STUDIES ON PARTICLE ASSOCIATED MICROORGANISMS IN COCHIN ESTUARY AND THEIR RESPONSE TO METAL POLLUTION

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Studies on Particle Associated Microorganisms in Cochin Estuary and their response to metal pollution

Ph.D. Thesis under the Faculty of Environmental Studies

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Certificate

This is to certify that the research work presented in this thesis entitled **"Studies on Particle Associated Microorganisms in Cochin Estuary and their response to metal pollution"** is based on the original work done by Ms. Sheeba V A (Reg. No. 4062), under my supervision at CSIR-National Institute of Oceanography, Regional Centre, Kochi, 682018, in partial fulfillment of the requirements for the award of degree of **Doctor of Philosophy** in **Environmental Sciences** under the Faculty of **Environmental Studies**, Cochin University of Science and Technology, Kochi, 682039 and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition in any universities or institutes. All the relevant corrections and modifications suggested by the audience and recommended by the doctoral committee of the candidate during the pre-synopsis seminar have been incorporated in the thesis.

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Declaration

The research work presented in this thesis entitled **"Studies on Particle Associated Microorganisms in Cochin Estuary and their response to metal pollution"** submitted in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy, is a bonafide record of research work done by me under the supervision of Dr. P. S. Parameswaran, Chief Scientist & Scientist-in-Charge (Rtd), CSIR-National Institute of Oceanography, Regional Centre, Kochi 682018. No part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition in any other similar title or recognition in any universities or institutes.

Kochi –18
February 2018

Sheeba V. A.

Dedicated to

my dear parents and teachers

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Sheeba

Preface

Estuaries are the connecting link between the marine and fresh water regions, experiencing multitudes of complex physical, chemical and biological (including biochemical) processes, leading to several biogeochemical transformations / products. An estuary may be termed as a bioreactor because of its high productivity and heterotrophic microbial activity. The dynamic nature of estuaries due to continuous river discharge, tidal currents, resuspension etc. renders it turbid for most of the time. The particulate matter, also functioning as a hotspot of microbial activities, play significant role in orchestrating various biogeochemical processes in the estuary including vertical transport of organic matter, food web dynamics, trace metal cycling, remineralization of organic matter, etc. Hence the studies on chemical and biochemical characterization of particulate matter and diversity and eco-physiology of associated microorganisms would help to expand our knowledge of understanding its role in the ecosystem functioning in the environment.

Estuaries were subjected to various stresses of anthropogenic origin including metal pollution from industries, land runoff, harbor activities etc. Although the metal pollution and its impact on higher forms of life in world estuaries are well studied, its impact on the diversity and eco-physiology of the particulate matter (PM) associated bacteria are least understood. The present study focuses on understanding the effect of metal pollution on the particulate matter composition and its impact on diversity, metal resistance and enzyme expression profile of particle associated microorganism in Cochin estuary situated along the southwest coast of India.

The thesis is divided into seven chapters. The introductory chapter (Chapter 1) provides a general description of the study area as well as the relevance, aims and objectives of the study being undertaken. A literature survey, consolidating the existing knowledge based on the previous reports relevant to the present study is presented in Chapter 2. The spatio-temporal variations in the chemical and biochemical characteristics of particulate matter in Cochin estuary and the community structure of the particulate matter -associated archaea and bacteria are discussed in Chapter 3. The isolation and molecular taxonomy of heterotrophic bacteria are discussed in Chapter 4 while their metal tolerance levels and enzyme expression profile are discussed in Chapter 5 and 6 respectively. Chapter 7 provides the summary and conclusions of the study.

