaction throughout the medium. Organisms that cannot break down the carbohydrate aerobically or anaerobically, produce an alkaline reaction in the tube. Hugh and Leifson's medium can also be used for recording gas production and motility.

	OF medium	
1	Dextrose (was filter sterilized and added to the medium later)	1 g
2	Peptone	0.2 g
3	KH_2PO_4	0.03 g
4	Agar	1.5 g
5	Bromothymol blue	0.002 g
6	50% SW	100 mL

Tubes containing OF medium were stab inoculated with cultures and incubated at RT for 48h.
The observations were inferred from the following table

	Observation	Report
1.	Bottom to top yellow/ Bottom yellow	Fermentative (with or without gas)
2.	Yellow only on top	Oxidative
3.	Blue color	Alkaline
4.	Growth, no color	Growth only
5.	No Growth	Inert

1.4) Metabolic diversity of bacterial isolates

1.4.1. Screening for Amylase

Amylolytic medium

Nutrient Agar	28 g
Starch	2 g
50% SW	1000 mL
pH	7.5-7.8

Culture was spot inoculated on the medium and incubated for 24 –48h at RT.
On addition of iodine to the plate, the whole plate turns dark blue except for yellow/colourless halos around the colonies indicating amylase production.

1.4.2. Screening for Protease

a) Proteolytic medium

Nutrient Agar	28 g
Caesin	2 g
50% Sea water	1000 mL
pH	7.5-7.8

b) $HgCl_2$ solution

$HgCl_2$	15 g
HCl	20 mL
DW	100 mL

Culture was spot inoculated on the proteolytic medium and incubated for 24 –48h at RT.
After the colonies have grown, overlay the plate with $HgCl_2$ solution
Observe for the clearance zone around the colony.

1.4.3. Screening for Lipase

Lipolytic medium

Peptone	10 g
NaCl	5 g
$CaCl_2$	0.1 g
*Tween	10 mL
Agar	15 g
50% SW	1000 mL
pH	7.0-7.4

* Tween was autoclaved separately and added to the medium just before pouring
Culture was spot inoculated on the medium and incubated for 24 –48h at RT.
Observe for precipitate around the colony.

1.4.4. Screening for Phosphatase

Phosphatase medium

Nutrient Agar	28.0 g
50% SW	120 mL

After autoclaving and just before pouring the substrate, filter sterilized *p*-nitrophenyl phosphatase (sigma) was added to the medium so as to obtain a final concentration of 0.02%.
Culture was spot inoculated on the medium and incubated for 24 –48h at RT
Presence of a greenish yellow color around the colony is indicative of phosphatase production

1.4.5. Screening for DNase

DNase test medium

DNase test agar	5.04 g
Toluidine blue	0.012 g
50% SW	120 mL
pH	7.5-7.8

Culture was spot inoculated on the medium and incubated for 24 –48h at RT.
Presence of a clearance zone indicated DNase production

1.4.6. Classification of BiologTM EcoPlate carbon sources and associated guild groupings according to Christian and Lind (2007).

Guild (Abbrev.)	Substrate	Well no.
Amino acids (AA) (n=6)	L-Arginine	A4
	L-Asparagine	B4
	Glycyl-L-glutamic acid	F4
	L-Phenylalanine	C4
	L-Serine	D4
	L-Threonine	E4
Amines and amides (AM) (n=2)	Phenylethylamine	G4
	Putrescine [a]	H4
Carboxylic acids (CA) (n=9)	D-Galacturonic acid	B3
	D-Glucosaminic acid	F2
	2-Hydroxybenzoic acid	C3
	4-Hydroxybenzoic acid	D3
	γ-Hydroxybutyric acid	E3
	Itaconic acid	F3
	α-Ketobutyric acid	G3
	D-Malic acid	H3
	Pyruvic acid methyl ester	B1
Carbohydrates (CH) (n=10)	N-Acetyl-D-glucosamine [d]	E2
	D-Cellobiose	G1
	i-Erythritol	C2
	D-Galactonic acid γ-lactone	A3
	Glucose-1-phosphate	G2
	D,L-α-Glycerol phosphate	H2
	α-D-Lactose	H1
	D-Mannitol	D2
	β-Methyl-D-glucoside	A2
	D-Xylose	B2
Polymers (P) (n=4)	α-Cyclodextrin	E1
	Glycogen	F1
	Tween 40 [b]	C1
	Tween 80 [c]	D1

* [a] Putrescine breakdown product of the amino acid ornithine by removal of CO_2
[b] Polyoxyethylene sorbitan monoplamitate
[c] Polyoxyethylene sorbitan monooleate
[d] Amide between glucosamine (saccharide) and acetic acid

Appendix I (contd/....)

1.5) Screening for Siderophores

1.5.1. Modified M9 Solution

Modified M9 solution was prepared from three solutions which were sterilized separately before mixing.
Solution 1: Buffer solution

Salt solution	750 mL
KH_2PO_4	0.3 g
NaCl	0.5 g
NH_4C1	1.0 g
Piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES)	30.24 g
Salt solution	750 mL

PIPES was dissolved in 750 mL of salt solution.
The pH was adjusted to 6.8 with 50% KOH, and water was added to bring the final volume to 800 mL.
The solution 1 was autoclaved and cooled to 50 °C

Solution 2

DW	70 mL
Glucose	2 g
Mannitol	2 g
$MgSO_4.7H_20$	493 mg
$CaCl_2$	11 mg
$MnSO_4.\ H_20$	1.17 mg
H_3BO_3	1.4 mg
$CuSO_4.5H_20$	0.04 mg
$ZnSO_4.7H_20$	1.2 mg
$Na_2MoO_4.2H_20$	1.0 mg

The solution 2 was autoclaved and cooled to 50 °C

Solution 3

casamino acids10%(w:v) 30 mL
Dissolve casamino acids in autoclaved DW and filter sterilize.

Solution (2) was to buffer solution (1) along with solution (3)

1.5.2. Chrome Azurol solution

Solution 1

Hexadecyltrimethylammonium bromide (HDTMA)	21.9 mg
DW	25 mL

HDTMA was dissolved in DW while stirring constantly over low heat

Solution 2

$FeCl_3.6H_2O$ (1mM) in 10mM HCl	1.5 mL
CAS (2mM)	7.5 mL

Mix $FeCl_3.6H_2O$ and CAS

Solution 3

Solution (2) was slowly added to Solution (1) while stirring.
Transfer mixture to 100 mL of volumetric flask

Solution 4

(2-[N-morpholino]ethanesulfonic acid)	9.76g
MES buffer	
DW	50 mL

Adjust the ph to 5.6 with 50% of KOH (potassium hydroxide)

Solution 5

Mix Solution (3) and Solution (4)
Bring volume up to 100 mL with DW
87.3 mg of 5-sulfosalicylic acid was added just before use

1.6 Other biological parameters

1.6.1 For Chlorophyll estimation

Reagents

90% Acetone

Acetone	90 mL
Distilled water	10 mL

1.6.2 For estimating phytoplankton abundance

Reagents

Lugol's iodine solution

Iodine	10.0g
Potassium iodide	20.0g
Glacial acetic acid (10%)	200 mL

1.7) Laboratory experiments

1.7.1 Artificial seawater (ASW) (Atlas, 2004).

DW	1000 mL
NaCl	24.7 g
$MgSO_4.7H_2O$	6.3 g
$MgCl_2.6H_2O$	4.6 g
$CaCl_2$	1.0 g
KCl	0.7 g
$NaHCO_3$	0.2 g

1.7.2 Ferrozine-MPN assay medium

ASW	1000 mL
HEPES	4.766 g
Ferrozine	0.173 g
$Na_2MoO_4.2H_2O$	0.084 g
Ferric citrate	0.124 g
pH	7.5
Glucose*	

*100 µL of 10% filter sterilized glucose solution added to 300 mL media after autoclaving

1.7.3 Ferrozine medium I

ASW	1000 mL
HEPES	4.766 g
Ferrozine	0.173 g
$Na_2MoO_4.2H_2O$	0.084 g
pH	7.0

Appendix II : Biochemical and Chemical parameters

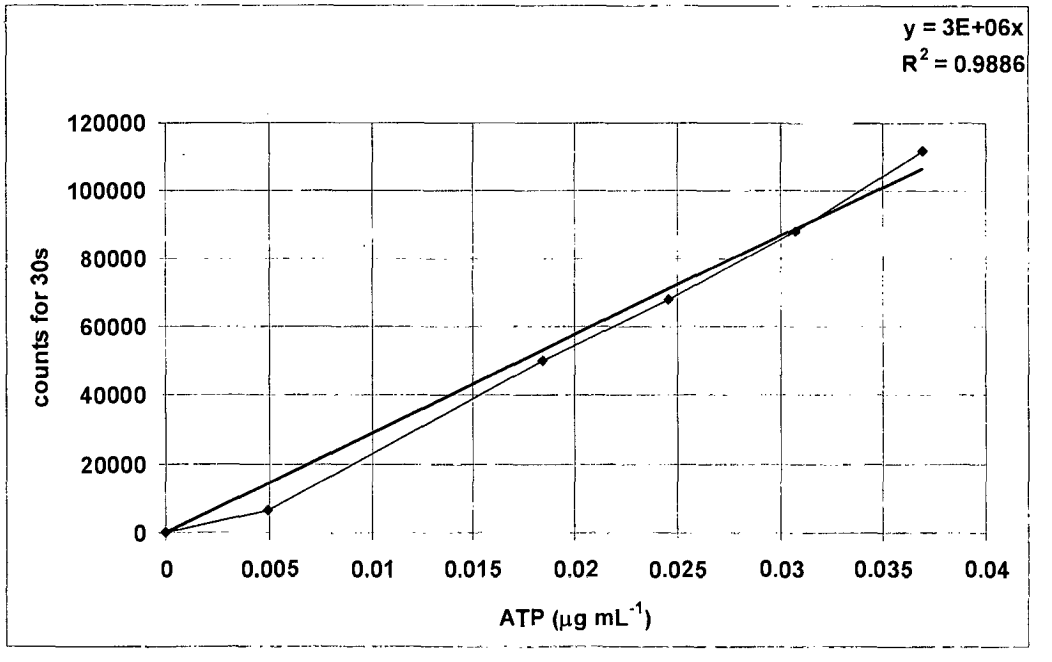
2) Biochemical and Chemical parameters

2.1 Estimation of ATP

Reagents

Tris buffer	
Tris (hydroxymethyl) amino methane	0.75 g
DW	200 mL
pH	7.7-7.8
pH was adjusted with 20% HCl	
Firefly extract preparation	
luciferin-luciferase enzyme (Sigma, FLE50)	50 mg
Autoclaved DW	5 mL
Age the enzyme at 4(±2)°C for 3h	

Typical example of Standard Curve for ATP estimation using standard ATP (Sigma)



2.2 Estimation of Total Carbohydrates

Estimation of carbohydrates was done by using phenol-sulphuric acid methods, (Kochert, 1978) the test was based on the formation of yellow color, and where concentrated sulphuric acid was added to the sample mixed with phenol solution. Carbohydrates were dehydrated by the sulphuric-acid to form furfural and variety of other degradation products.