Contents

Chapter 1

INTRODUCTION..01 -08

1.1 General introduction.. 01
1.2 Objectives 08

Chapter 2

REVIEW OF LITERATURE...09 - 38

2.1 Chemical and biochemical properties of particulate matter 10
2.2 Particle associated microorganisms 12
2.2.1 Environmental influence on particulate associated microorganisms 19
2.3 Heavy metals 21
2.4 Heavy metal pollution in estuaries 22
2.4.1 Heavy metal pollution studies in world estuaries..................... 23
2.4.2 Heavy metal pollution studies in Indian estuaries 25
2.4.3 Heavy metal pollution in Cochin estuary 27
2.5 Impact of metal- microbe interactions on environment and human health 31
2.5.1 Microbial community structure... 31
2.5.2 Enzymes and Biogeochemical cycles 32
2.5.3 Metal – Microbe interaction, Microbial resistance and Bioremediation 34
2.5.4 Metal pollution in estuaries and its impact on food chain and human health 36

Chapter 3

CHEMICAL AND BIOCHEMICAL CHARACTERIZATION AND MICROBIAL COMMUNITY STRUCTURE OF PM IN CE........ 39 - 73

3.1 Introduction.. 39
3.2 Materials and Methods 40
3.2.1 Sample collection and preservation.. 40
3.2.2 Analysis of environmental variables 42
3.2.3 Biochemical characterization of PM 43
3.2.4 Heavy metal analysis of water and PM samples 45
3.2.5 Abundance of archaea and bacteria using Fluorescent in situ hybridization (FISH) 46
3.2.6 Community structure of archaea and bacteria using DGGE..... 47
3.2.7 Statistical Analysis 50
3.3 Results 50

3.3.1 Environmental variables and dissolved heavy metals 50
3.3.2 Biochemical and chemical characterization of PM 52
3.3.3 Microbial abundance and community structure 57
3.3.4 Environmental influence on microbial distribution.................. 64
3.4 Discussion 67

Chapter 4
ISOLATION AND IDENTIFICATION OF PARTICLE-ASSOCIATED BACTERIA .... 75 - 92
4.1 Introduction 75
4.2 Methodology 76
4.2.1 Sample collection and preservation: 76
4.2.2 Isolation and identification of particle-associated bacteria............. 78
4.2.3 Data analysis 80
4.3 Results 81
4.4 Discussion 90

Chapter 5
METAL TOLERANCE PROFILE OF PARTICLE ASSOCIATED BACTERIA .... 93 - 106
5.1 Introduction 93
5.2 Methodology 94
5.2.1 Heavy metal tolerance of PAB: 94
5.3 Results 96
5.3.1 Zinc Tolerance 96
5.3.2 Cadmium Tolerance 97
5.3.3 Copper Tolerance 98
5.3.4 Nickel Tolerance 100
5.4 Discussion 103

Chapter 6
ENZYME EXPRESSION PROFILE OF PARTICLE-ASSOCIATED BACTERIA .... 107 - 120
6.1 Introduction 107
6.2 Methodology 110
6.2.1 Bacterial isolates for the study 110
6.2.2 Preparation of crude enzyme for the assay 110
6.2.3 Enzyme assay 111
6.2.4 Statistical analyses 112
6.3 Results 112
6.3.1 Alkaline Phosphatase activity 113
6.3.2 β–glucosidase activity 113

6.3.3 Aminopeptidase activity 114
6.3.4 Lipase activity 114
6.4 Discussion...117

Chapter 7
SUMMARY AND CONCLUSION...121 - 128

References ....129 - 175

Appendix 177

Publications ....179 - 208

List of Tables

Table 2.1 Diversity of Particle associated bacteria from various environments 16

Table 2.2 Phylum level classification of Particle associated bacteria reported from various studies 17

Table 2.3 Diversity of Particle associated archaea from various environment. 19

Table 2.4 Heavy metals accumulated in the Particulate matter from different world estuaries. 24

Table 2.5 Heavy metals accumulated in the Particulate matter from different Indian estuaries 26

Table 2.6 Highest value of heavy metals reported from the sediment of CE irrespective of seasons. 30

Table 2.7 Highest value of heavy metals reported in dissolved form from CE irrespective of seasons. 30

Table 2.8 Highest value of heavy metals reported in Particulate matter of CE irrespective of seasons. 31

Table 3.1 Trace metal extracted from the standard reference material PACS-2 (n=4) 46

Table 3.2 Details of PCR primers used in the study 49

Table 3.3 Environmental variables and heavy metal of water column in study area during wet and dry season 52

Table 3.4 Biopolymeric carbon (BPC) content of particulate matter. 54

Table 3.5 Biochemical and chemical characterization of particulate matter 55

Table 3.6 Number of Operational Taxonomic Units (OTUs) obtained from DGGE profile 60

Table 3.7 Shannon-Weiner Diversity Index of Planktonic and PM attached bacteria and archaea 62

Table 3.8 Sediment quality guidelines by Long (1995) 68

Table 4.1 The details of the identity of PAB isolated from the downstream of the CE and MMRF number assigned to them 81

Table 4.2 The details of the identity of PAB isolated from the upstream of the CE and MMRF number assigned to them 83

Table 5.1 Concentration ranges of various metals used for the study 96

Table 5.2 PAB showed maximum tolerance index against Zn, Cd, Cu and Ni in upstream and downstream of the estuary. 101

Table 6.1 Technical details of fluorescent substrates and buffers used in each assay and the excitation and emission maxima of each dye released 112

Table 6.2 The range of enzyme activity of the PAB 113

Table 6.3 PAB showing high (>10 $\mu M^{-1} ml^{-1} h^{-1}$) range of enzyme activities in CE 115

Table 6.4 Analysis of variance (ANOVA) between the PAB enzymes from downstream and upstream of CE 116

List of Figures

Figure 1.1 Size spectra of the major particulate and dissolved organic constituents in aquatic systems 03

Figure 1.2 a) Vembanad Lake in South-West India b) enlarged view of Cochin estuary... 06

Figure 2.1 Schematic representation of various role of particle attached bacteria in estuaries 14

Figure 2.2 Zn and Cd concentrations reported in the Cochin estuary during the past years. 29

Figure 2.3 Heavy metal efflux systems 36

Figure 3.1 Study Area of Cochin Estuary andsampling stations.......... 41

Figure 3.2 Schematic representation of analysis strategies 42

Figure 3.3 Representative image of Planktonic Archaea (A) and PM- associated Archaea (B) using FISH 57

Figure 3.4 Whisker box plot showing the abundance of Archaea........ 58

Figure 3.5 Whisker box plot showing the abundance of Bacteria........ 59

Figure 3.6 Representative image of Planktonic Bacteria (A) and PM- associated Bacteria (B) using FISH 59

Figure 3.7 DGGE profile of Particle attached archaea and Planktonic archaea during wet and dry season................... 60

Figure 3.8 DGGE profile of Particle-attached bacteria and Planktonic bacteria during wet and dry season 61

Figure 3.9 Cluster analysis of Planktonic and PM-attached archaea during wet and dry season 62

Figure 3.10 Cluster analysis of Planktonic and PM-attached bacteria during the wet and dry season. 63

Figure 3.11 Schematic diagram showing the two-tailed correlation of the abundance and diversity index of archaea and bacteria with heavy metal concentration and biochemical characteristics of PM. 65

Figure 3.12 Redundancy analysis showing the influence of hydrographic variables and heavy metal concentrations on the abundance and diversity of Planktonic and PM associated archaea and bacteria 66

Figure 4.1 Map of northern part Cochin estuary showing the upstream and downstream, with details of station locations 77
Figure 4.2 Major group of PAB at phylum level isolated from the downstream (A) and upstream (B) of the CE 85
Figure 4.3 Venn diagram showing the similarity between the diversity of PAB isolated from downstream and upstream 86
Figure 4.4 Genus level diversity of PAB isolated from upstream and downstream 87
Figure 4.5 Rooted neighbor-joining phylogenetic tree based on 16S rRNA gene sequences retrieved from the DNA of the isolated cultures from the estuary. 88
Figure 5.1 Representative image showing the total length (TL) of streak and growth length (GL) of heterotrophic bacteria in plate diffusion assay 95
Figure 5.2 Tolerance of PAB from upstream and downstream towards increasing concentrations of Zn. 97
Figure 5.3 Tolerance of PAB from upstream and downstream towards increasing concentrations of Cd. 98
Figure 5.4 Tolerance of PAB from upstream and downstream towards increasing concentrations of Cu. 99
Figure 5.5 Representative images showing Cu tolerance assay of PAB 99
Figure 5.6 Tolerance of PAB from upstream and downstream towards increasing concentrations of Ni. 100
Figure 5.7 Venn diagram showing the Multiple Metal Resistance (MMR) of PAB 102
Figure 6.1 Schematic representation of the interaction of heavy metals with microbial enzymes 109
Figure 6.2 The alkaline phosphatase, amino peptidase, β- glucosidase and, lipase activities of PAB isolated from downstream and upstream of CE 116