Reagents

5% TCA solution

Trichloroacetic acid	5g
DW	100 mL

5% phenol solution

Phenol crystals	5 g
DW	100 mL

H_2SO_4 (95.5%, specific gravity 1.82)

DW

A known amount of sediment sample was weighed in triplicate and 1.5 mL of 5% of TCA solution was added to the tubes.

The tubes for heated for 3 h in boiling water bath at 80-90°C and cooled.

The tubes were centrifuge at 5000 x g for 5 min and supernatant was collected.

0.5 mL of the clear supernatant was mixed with 0.5 mL of distilled water to form 1mL of treated sample solution (TSS).

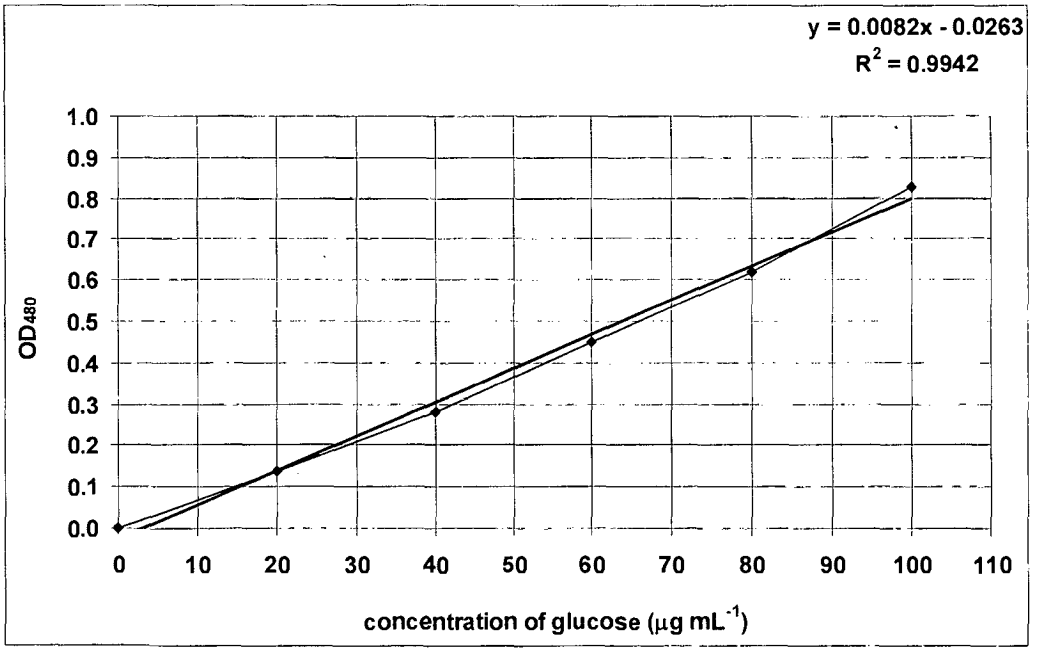
1 ml of TSS was added to 1 mL of 5% phenol reagent and mixed.

5 mL of conc. H_2SO_4 was added and kept in dark for 30 min.

The OD was measured at 480 nm using a spectrophotometer (Jasco).

The standard curves were prepared using glucose as standard.

Standard Curve for carbohydrate estimation using glucose as standard



Appendix II (contd/....)

2.3 Estimation of Total Lipids

The test is based on the oxidation of acid dichromate, which is followed by a decrease in the dichromate color. The extraction of reaction mixture has an inverse relationship based on the decrease of dichromate color.

Reagents

0.5% dichromate in conc. H_2SO_4

$K_2Cr_2O_4$	0.75 g
DW	10 mL
H_2SO_4 (95.5%, specific gravity 1.82)	500 mL

Organic solvent

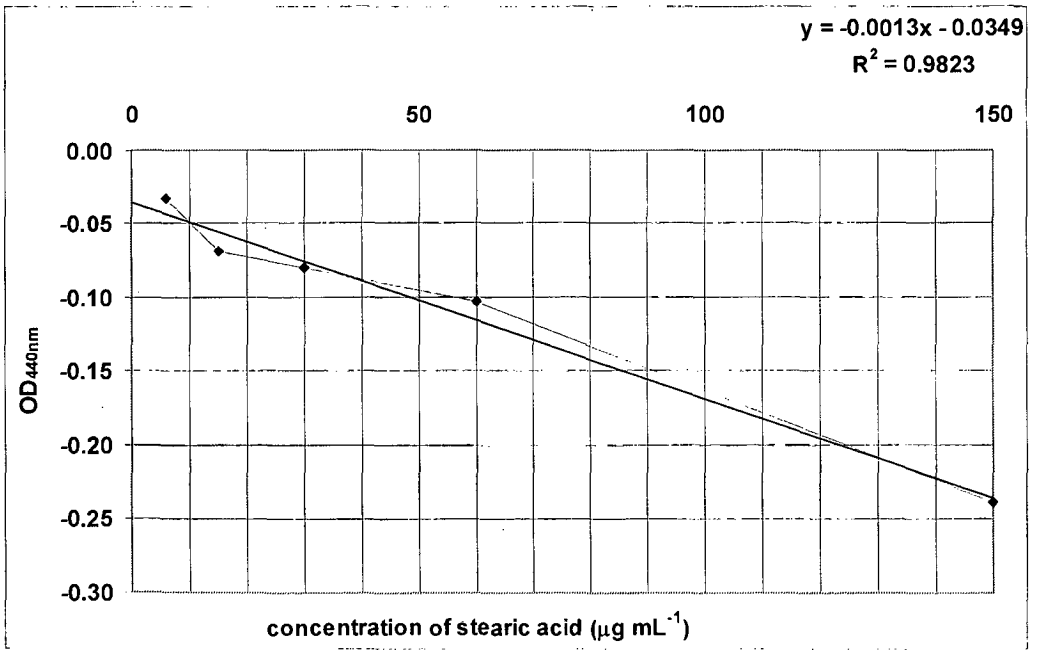
$CHCl_3$	200 mL
CH_3OH	400 mL
DW	160 mL

Analytical grade chloroform $CHCl_3$

DW

A known amount of sediment sample was weighed in triplicate.
8 mL of organic solvent was added to these tubes and mixed.
The tubes were homogenized at 9000 x g for 1 min and centrifuge at 5000 x g for 5 min.
The supernatant was added to a separating funnel followed by 2 mL of $CHCl_3$ and 2 mL DW
The funnels were shaken thoroughly, allowed to stand for some time. After clear separation of the two layers, the lower layer was collected in an evaporating flask
The flasks were evaporated to dryness using rotary vacuum evaporator.
To the dried lipid sample, 2 mL 0.15% acid dichromate was added and the flasks were heated in boiling water bath for 15 min.
The flasks were cooled thoroughly.
4.5 mL DW was added to the flasks, mixed and kept aside to cool.
The OD was measured at 440 nm using a spectrophotometer (Jasco).
The standard curves were prepared using stearic acid as standard.

Standard Curve for lipid estimation using stearic acid as standard



Appendix II (contd/....)

2.4 Estimation of Total Proteins

Reagents

Reagent A (2% Na_2CO_3 in 0.1 N NaOH)

Na_2CO_3	2g
0.1N NaOH	100 mL

Reagent B (0.5% $CuSO_4$ in 1% sodium potassium tartarate solution)

a)	$CuSO_4$	0.5 g
	DW	50 mL
b)	Na-K tartarate	1 g
	DW	50 mL
	Mix a and b together	

Reagent C

Reagent A	50 mL
Reagent B (Prepare fresh)	1 mL

Folin's reagent (1:1 dilution)

Folin ciocalteau phenol reagent	5 mL
DW	5 mL

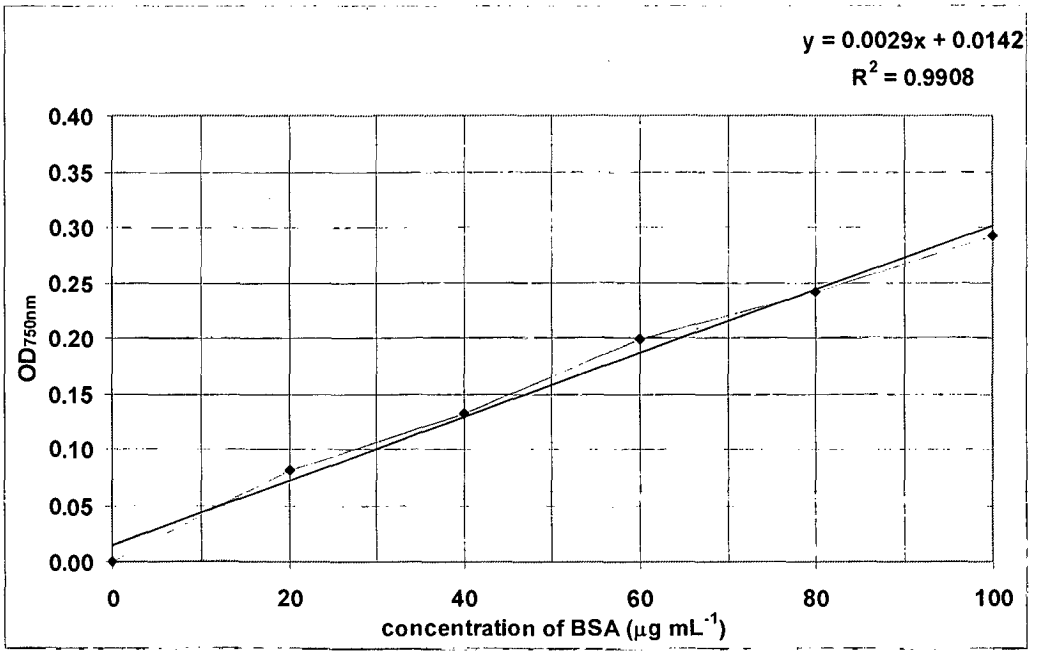
1N NaOH

NaOH	4 g
DW	100 mL

DW

A known amount of sediment sample was weighed in triplicate and 2 mL of 1N NaOH was added to these tubes.
After mixing the tubes were placed in boiling water bath for 5 min at 100°C.
The tubes were cooled and centrifuged at 5000 x g for 5 min for clear supernatant.
0.5 mL of the supernatant was mixed with 0.5 mL DW, and 5 mL Reagent C was added to it.
The tubes were mixed and kept in dark for 10 min.
After 10 min, 0.5 mL Folin Ciocalteau reagent (1:1 dilution) was added to the tubes.
The tubes were mixed and again kept in the dark for 20 min.
The OD was measured at 750 nm using a spectrophotometer (Jasco)
The standard curves were prepared using bovine serum albumin as standard.
The stock solution of 1mg/mL was made with the known concentration of BSA.