List of Abbreviations

PM	-	Particulate Matter
POM	-	Particulate Organic Matter
DOM	-	Dissolved Organic Matter
PAB	-	Particle Associated Bacteria
PAA	-	Particle Associated Archaea
PLB	-	Planktonic Bacteria
PLA	-	Planktonic Archaea
RDA	-	Redundancy analysis
CE	-	Cochin Estuary
MMR	-	Multiple Metal Resistance
ANOVA	-	Analysis of variance
DGGE	-	Denaturing Gradient Gel Electrophoresis
FISH	-	Fluorescent Insitu Hybridisation
OTU	-	Operational Taxonomic Unit
EDTA	-	Ethylene Diamene Tetra Acetic acid
TAE	-	Tris - Acetic acid-EDTA buffer
TE	-	Tris- EDTA buffer
SDA	-	Sodium Dodecyl Sulphate
TCA	-	Trichloro Acetic Acid
SPM	-	Suspended Particulate Matter
POC	-	Particulate Organic Carbon
PON	-	Particulate Organic Nitrogen
BPC	-	Biopolymeric carbon
CHO	-	Carbohydrate
PRT	-	Protein
LPD	-	Lipid
APDC	-	Ammonium pyrrolidine dithiocarbamate
MUF	-	4-methylumbelliferyl
AMC	-	7-amino-4-methylcoumarin

MMRF	-	Marine Microbial Reference Facility
ICP-OES	-	Inductively Coupled Optical Emission Spectrometry
BLAST	-	Basic Local Alignment Search Tool
NCBI	-	National Center for Biotechnology Information
PCR	-	Polymerase Chain Reaction
H’	-	Shannon Wiener Diversity index
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
PN	-	Particulate Nitrogen
MIBK	-	Methyl Isobutyl Ketone

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Chapter 1

INTRODUCTION

Contents

1.1 General Introductino
1.2 Objectives

1.1 General Introduction

Estuaries are transition zones connecting both fresh- and marine waters and are greatly influenced by terrestrial discharge and oceanographic processes. A classical definition of the estuary, still quoted frequently (Cameron and Pritchard, 1963) is: *"an estuary is a semi-enclosed coastal body of water which has a free connection with the open sea and within which sea water is measurably diluted with fresh water derived from land drainage"*. There are nearly 1200 estuaries across the world among these, 30 estuaries are in India (ESRI, DeLorme). Majority of the major estuaries in India are located on the west coast, especially in the coastal regions of Gujarat. The major estuaries in other coastal states include Narmada in Maharashtra, Mandovi-Zuari in Goa, Karwar in Karnataka, Cochin in Kerala, Cauvery in Tamil Nadu, Godavari, Krishna and Pennar in Andhra Pradesh, Mahanadi in Odisha and Ganges delta in West Bengal (Centre for Coastal Zone Management and Coastal Shelter Belt and ENVIS). Majority of the urban cities and industrial belts in India and abroad are built along the banks of estuaries and hence contributes substantially to the economic

and cultural growth of any country (Zwolsman, 1994). It is predicted that nearly 75 % of the world population would be moving towards the peripheries of coastal ecosystems in future (Little et al., 2017; Von Glasow et al., 2013). The increasing growth of industrialization and human population density imparts more pressure on the estuarine and coastal ecosystems which makes them a more vulnerable habitat.