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



Appendix II (contd/....)

2.5 Estimation of Total Organic carbon

Reagents

H_3PO_4 (85%)	**10 mL**
NaF	**0.2 g for each sample**
Acid Mixture:	
Ag_2SO_4	2.5 g
H_2SO_4 (conc)	1000 mL
Std. 1N $K_2Cr_2O_7$	
$K_2Cr_2O_7$	49.04 g
DW	1000 mL
0.5N Ferrous ammonium sulphate solution (Mohr's Salt)	196.1 g
DW	18 mL
H_2SO_4 (conc)	20 mL
DW	1000 mL

Mohr's salt was dissolved in 18 mL distilled water containing conc. H_2SO_4.
The mixture was diluted 1L with DW.

Diphenylamine indicator	
Diphenylamine	0.5 g
DW	20 mL
H_2SO_4 (conc)	1000 mL

A 0.5 g of dried, sieved and finely ground sediment sample was weighed in triplicates.
10 mL of 1N $K_2Cr_2O_7$ and 20 mL of concentrated acid mixture was added to the weighed sediment and allowed to stand for 30 min.
The following was added in the sequence – 200 mL DW, 10 mL H_3PO_4(85%), 0.2 g NaF and 0.5 mL diphenylamine indicator and titrated against Mohr's salt till one drop end point i.e. Brilliant green.
The standard curves were prepared using dextrose (Merck) as standard.

Calculations:
%C = 10 (1- T/S) x F
where, T = titre value of standard / sample
S = titre value of blank
F = (1.0N) x 12/4000 x 100/0.5

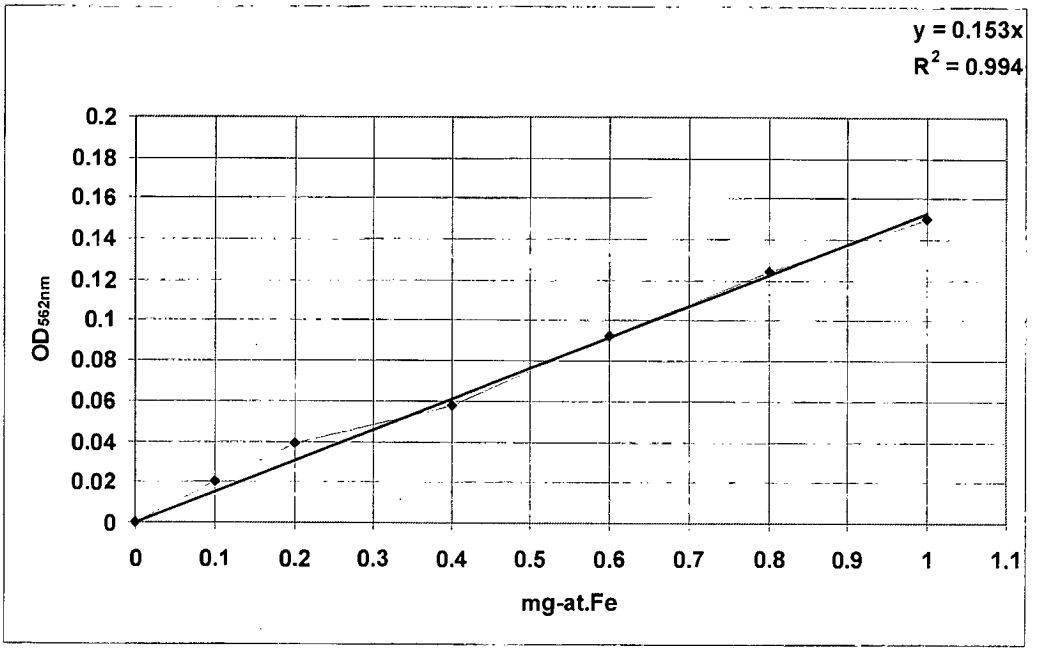
Appendix II (contd/....)

2.6. Estimation of Iron

Reagents

0.1% Ferrozine reagent	
Ferrozine	0.1 g
HEPES buffer (50mM)	100 mL
50mM HEPES buffer	
HEPES buffer	1.19 g
Distilled water	100 mL
0.25 M Hydroxylamine hydrochloride	
Hydroxylamine hydrochloride	1.73 g
HCl (0.25 M)	100 mL

Standard Curve for iron estimation using $FeSO_4.7H_2O$ as standard



Appendix III : Tables

Tables for Chapter 4

Table 4AT1: Spearman rank (rs) correlation between bacterial and biochemical parameters in the three transects pooled for the five sampling periods.

Interrelated parameters		Transect		
		Northern (df = 182)	Central (df = 354)	Southern (df = 179)
Depth	TC	ns	***-0.17****	**-0.36****
Depth	TVCa	ns	**-0.21****	-0.18*
Depth	TVCan	ns	-0.11*	ns
Depth	ATP	ns	0.16***	ns
Depth	CHO	ns	-0.11*	ns
Depth	LIP	ns	ns	0.17*
Depth	PROT	ns	***-0.14****	***-0.22****
Depth	LOM	ns	***-0.15****	ns
TC	TVCa	**0.66****	**0.81****	**0.44****
TC	TVCan	**0.74****	**0.74****	**0.35****
TC	RC-0.001	0.16*	**0.49****	ns
TC	RC-0.01	0.17*	**0.47****	ns
TC	RC-10	ns	**0.41****	**0.30****
TC	ATP	**0.31****	**0.21****	ns
TC	CHO	**-0.49****	ns	-0.15*
TC	LIP	**0.58****	**0.21****	**0.39****
TC	PROT	***0.25****	**0.24****	***0.23****
TC	LOM	ns	0.13*	0.15*
TVCa	TVCan	**0.67****	**0.75****	***0.24****
TVCa	RC-0.001	ns	**0.60****	**0.27****
TVCa	RC-0.01	ns	**0.58****	***0.25****
TVCa	RC-10	ns	**0.48****	**0.28****
TVCa	ATP	0.17	ns	ns
TVCa	CHO	**-0.44****	ns	ns
TVCa	LIP	**0.40****	ns	**0.30****
TVCa	PROT	***0.22****	ns	0.16*
TVCan	RC-0.001	0.15*	**0.47****	ns
TVCan	RC-0.01	ns	**0.44****	ns
TVCan	RC-10	ns	**0.29****	ns
TVCan	ATP	***0.22****	0.13	ns
TVCan	CHO	**-0.44****	ns	***-0.21****
TVCan	LIP	**0.38****	ns	**0.39****
TVCan	PROT	ns	ns	0.16*
TVCan	LOM	***-0.20****	ns	ns
RC-0.001	RC-0.01	**0.93****	**0.90****	**0.90****
RC-0.001	RC-10	**0.91****	**0.66****	**0.78****
RC-0.001	ATP	**-0.16****	ns	***-0.19****
RC-0.001	CHO	ns	***0.16****	ns
RC-0.001	LIP	**0.49****	ns	**0.34****
RC-0.001	PROT	**-0.39****	ns	***-0.25****
RC-0.001	LOM	ns	0.12*	-0.19*
RC-0.01	RC-10	**0.90****	**0.78****	**0.75****
RC-0.01	ATP	**-0.26****	ns	-0.19*
RC-0.01	LIP	**0.44****	ns	**0.36****
RC-0.01	PROT	**-0.33****	ns	***-0.24****
RC-10	ATP	-0.18*	ns	ns
RC-10	LIP	**0.50****	**0.31****	0.32*
RC-10	PROT	**-0.42****	0.13*	-0.18*
RC-10	LOM	-0.17*	ns	-0.15*
ATP	CHO	-0.15*	ns	ns
ATP	LIP	ns	**0.26****	-0.18
ATP	PROT	ns	**0.22****	ns
ATP	LOM	ns	***0.15****	ns
CHO	LIP	**-0.47****	-0.12*	**-0.52****
CHO	PROT	ns	**0.30****	**0.27****
CHO	LOM	**0.78****	**0.86****	**0.71****
LIP	PROT	ns	**0.28****	ns
LIP	LOM	ns	***0.17****	**-0.22****
PROT	LOM	**0.66****	**0.74****	**0.85****

Significance at p≤0.001 is denoted by – bold ***, at 0.01- by italic **, at 0.05 –by regular fonts & * , and non significant by ns.

Table 4AT2: Analyses of variance showing significant variation between stations and season at the central transect.

A) with respect to bacterial parameters

Source of variation	dF	p value					
		TC	TVCa	TVCan	RC-0.001	RC-0.01	RC-10
Month (M)	4	***	***	***	**	**	**
Station (S)	4	***	***	***	**	**	**
Depth (D)	4	***	***	***	**	**	**
MxS	16	***	***	***	**	**	**
MxD	16	***	***	***	**	**	**
SxD	16	***	***	***	**	**	**
MxSxD	64	***	***	***	**	**	**

B) with respect to biochemical parameters

Source of variation	dF	p value				
		ATP	CHO	LIP	PROT	LOM
Month (M)	4	***	**	***	**	**
Station (S)	4	***	**	***	**	**
Depth (D)	4	***	**	***	**	**
MxS	16	***	**	***	**	**
MxD	16	***	**	***	**	**
SxD	16	***	**	***	**	**
MxSxD	64	***	**	***	**	**

* Month : February, May, August, November, April = 5
Spatial : Stations : dune, berm, high tide, mid tide and low tide = 5
: Depth : (0-5), (5-10), (10-15), (15-20), (20–40)cm = 5

Significance at p≤0.001 is denoted by – bold ***, at 0.01- by italic **, at 0.05 –by regular fonts & *, and non significant by ns. df = 254

Appendix III (contd/....)

Tables for Chapter 5

Table 5AT1: One way ANOVA showing variations between the stations Dune, Berm, Mid tide of the central transect.

Parameter	Stations								
	Stn B and Stn D			Stn B and Stn M			Stn D and Stn M		
	P value	F	F critical	P value	F	F critical	P value	F	F critical
TC	0.043	4.62	4.30	0.000	18.41	4.30	0.017	6.63	4.30
TVCa	0.092	3.11	4.30	0.003	10.71	4.30	0.053	4.20	4.30
TVCan	0.780	0.08	4.30	0.006	9.21	4.30	0.010	8.02	4.30
RC-0.001	0.154	2.18	4.30	0.024	5.89	4.30	0.003	10.76	4.30
RC-0.01	0.860	0.03	4.30	0.001	14.75	4.30	0.017	6.67	4.30
RC-10	0.138	2.36	4.30	0.958	0.00	4.30	0.243	1.44	4.30
ATP	0.552	0.36	4.30	0.009	8.09	4.30	0.011	7.67	4.30
CHO	0.155	2.17	4.30	0.026	5.72	4.30	0.003	11.08	4.30
LIP	0.085	3.25	4.30	0.011	7.70	4.30	0.001	15.84	4.30
PROT	0.591	0.30	4.30	0.000	18.53	4.30	0.000	138.46	4.30
LOM	0.337	0.97	4.30	0.003	11.29	4.30	0.000	20.97	4.30
Tcarb	0.138	2.36	4.30	0.001	45.80	5.99	0.000	60.74	5.99
TOC	0.055	5.65	5.99	0.032	7.74	5.99	0.006	16.70	5.99
Fe(II)	0.035	5.03	4.30	0.495	0.48	4.30	0.283	1.21	4.30
FeA	0.121	2.60	4.30	0.058	4.02	4.30	0.103	2.90	4.30
FeM	0.468	0.55	4.30	0.661	0.20	4.30	0.984	0.00	4.30
Ilmenite	0.000	53.75	5.99	0.000	86.13	5.99	0.001	35.37	5.99
BIOLOG (Eco)	0.016	10.99	5.99	0.035	7.36	5.99	0.879	0.03	5.99
Enzyme diversity	0.600	0.29	4.75	0.464	0.57	4.75	0.902	0.02	4.75
Generic diversity	0.150	2.29	4.49	0.022	6.40	4.49	0.346	0.94	4.49

Appendix III (contd/....)

Table 5AT2: Correlation between different environmental parameters at dune, berm and mid tide station. The Spearman rank (rs) correlation analysis was carried out to test correlation among bacterial and biochemical parameters at the three sites.

Interrelated parameters		Correlation values at different stations		
		Stn D	Stn B	Stn M
TC	TVCa	ns	**0.93*** **	**0.83*** **
TC	TVCan	***0.73****	**0.86*** **	**0.95*** **
TC	RC-0.001	ns	0.58*	ns
TC	RC-10	ns	***0.75****	ns
TC	IB	ns	**0.96*** **	**0.87*** **
TC	ATP	ns	0.70*	***0.72****
TC	LIP	***-0.76****	***0.80****	0.62*
TC	Tcarb	ns	-0.63*	ns
TC	FeA	-0.66*	ns	-0.66*
TC	FeM	-0.65*	ns	0.67*
TC	IIm	-0.68*	***0.77****	***0.77****
TVCa	TVCan	0.60*	**0.88*** **	**0.89*** **
TVCa	RC-0.001	ns	***0.65****	ns
TVCa	RC-10	ns	***0.82****	ns
TVCa	IB	ns	**0.95*** **	***0.80****
TVCa	ATP	***0.73****	ns	**0.84*** **
TVCa	LIP	ns	**0.83*** **	ns
TVCa	FeA	ns	ns	***-0.79****
TVCa	FeM	ns	ns	***0.81****
TVCa	IIm	**-0.86*** **	***0.73****	**0.86*** **
TVCan	RC-0.01	ns	***-0.78****	ns
TVCan	RC-10	ns	**0.84*** **	ns
TVCan	IB	-0.70*	***0.80****	**0.85*** **
TVCan	ATP	ns	0.58*	***0.77****
TVCan	CHO	**-0.89*** **	ns	ns
TVCan	LIP	**-0.94*** **	ns	0.60*
TVCan	PROT	-0.66*	ns	ns
TVCan	LOM	**-0.89*** **	ns	ns
TVCan	Tcarb	-0.65*	ns	ns
TVCan	FeA	***-0.75****	ns	***-0.75****
TVCan	FeM	***-0.81****	ns	***0.75****
TVCan	IIm	-0.64*	**0.85*** **	**0.83*** **
RC-0.001	RC-0.01	**0.99*** **	ns	***0.77****
RC-0.001	RC-10	**-0.99*** **	**0.90*** **	ns
RC-0.001	IB	ns	***0.71****	ns
RC-0.001	IR	**-0.91*** **	ns	ns
RC-0.001	ATP	**0.86*** **	ns	0.59
RC-0.001	CHO	ns	*0.78***	***0.74****
RC-0.001	PROT	ns	**0.89*** **	ns
RC-0.001	LOM	ns	**0.84*** **	***0.78****
RC-0.001	TOC	0.65	ns	0.69*
RC-0.001	Tcarb	-0.58	ns	**-0.85*** **
RC-0.001	Fe(II)	**-0.88*** **	ns	*0.76***
RC-0.001	FeA	ns	ns	**-0.86*** **
RC-0.001	FeM	ns	***-0.74****	**0.85*** **
RC-0.001	IIm	ns	ns	0.68*
RC-0.01	RC-10	**-0.99*** **	ns	ns
RC-0.01	IR	**-0.95*** **	***-0.80****	ns
RC-0.01	ATP	***0.80****	ns	ns
RC-0.01	CHO	ns	ns	0.67
RC-0.01	LOM	ns	ns	0.68
RC-0.01	TOC	0.61*	0.91*	**0.99*** **
RC-0.01	Tcarb	ns	ns	***-0.77****
RC-0.01	Fe(II)	**-0.89*** **	ns	ns
RC-0.01	FeA	ns	0.63*	ns
RC-0.01	FeM	ns	-0.61*	ns
RC-0.01	IIm	ns	**-0.84*** **	ns
RC-10	IB	ns	***0.80****	-0.67
RC-10	IR	**0.93*** **	ns	**-0.98*** **

Significance at p≤0.001 is denoted by – bold ***, at 0.01- by italic **, at 0.05 –by regular fonts & *, and non significant by ns. N=12 *contd/-*

Table 5AT2: Correlation between different environmental parameters at dune, berm and mid tide station .....*contd/-*

Interrelated parameters		Correlation values at different stations		
		Stn D	Stn B	Stn M
RC-10	ATP	-0.84***	***ns***	***ns***
RC-10	PROT	ns	***0.62****	***0.73****
RC-10	LIP	ns	ns	-0.60*
RC-10	LOM	ns	***0.59****	ns
RC-10	Fe(II)	**0.93*****	ns	ns
IB	ATP	ns	ns	*0.81**
IB	CHO	***0.76****	0.60*	ns
IB	PROT	***0.73****	0.60*	ns
IB	LIP	**0.84*****	**0.88*****	***0.76****
IB	LOM	***0.79****	0.63*	ns
IB	Tcarb	0.58*	ns	ns
IB	Fe(II)	-0.67*	ns	ns
IB	FeA	0.64	ns	ns
IB	Ilm	ns	0.62*	**0.85*****
IR	IB	ns	ns	***0.81****
IR	ATP	-0.60*	0.60*	0.60*
IR	CHO	ns	-0.60*	ns
IR	LIP	ns	ns	0.69*
IR	PROT	ns	-0.70*	-0.68*
IR	LOM	ns	-0.64*	ns
IR	TOC	ns	-0.61*	ns
IR	Fe(II)	**0.89*****	ns	ns
IR	FeM	-0.70*	**0.88*****	ns
IR	Ilm	ns	**0.85*****	0.66*
ATP	CHO	ns	ns	0.59*
ATP	LOM	ns	ns	0.62*
ATP	TOC	0.59*	ns	ns
ATP	Tcarb	-0.59*	-0.68*	ns
ATP	Fe(II)	***-0.73****	ns	ns
ATP	FeA	ns	ns	***-0.78****
ATP	FeM	ns	ns	***0.82****
ATP	Ilm	ns	***0.73****	0.92*
CHO	PROT	***0.76****	**0.92*****	ns
CHO	LIP	**0.87*****	0.64*	ns
CHO	LOM	**0.99*****	**0.99*****	**0.99*****
CHO	TOC	-0.69*	ns	0.58
CHO	Tcarb	***0.77****	ns	ns
CHO	FeA	***0.73****	ns	ns
CHO	FeM	0.64*	**-0.85*****	ns
CHO	Ilm	ns	ns	0.59*
LIP	PROT	*0.78**	ns	ns
LIP	LOM	**0.89*****	0.65*	ns
LIP	TOC	-0.64*	ns	ns
LIP	Tcarb	***0.74****	-0.61*	ns
LIP	Fe(II)	ns	***0.72****	ns
LIP	FeM	0.69*	ns	ns
LIP	FeA	**0.84*****	ns	ns
PROT	LOM	***0.82****	**0.97*****	ns
PROT	TOC	**-0.84*****	ns	ns
PROT	Tcarb	**0.88*****	ns	**-0.88*****
PROT	FeA	***0.71****	ns	-0.60*
PROT	FeM	ns	**-0.94*****	ns
LOM	TOC	***-0.73****	ns	0.59*
LOM	Tcarb	***0.81****	ns	ns
LOM	Fe(II)	ns	ns	0.60
LOM	FeA	***0.75****	ns	ns
LOM	FeM	0.62*	**-0.90*****	0.59*
LOM	Ilm	ns	ns	0.63*
TOC	FeM	ns	-0.59*	ns
TOC	FeA	***-0.77****	**0.82*****	ns