Every estuary has its own characteristic features in terms of morphology, climatic zone, tides, chemical and biological processes, and it supports a wide range of organisms (Flemer and Champ, 2006). Estuaries are ecotone areas of marine and freshwater ecosystems and most of the physical, chemical, and biological processes are specific to this habitat. Estuaries are very dynamic and productive ecosystem, in which various material transformations occur (Dolbeth et al., 2007; Schlesinger and Bernhardt, 2013). The estuaries are more dynamic and the suspended particulate matter formation through re-suspension of sediment particles and continuous aggregation and disaggregation of organic matter makes them more turbid than the adjoining sea or river (Gitelson et al., 2007; Turner and Millward, 2002; Volkman and Tanoue, 2002). The estuaries facilitate the processing of the organic matter carried by rivers, before the delivery to the coastal waters. A diverse group of organisms extending from microorganisms to mammals are present in estuaries. The hydrodynamic conditions, the point and nonpoint pollutions, diversity of organisms, food web dynamics and biogeochemical cycles of estuaries have been the topic of various research programs across the globe.

The chemically and biologically complex particulate matter (PM) found in the estuaries function as the hotspot of many biogeochemical reactions. These particles are formed by various physical (erosion, breakup, resuspension), chemical (precipitation, flocculation) or biological (aggregation of small organisms by their secretions, exopolysaccharides production etc) processes (Alldredge et al., 1993; Riley et al., 1964). PM comprises mainly of living and non-living fractions originated from freshwater, estuarine and marine environments (Lee et al., 2017). The non-living fraction includes clay, silt, sand particles, minerals, dead materials or detritus, fecal matters etc. The living fractions are composed of live phytoplankton, diatoms, prokaryotes such as bacteria, archaea, fungi, virus etc (Alldredge et al., 1993; Fowler and Knauer, 1986; Simon et al., 2014; Volkman and Tanoue, 2002), which dictates the nutritional value of PM (Lee et al., 2017; Mayzaud et al., 1984; Navarro et al., 1993). The size range of major constituents of PM is shown in Fig.1.1.

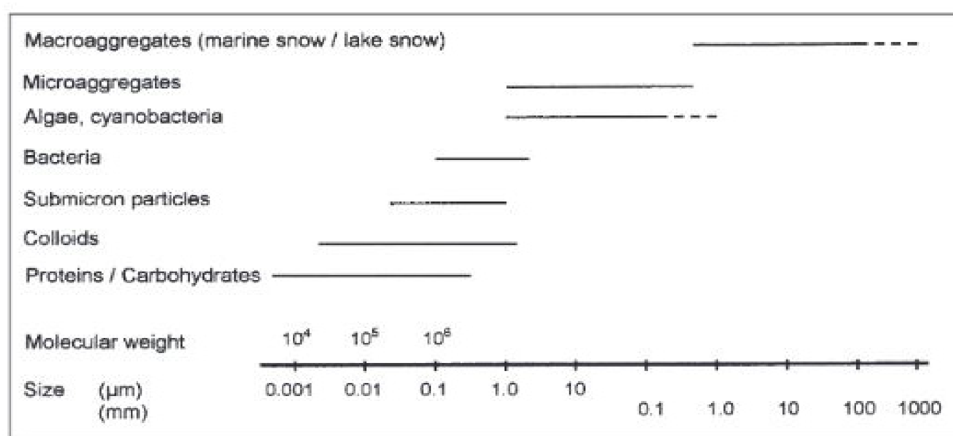

Fig. 1.1. Size spectra of the major particulate and dissolved organic constituents in aquatic systems (Kirchman, 2010; Simon et al., 2002).

Several microorganisms are also found associated with suspended particles as in any other environment with varying diversity and function depending upon the chemical and biochemical composition of PM. The PM associated microorganisms play important role in the estuarine processes. Major examples include increasing the nutritional quality of organic matter consumed by the protozoans, biogeochemical cycling of carbon, nitrogen, sulfur etc by the processes of remineralization, carbon sequestration, vertical transport of POM, nitrification and denitrification, sulfur assimilation etc. (Cavan et al., 2017; Graf et al., 1995; Turner, 2015). It is reported that the PM-associated bacteria are more active than the free-living ones (Grossart et al., 1998; Smith et al., 1995). They secrete a variety of hydrolytic enzymes to break down the high molecular weight polymers in the PM into lower molecular weight soluble compounds, Dissolved Free and Combined Aminoacids (DFAA and DCAA) and inorganic nutrients (phosphate, ammonium, nitrate, silicate) (Simon et al., 2002), readily available for food web and biogeochemical cycling. The surface of most of the particles in the aquatic environment is negatively charged, which is contributed by anionic polymers like exopolysaccharides secreted by particle attached microorganisms (Biber et al., 1994; Gibbs, 1983). The cationic metal ions attach these particles electro-statically and influence the chemical characteristics, microbial diversity and biogeochemical reactions in the PM.