Significance at p≤0.001 is denoted by – bold ***, at 0.01- by italic **, at 0.05 –by regular fonts & *, and non significant by ns. N=12 *contd/-*

Table 5AT2: Correlation between different environmental parameters at dune, berm and mid tide station .....*contd/-*

Interrelated parameters		Correlation values at different stations		
		Stn D	Stn B	Stn M
Tcarb	TOC	-0.99***	ns	*-0.76***
Tcarb	Fe(II)	ns	-0.83***	-0.66*
Tcarb	FeA	*0.82***	ns	0.71*
Tcarb	FeM	ns	ns	-0.68*
Tcarb	Ilm	ns	-0.65*	ns
Fe(II)	FeA	ns	0.58*	*-0.73***
Fe(II)	FeM	ns	ns	0.70*
FeA	FeM	ns	ns	-0.99***
FeA	Ilm	ns	ns	-0.84***
FeM	Ilm	*0.77***	ns	0.85***
Depth	TC	ns	-0.87***	-0.89***
Depth	TVCa	ns	-0.86***	-0.92***
Depth	TVCan	*0.72***	-0.63	-0.93***
Depth	RC-0.001	0.70*	*-0.79***	-0.61*
Depth	RC-0.01	0.62*	ns	ns
Depth	RC-10	-0.59*	*-0.75***	ns
Depth	IB	ns	-0.96***	-0.85***
Depth	ATP	*0.82***	ns	-0.89***
Depth	CHO	*-0.72***	*-0.76***	ns
Depth	PROT	*-0.76***	*-0.77***	ns
Depth	LIP	*-0.72***	-0.89***	ns
Depth	LOM	*-0.75***	*-0.80***	ns
Depth	TOC	0.90***	ns	ns
Depth	Tcarb	-0.93***	ns	ns
Depth	FeA	*-0.79***	ns	0.89***
Depth	FeM	ns	ns	-0.90***
Depth	Ilm	ns	ns	-0.95***

Significance at p≤0.001 is denoted by – bold ***, at 0.01- by italic **, at 0.05 –by regular fonts & *, and non significant by ns. N=12 *contd/-*

Appendix III (contd/....)

Tables for Chapter 6

Table 6AT1: Correlation analyses between various parameter studied in the microcosm

		Microcosms			
Interrelated parameters		**C-1**	**C-2**	**Exp-1**	**Exp-2**
Time	Fe(II)	-0.550	0.209	0.952***	0.964***
Time	Fe(III)	0.749**	-0.038	0.987***	0.99***
Time	ATP	0.083	-0.540	0.836***	0.450
Time	TC	0.936***	0.654*	-0.394	0.440
Time	Chl *a*	-0.304	-0.435	0.656*	0.060
Time	Phytoplankton cell abundance	-0.358	-0.446	0.853***	0.768**
Time	Algal filaments	-0.297	0.953***	0.956***	0.838***
Fe(II)	Fe(III)	-0.806***	-0.835***	0.955***	0.958***
ATP	Fe(II)	0.042	-0.146	0.87***	0.502
ATP	Fe(III)	0.059	0.310	0.899***	0.549
TC	Fe(II)	-0.436	0.491	-0.398	0.449
TC	Fe(III)	0.599*	-0.382	-0.342	0.452
TC	ATP	0.173	-0.141	-0.084	0.271
Chl *a*	Fe(II)	0.368	-0.121	0.683*	0.131
Chl *a*	Fe(III)	-0.508	0.310	0.722**	0.137
Chl *a*	Algal filaments	0.693**	-0.416	0.463	-0.117
Chl *a*	ATP	-0.342	0.497	0.886***	0.787
Chl *a*	TC	-0.336	-0.239	0.104	0.321
Phytoplankton cell abundance	ATP	0.031	0.601*	0.921***	0.859***
Phytoplankton cell abundance	TC	-0.265	-0.363	-0.155	0.459
Phytoplankton cell abundance	Chl *a*	0.814***	0.945***	-0.88***	0.607
Phytoplankton cell abundance	Algal filaments	0.761**	-0.421	0.672*	0.459
Phytoplankton cell abundance	Fe(II)	0.66*	-0.233	0.849***	0.822***
Phytoplankton cell abundance	Fe(III)	-0.712**	0.482	0.872***	0.812***
Algal filaments	Fe(II)	0.395	0.297	0.867***	0.678
Algal filaments	Fe(III)	-0.408	-0.132	0.923***	0.818***
Algal filaments	ATP	-0.009	-0.567*	0.677*	0.226
Algal filaments	TC	-0.230	0.574*	-0.424	0.241

Fe(II) (ferrous), Fe(III) (ferric), TC (Total bacterial counts), ATP (Adenosine triphosphate), Chl a (Chlorophyll a). N=12, df=11,

Significance $p \leq 0.05$ is reported in *, $p \leq 0.01$ is reported in **, $p \leq 0.001$ is reported in ***

Tables for Chapter 7

Table 7AT1: Table showing taxonomic breakdown of bacterial V6 tags of taxa grouped under minor order. Table shows Phylum Class Order distribution for taxonomically assigned tags that occurred less than 100 times in berm sediments (100% = 14893 tags).

Sr. No.	Taxa	Frequency of occurrence (%)
1	Proteobacteria Gammaproteobacteria unk	0.66
2	Firmicutes Clostridia Clostridiales	0.55
3	Verrucomicrobia Verrucomicrobiae Verrucomicrobiales	0.52
4	Proteobacteria Alphaproteobacteria Sphingomonadales	0.46
5	Proteobacteria Gammaproteobacteria Xanthomonadales	0.42
6	Proteobacteria Gammaproteobacteria Chromatiales	0.32
7	Acidobacteria Acidobacteria unk	0.31
8	Proteobacteria Alphaproteobacteria unk	0.30
9	WS3 unk unk	0.29
10	Actinobacteria Actinobacteria Acidimicrobiales	0.28
11	Nitrospira unk unk	0.27
12	Gemmatimonadetes unk unk	0.26
13	Nitrospira Nitrospira Nitrospirales	0.26
14	Firmicutes Bacilli Lactobacillales	0.24
15	Actinobacteria unk unk	0.23
16	Proteobacteria Deltaproteobacteria Bdellovibrionales	0.20
17	Chloroflexi unk unk	0.18
18	Unassigned	0.17
19	Proteobacteria Alphaproteobacteria Rhodobacterales	0.15
20	Thermomicrobia unk unk	0.15
21	Chloroflexi Anaerolineae Anaerolineales	0.13
22	Chlorobi unk unk	0.10
23	TM7 unk unk	0.10
24	Proteobacteria Alphaproteobacteria Caulobacterales	0.09
25	Chlamydiae Chlamydiae Chlamydiales	0.08
26	Planctomycetes unk unk	0.08
27	Chloroflexi Anaerolineae Caldilineaceae	0.07
28	Verrucomicrobia Spartobacteria Chthoniobacterales	0.07
29	Chloroflexi Caldilineae Caldilineales	0.06
30	OP5 unk unk	0.05
31	Chloroflexi Chloroflexi Chloroflexales	0.04
32	Firmicutes unk unk	0.04
33	Proteobacteria Gammaproteobacteria Enterobacteriales	0.03
34	Proteobacteria Gammaproteobacteria Pseudomonadales	0.03
35	Verrucomicrobia Opitutae Opitutales	0.03
36	Cyanobacteria unk unk	0.03
37	Proteobacteria Gammaproteobacteria Thiotrichales	0.03
38	TM6 unk unk	0.03
39	Deinococcus-Thermus Deinococci Thermales	0.02
40	Firmicutes Bacilli unk	0.01
41	Firmicutes Clostridia Thermoanaerobacteriales	0.01
42	Firmicutes Clostridia unk	0.01
43	OD1 unk unk	0.01
44	OD10 unk unk	0.01
45	Proteobacteria Gammaproteobacteria Legionellales	0.01

*Unk - unknown

Table 7AT2: Table showing taxonomic breakdown of bacterial V6 tags of taxa grouped under minor order. Table shows Phylum Class Order distribution for taxonomically assigned tags that occurred less than 100 times in bay sediments (100% = 15349 tags).

Sr. No.	Taxa	Frequency of occurrence (%)
1	Thermomicrobia unk unk	0.62
2	Bacteroidetes unk unk	0.61
3	Chloroflexi Caldilineae Caldilineales	0.55
4	Firmicutes Bacilli Bacillales	0.53
5	Lentisphaerae unk unk	0.50
6	Chloroflexi Anaerolineae Anaerolineales	0.37
7	Nitrospira unk unk	0.31
8	Gemmatimonadetes Gemmatimonadetes Gemmatimonadales	0.27
9	Proteobacteria Gammaproteobacteria Methylococcales	0.26
10	Spirochaetes Spirochaetes Spirochaetales	0.25
11	Proteobacteria Betaproteobacteria Burkholderiales	0.24
12	Proteobacteria Gammaproteobacteria Alteromonadales	0.23
13	Unassigned	0.19
14	Proteobacteria Gammaproteobacteria Oceanospirillales	0.18
15	Firmicutes Erysipelotrichi Erysipelotrichales	0.18
16	Proteobacteria Gammaproteobacteria Legionellales	0.18
17	Proteobacteria Alphaproteobacteria Rhodospirillales	0.16
18	Firmicutes Bacilli Lactobacillales	0.14
19	OP8 unk unk	0.13
20	Bacteroidetes Bacteroidia Bacteroidales	0.13
21	Chlamydiae Chlamydiae Chlamydiales	0.12
22	Cyanobacteria True Cyanobacteria unk	0.10
23	Actinobacteria Actinobacteria Coriobacteriales	0.10
24	Verrucomicrobia Opitutae Opitutales	0.09
25	Planctomycetes unk unk	0.08
26	Proteobacteria Gammaproteobacteria Vibrionales	0.08
27	Fusobacteria Fusobacteria Fusobacteriales	0.07
28	Firmicutes unk unk	0.07
29	Firmicutes Clostridia unk	0.07
30	Actinobacteria unk unk	0.06
31	Actinobacteria Actinobacteria Acidimicrobiales	0.06
32	Proteobacteria Gammaproteobacteria Enterobacteriales	0.05
33	Deinococcus-Thermus Deinococci Deinococcales	0.05
34	Proteobacteria Gammaproteobacteria Pseudomonadales	0.04
35	Chlorobi unk unk	0.04
36	OD11 unk unk	0.03
37	Verrucomicrobia Spartobacteria Chthoniobacterales	0.03
38	TM6 unk unk	0.03
39	Proteobacteria Betaproteobacteria unk	0.03
40	Fibrobacteres unk unk	0.03
41	BRC1 unk unk	0.03
42	Proteobacteria Gammaproteobacteria Pasteurellales	0.02
43	Proteobacteria Deltaproteobacteria Desulfovibrionales	0.02
44	Proteobacteria Deltaproteobacteria Bdellovibrionales	0.02
45	Proteobacteria Alphaproteobacteria Sphingomonadales	0.02
46	Proteobacteria Alphaproteobacteria Parvularculales	0.02
47	Cyanobacteria True Cyanobacteria Prochlorales	0.02
48	Chloroflexi Anaerolineae Caldilineaceae	0.02
49	WS1 unk unk	0.01
50	TM7 unk unk	0.01
52	Proteobacteria Epsilonproteobacteria Campylobacterales	0.01
53	Proteobacteria Betaproteobacteria Methylophilales	0.01
54	Proteobacteria Alphaproteobacteria Rickettsiales	0.01
55	OD1 unk unk	0.01
56	Lentisphaerae Lentisphaeria unk	0.01
57	Lentisphaerae Lentisphaeria Lentisphaerales	0.01
58	OP3 unk unk	0.01
59	Cyanobacteria True Cyanobacteria Pleurocapsales	0.01

*Unk – unknown

Contd/-....