Heavy metal pollution is a major problem reported from many world estuaries (Clark et al., 1989; Palma et al., 2015). The level of heavy metals would be higher in the sediments and particulate matters than their dissolved concentration in estuaries. Previous studies have shown that the

heavy metal pollution may adversely affect the diversity and activities of planktonic and sediment-associated bacteria in the estuarine and marine environment (Jiya et al., 2011;Yao et al., 2017;Zampieri et al., 2016). However, the impact of heavy metal pollution on PM associated microorganisms hitherto remains unstudied. Microorganisms interact with the metals through various mechanisms like biosorption, efflux pump, bioaccumulation in intracellular components or extracellular components etc. Metals accumulated in the particulate matter of the estuary may lead to serious environmental concern because PM can transport metals to nonpolluted regions. The PM can be transported physically by water currents and biologically into higher trophic levels or other parts of the estuary via consumption by detritivores. Although heavy metal pollution and its impact on PM associated bacteria are of local, their implications are of global nature. The major examples of the global impacts of heavy metal pollution in PM includes the emergence of metal resistant genes and their sharing for conferring resistance against antibiotics and their global transportation and impairment of nitrogen cycle leading to the enhanced release of nitrous oxide (Gullberg et al., 2014; Pal et al., 2017; Sobolev and Begonia, 2008). On the other side, the studies on metal resistant bacteria and understanding their metal resistance mechanisms would also help in appropriate designing of microbial technology for bioremediation of heavy metal polluted environments. In the present thesis, the impact of heavy metal pollution on the diversity and activities of PM-associated bacteria and archaea in Cochin estuary of Vembanad lake is reported for the first time.

Vembanad lake (Fig.1.2) is the largest lake system on the south-west coast of India (09^0 00' – 10^0 40' N Latitude and 76^0 00' -77^0 30' E Longitude) and is spread over 4 districts Kerala state, viz., Alappuzha, Kottayam, Ernakulam, and Thrissur. The importance of this lake is next only to the Arabian Sea in supporting the livelihood of coastal communities and is one of the three Ramsar sites in Kerala. In all 7 rivers (Achenkovil, Manimala, Meenachil, Muvattupuzha, Pamba, Chalakudy and Periyar river) drain into the Vembanad Lake, which adds to a total drainage area of 15,770 sq km (40 % of the area of the State), and its annual surface runoff of 21,900 Mm^3 (~30 % of the total surface water resource of Kerala) drain into this huge lake every year.