Tables for Chapter 8

Table 8AT1: Evolution of interrelationships between bacterial and biochemical parameters during different phases of simulated mining at Kalbadevi beach (df = 59).

Interrelated parameters		Correlation coefficients during different phases		
		PI	PII	PIII
TC	Depth	-0.334	ns	-0.323
TC	TVCa	0.323	**0.579*** **	**0.589*** **
TC	TVCan	**0.458*** **	**0.428*** **	**0.478*** **
TC	ATP	0.295	ns	ns
TC	RC-0.001	0.287	**0.435*** **	**0.442*** **
TC	RC-0.01	ns	ns	***0.398****
TC	RC-10	ns	**0.717*** **	**0.582*** **
TC	CHO	ns	ns	***0.384****
TC	LIP	ns	-0.27	ns
TC	PROT	ns	ns	**0.559*** **
TC	LOM	ns	ns	**0.517*** **
TVCa	Depth	ns	-0.28	ns
TVCa	TVCan	**0.722*** **	**0.83*** **	**0.544*** **
TVCa	RC-0.001	**0.573*** **	**0.846*** **	**0.454*** **
TVCa	RC-0.01	0.286		0.26
TVCa	RC-10	0.262	**0.696*** **	**0.665*** **
TVCa	PROT	ns	ns	**0.551*** **
TVCa	LOM	ns	ns	**0.406*** **
TVCan	ATP	0.255	ns	-0.261
TVCan	RC-0.001	**0.540*** **	**0.932*** **	ns
TVCan	RC-0.01	***0.37****	ns	**0.467*** **
TVCan	RC-10	ns	**0.563*** **	0.325
TVCan	CHO	ns	ns	**0.585*** **
TVCan	LIP	ns	ns	0.26
TVCan	PROT	ns	ns	**0.500*** **
TVCan	LOM	ns	ns	**0.655*** **
RC-0.001	RC-0.01	**0.499*** **	ns	ns
RC-0.001	RC-10	ns	**0.54*** **	**0.709*** **
RC-0.001	ATP	**0.437*** **	ns	ns
RC-0.001	CHO	ns	ns	0.378
RC-0.001	LIP	0.267	-0.263	ns
RC-0.001	PROT	ns	ns	**0.402*** **
RC-0.001	LOM	0.263	ns	***0.402****
RC-0.01	ATP	ns	**0.609*** **	***-0.338****
RC-0.01	CHO	***0.369****	***0.384****	***0.355****
RC-0.01	LIP	ns	**0.332** **	ns
RC-0.01	PROT	**0.429*** **	0.516	***0.364****
RC-0.01	LOM	**0.413*** **	0.47	**0.412*** **
RC-10	ATP	-0.32	0.271	ns
RC-10	PROT	0.312	ns	**0.523*** **
RC-10	LOM	ns	ns	***0.387****
ATP	CHO	ns	**0.74*** **	***-0.339****
ATP	LIP	ns	**0.708*** **	ns
ATP	PROT	ns	**0.625*** **	***-0.378****
ATP	LOM	ns	**0.79*** **	**-0.415*** **
CHO	PROT	**0.719*** **	**0.61*** **	**0.468*** **
CHO	LIP	**0.659*** **	**0.584*** **	ns
CHO	LOM	**0.969*** **	**0.961*** **	**0.918*** **
LIP	PROT	**0.465*** **	**0.45*** **	ns
LIP	LOM	**0.667*** **	**0.653*** **	ns
PROT	LOM	**0.864*** **	**0.798*** **	***0.778****

* PI – Pre-disturbance PII – Immediately after disturbance PIII – 24 hours after disturbance
Significance at p≤0.001 is denoted by – bold ***, at 0.01- by italic **, at 0.05 –by regular fonts & * , and non significant by ns

Appendix IV : Figures

Figures for chapter 5

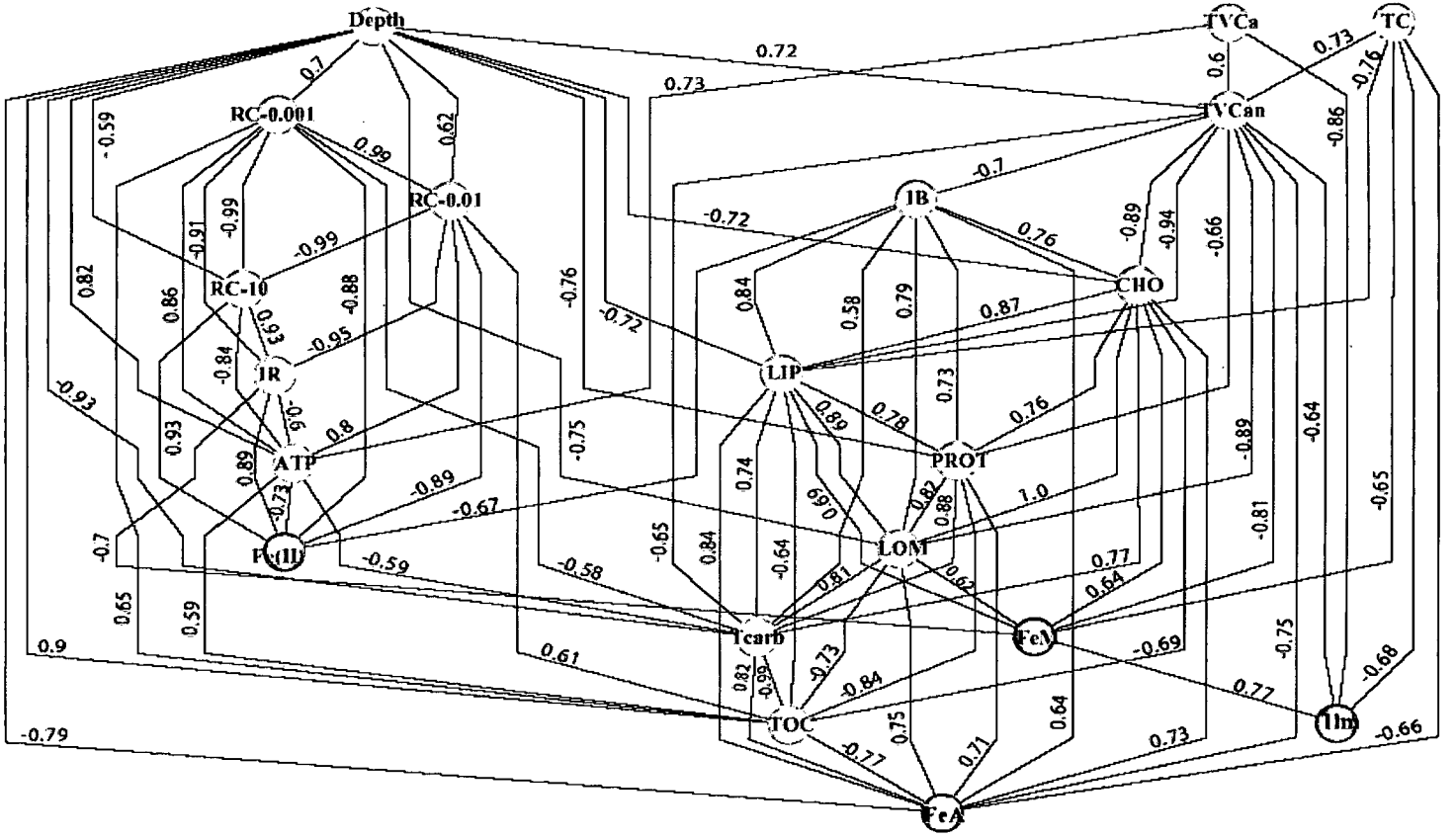


Fig 5AF1: Significant correlation between different environmental parameters at dune station using cytoscape software.

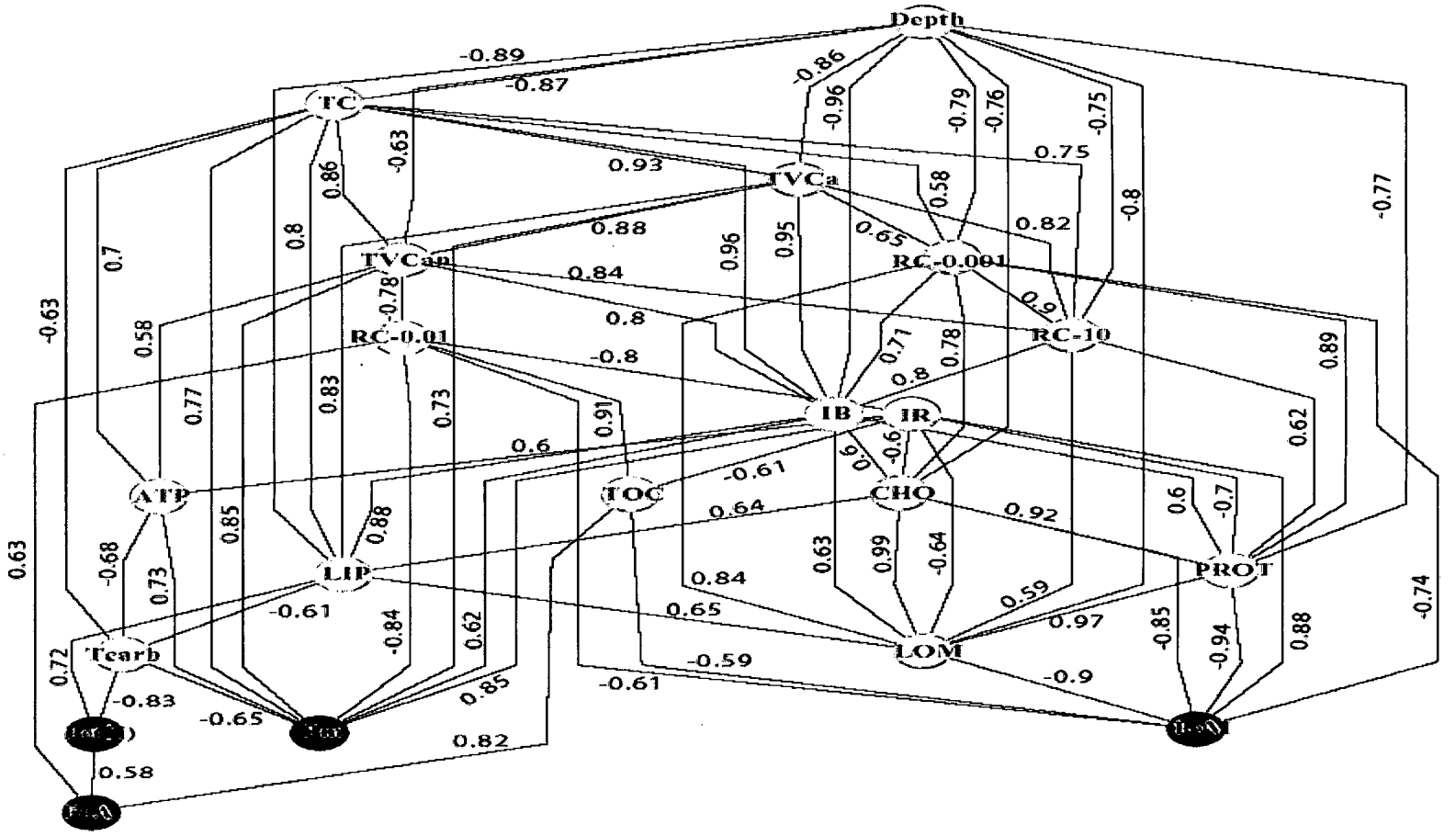


Fig 5AF2: Significant correlation between different environmental parameters at berm station using cytoscape software.

Appendix IV (contd/....)

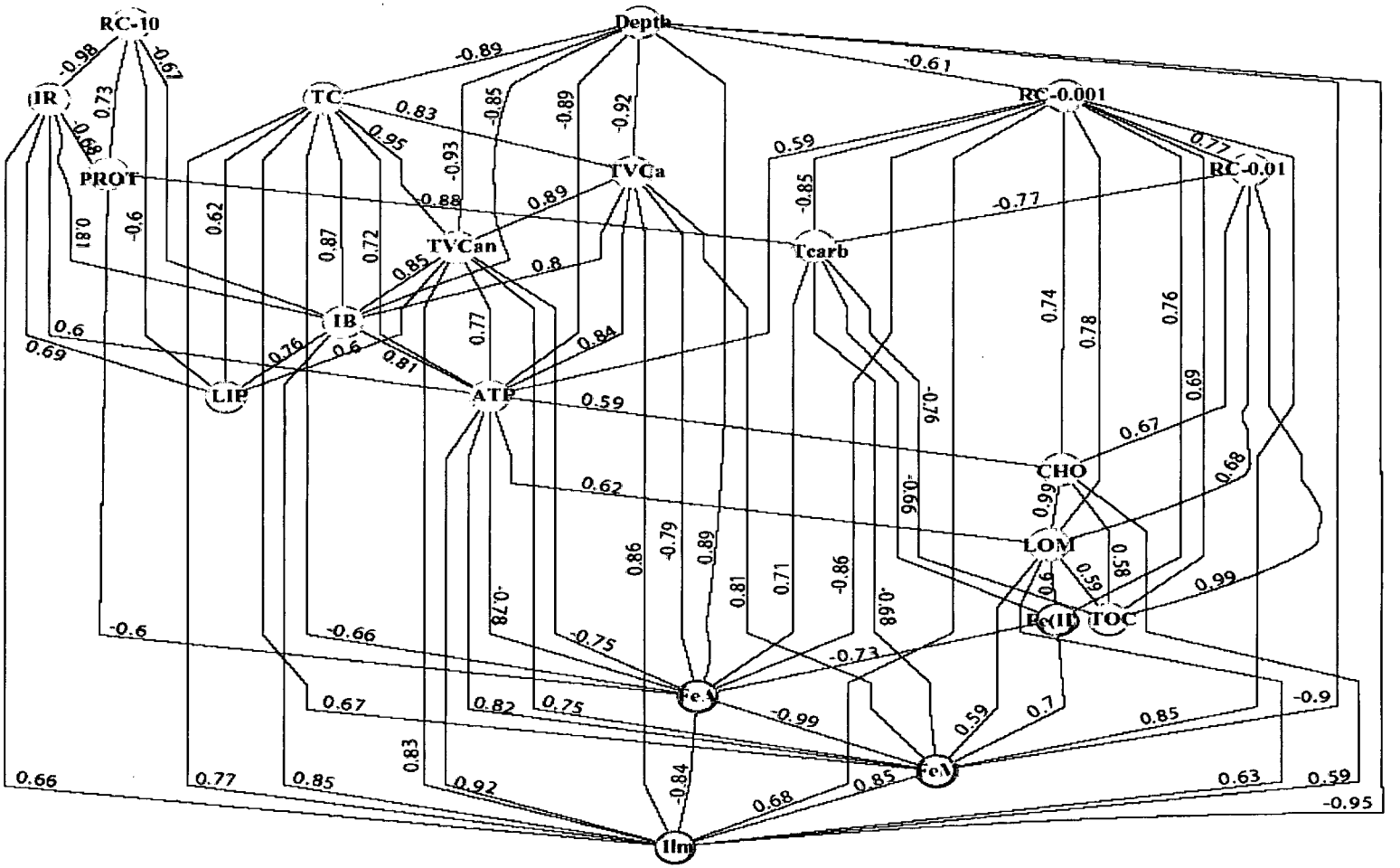


Fig 5AF3: Significant correlation between different environmental parameters at mid tide station using cytoscape software.

Appendix IV (contd/....)

Figures for chapter 6

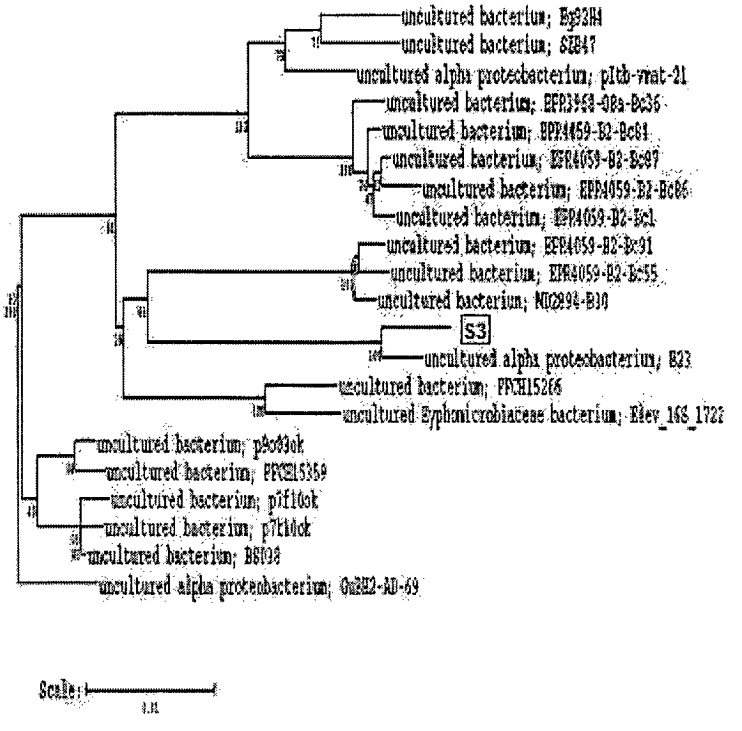


Fig 6AFI: Phylogenetic affinity of S3 isolate

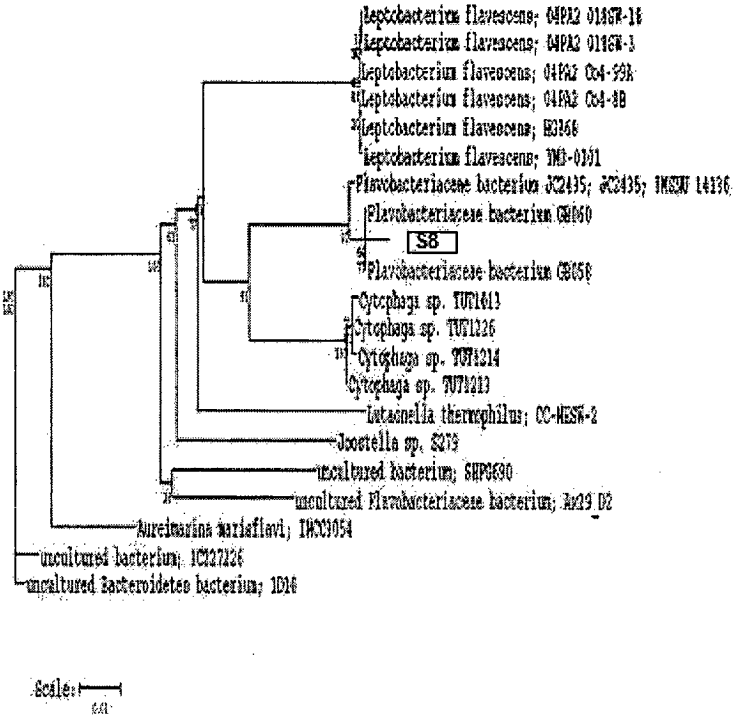


Fig 6AF2: Phylogenetic affinity of S8 isolate

Appendix IV (contd/....)

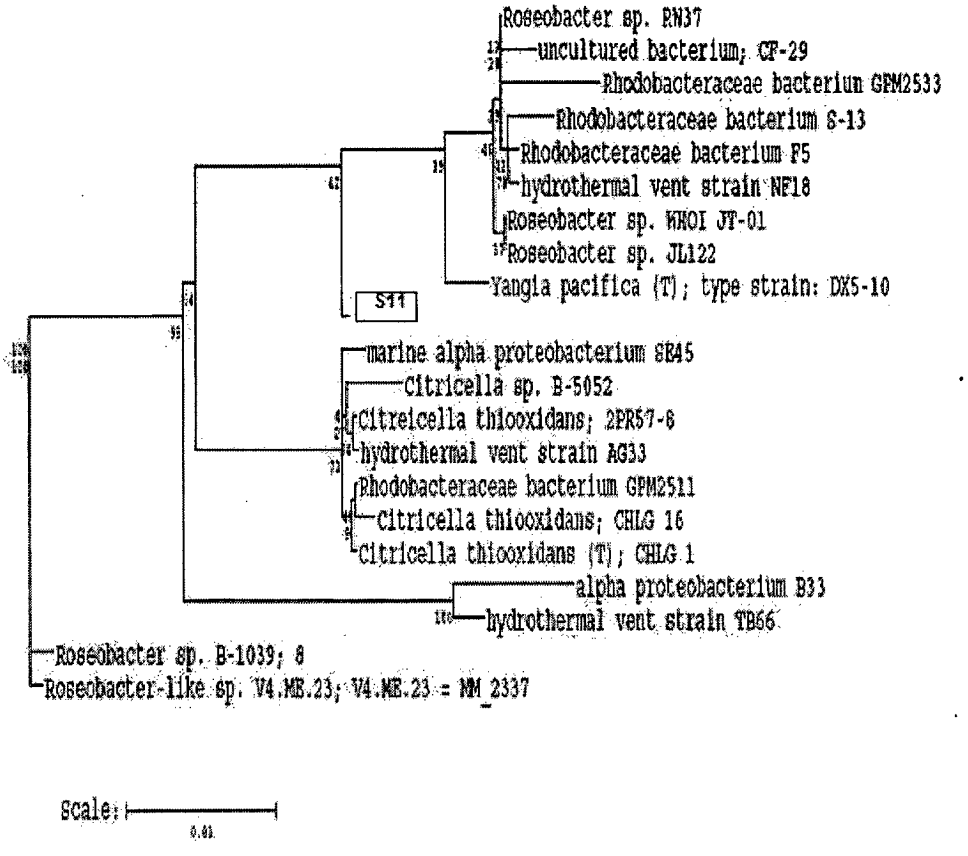


Fig 6AF3: Phylogenetic affinity of S11 isolate

List of Publications:

Papers published in SCI Journals:

1) Das, A., **Fernandes, C.E.G.,** Naik, S.S., Nagender Nath, B., Suresh, I., Mascarenhas-Pereira, M.B.L., Gupta, S.M., Khadge, N.H., Prakash Babu, C., Borole, D.V., Sujith, P.P., Valsangkar, A.B., Maurya, B.S., Biche, S.U., Sharma, R., and Loka Bharathi, P.A. (2010) Bacterial response to contrasting sediment geochemistry in the Central Indian Basin. **Sedimentology**, doi: 10.1111/j.1365-3091.2010.01183.x

2) Krishnan, K. P., **Fernandes, C.E.G.,** Fernandes, S. O., and P. A. Loka Bharathi (2006). Tolerance and immobilization of cobalt by some bacteria from ferromanganese crusts of the Afanasiy Nikitin seamounts. **Geomicrobiology Journal**, 23: 31-36.

3) **Fernandes, C.E.G.**, De Souza, M. J. B. D., Nair, S., and P. A. Loka Bharathi, (2005). Response of sedimentary nucleic acids to benthic disturbance in the Central Indian Basin. **Marine Georesources and Geotechnology**, 23:289-297.

4) Desa, E., Suresh, T., Matondkar, S. G. P., Desa, E., Goes, J., Mascarenhas, A., Parab, S. G., Shaikh, N., and **C.E.G. Fernandes** (2005). Detection of Trichodesmium bloom patches along the eastern Arabian Sea by IRS-P4/OCM ocean color sensor and by in-situ measurements. **Indian Journal of Marine Sciences**, 34(4): 374-386.

Papers published in Non SCI Journals:

1) **Fernandes C.E.G.**, Anindita Das, Sonali S. Naik, Rahul Sharma and P. A. Loka Bharathi (2007). Immediate effect of simulated sand mining on the variation of bacterial parameters in coastal waters of Kalbadevi bay, Ratnagiri. National Seminar on Exploration, Exploitation, Enrichment and Environment of Coastal Placer Minerals (PLACER 2007), 25-26, March 2007 organized by Central Mechanical engineering Research Institute, Durgapur and Central Mining Research Institute, Dhanbad at CMERI Campus, Durgapur, India, pp 270-277.

2) **Fernandes C.E.G.**, Anindita Das, Daphne G. Faria and P. A. Loka Bharathi (2005). Microbiology and Biochemistry of placer rich beach sediment: Short term response to small scale simulated mining. National Seminar on Development planning of Coastal placer minerals (Placer-2005), 26-27, October 2005 organized by Manonmanium Sundaranar University, Tirunelveli, Tamil Nadu and Central Marine Research Institute, Dhanbad, India, pp 248-255

3) Anindita Das, **Fernandes C.E.G.**, Sonali S. Naik, B. Nagender Nath and Loka Bharathi, P. A. (2005). Bacterial responses to contrasting geochemistry in the sediments of Central Indian Ocean Basin. National Seminar on Polymetallic Nodules, 29-30, September, 2005 organized by Regional Research laboratory, Bhubaneswar, Orissa, India, pp 1-8.

Poster presentation

1) Maria-Judith Gonsalves, **Fernandes C.E.G.**, Sheryl O. Fernandes, Sujith P.P, Anindita Das, Sonali Naik, P.A. Loka Bharathi (2009). Perspectives of microbial diversity in varied marine ecosystems in the Indian Ocean region. International Census of Marine Microbes 454 Users Spring Meeting, April 6-9th, 2009, Woods Hole, Massachusetts, USA.

2) Maria-Judith Gonsalves, **Fernandes C.E.G.**, P.A. Loka Bharathi (2008). Active bacterial populations associated with the deep sediments (~290 mbsf) of NGHP-01-01 site in the Kerala - Konkan (KK) Basin, West Coast of India. National Gas Hydrate Conference, New Delhi.

3) Sonali S. Naik, Manisha Fushe, Anindita Das, **Fernandes C.E.G.** and P. A. Loka Bharathi (2006). Evolution of culturable diversity of deep sea sediments: Effect of simulated disturbance. 7th Asia Pacific Marine Biotechnology Conference (7th APMBC - India '06), 2-5, November 2006 organized by National Institute of Oceanography, Goa, India. pp 210, P6-20

4) **Fernandes C.E.G.**, Maria Judith B. D'Souza, Shanta Nair and P. A. Loka Bharathi (2004). RNA/DNA ratio in the deep sea sediments of Central Indian Ocean Basin. Conference on Microbiology of the Tropical seas, 13-15, December, 2005 organized by National Institute of Oceanography, Goa, India. (MBGC(P)-04.

5) Ingole, B. S., **Fernandes C.E.G.** and C. Mohandass (2004) The sediment reworking activity of polychaete Nephthys sp. on the intertidal beach at Miramar, Goa and its influence on the interstital water and microbiota. Conference on Microbiology of the Tropical seas, 13-15, December, 2005 organized by National Institute of Oceanography, Goa, India. (MME(P)-08.

Presentation/Abstracts :

1) **Fernandes, C.E.G.,** Fernandes, S.O., Gonsalves M.J.B.D., Das A., Sujith P.P., Loka Bharathi P.A. (2010). Benthic bacterial diversity: iron ore dominated mangroves versus ilmenite-rich beach sediments. J. Frederick Grassle Science Symposium on the *Census of Marine Life* (Census of Marine Life – A Decade of Discovery 2010) Oct 4-6, The Royal Society, Central London, England.

2) Fernandes S.O., Gonsalves M.J.B.D., **Fernandes C.E.G**., Sujith P.P., Das A., Naik S., Loka Bharathi P.A. (2010). Insights on bacterial diversity in varied marine ecosystems in the Indian Ocean region with special reference to mangroves. International Conference on Aquatic Microbiology (Status, Challenges & Opportunities) Sept 2-4, CAS in Marine Biology, Annamalai University, Tamil Nadu, India.
"Best paper award" for the paper

3) **Fernandes C.E.G.**, Nisha Pillai, Sonali S. Naik, Anindita Das and P. A. Loka Bharathi (2006). Placer deposits reduce diversity and function: Examples from culturable bacteria of Kalbadevi beach, Ratnagiri. 7th Asia Pacific Marine Biotechnology Conference (7th APMBC - India '06), 2-5, November 2006 organized by National Institute of Oceanography, Goa, India. pp 53, S3-02.

4) Anindita Das, Sonali S. Naik, Neelam Sharma, Mohamed Riyas Theyyambattil, **Fernandes C.E.G.** and P. A. Loka Bharathi (2006). Abundance, diversity and activity of culturable bacteria in central Indian Ocean Basin. 7th Asia Pacific Marine Biotechnology Conference (7th APMBC - India '06), 2-5, November 2006 organized by National Institute of Oceanography, Goa, India pp 52, S3-01

5) S. G. Prabhu Matondkar, E. Desa, Sushma G. Parab, Nasreen Shaikh, and **Fernandes C.E.G.** (2003). Phytoplankton biomass and production in the Northern Arabian Sea during November 1999, March 2000 and November 2001. International workshop on "Biogeochemical Processes in the Northern Indian Ocean." 24-25, February, 2003 organized by National Institute of Oceanography, Goa, India and Center for Tropical Marine Ecology, University of Bremen, Germany

6) Sushma G. Parab, S.G. Prabhu Matondkar, Nasreen Shaikh, **Fernandes C.E.G.** and E.S. Desa (2003). Composition of phytoplankton in the Northern Arabian Sea. International workshop on "Biogeochemical Processes in the Northern Indian Ocean." 24-25, February, 2003 organized by National Institute of Oceanography, Goa, India and Center for Tropical Marine Ecology, University of Bremen, Germany