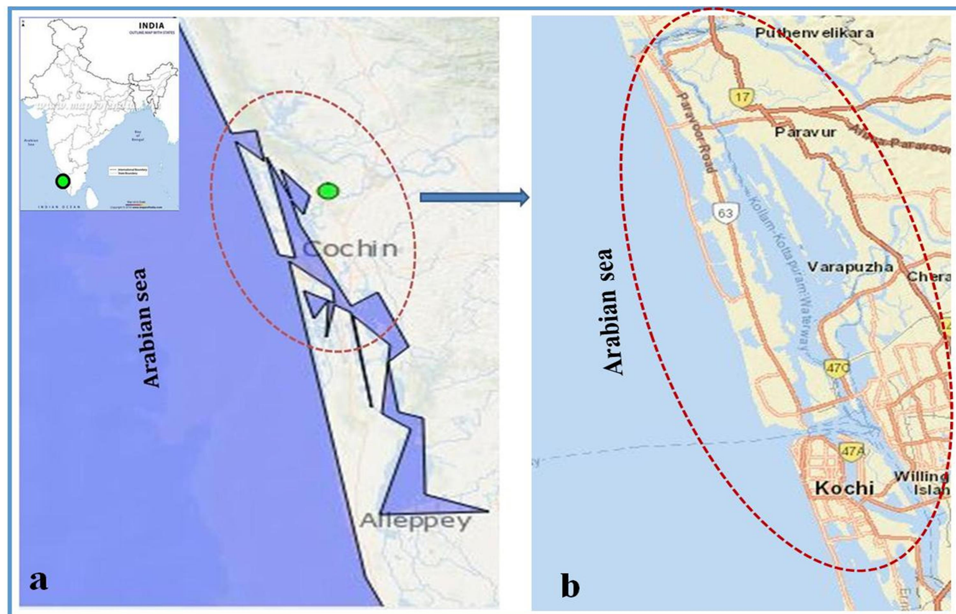

Fig. 1.2: **a)** Vembanad Lake in South-West India **b)** enlarged view of Cochin estuary (Source: Esri, DeLorme)

Cochin estuary is the part of Vembanad Lake, situated along the northern part of the lake, covering an area of ~ 25600 ha, extending from Thanneermukkam in the south to Azhikode in the north (9° 30' to 10° 12' N and 76° 10' to 76° 29' E). The nutrient composition of the estuary is greatly influenced by the anthropogenic and terrestrial inputs from six rivers, seawater influx from two bar mouths Azheekode (250m width) and Cochin (400m width) and the prolonged monsoon season (Menon et al., 2000). It is estimated that significant fractions of ~260 million litres of industrial effluents and ~235 million litres of sewage being produced per day in the industrial belt and residential areas respectively of Cochin city reach the estuary through the river Periyar (Chakraborty et al., 2014; Menon et al., 2000), land runoff and other nonpoint source sources (Jeyaprasad, 2010). The spectrum of pollutants received by CE includes metals, polyaromatic hydrocarbons and other organic pollutant & nutrients (Balachandran et al., 2006; Lallu et al., 2014; Martin et al., 2012; Ramzi et al., 2017). Among these, the concentration of heavy metals in the surficial sediments of CE has been increasing steadily ever since the beginning of industrialization in the region in the 1980s (Balachandran et al., 2006; Bindu et al., 2015; Ouseph, 1992; Salas et al., 2017; Venugopal et al., 1982). Accumulation of heavy metals in the sediment and their impact on trophic-level dynamics are the topic of research ever since (Martin et al., 2012; Mohan et al., 2012).

The present thesis aims at monitoring the chemical and biological characteristics of PM in heavy metal polluted and non-polluted regions of Cochin estuary with a view to understand its impact on the diversity and activities of associated microorganisms. Cochin estuary is the ideal site for

studying the effect of heavy metal pollution on PM associated microorganisms due to various reasons including monsoon dependent variation in the flushing rate and availability of sampling stations with metal pollution gradient which is well documented in previous studies. The present study forms the first multidisciplinary attempt at understanding the heavy metal accumulation in PM and its impact on microbial diversity and activity from Cochin estuary. The objectives of the present thesis are as follows.

1.2 Objectives

1) Chemical and biochemical characterization of PM and its effect on community structure of planktonic and particle-associated bacteria and archaea in CE
2) Isolation and Identification of particle-associated bacteria
3) Metal tolerance profile of particle-associated bacteria
4) Enzyme expression profile of particle-associated bacteria

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Chapter 2

REVIEW OF LITERATURE

Contents

2.1 *Chemical and biochemical properties of particulate matter*
2.2 *Particle associated microorganisms*
2.3 *Heavy metals*
2.4 *Heavy metal pollution in estuaries*
2.5 *Impact of metal: microbe interactions on environment and human health*

Microorganisms exist either as planktonic or in association with other living organisms or nonliving materials in the marine / estuarine environment. There exists a pronounced difference in the diversity and activities of planktonic (free living) and the above associated microorganisms, which in turn, are dictated by the chemical and biochemical properties of their micro niches (Acinas et al., 1999; Bidle and Fletcher, 1995; DeLong et al., 1993; Grossart et al., 2006). It has been reported in several studies that the microorganisms associated with particulate matter are more metabolically active than their planktonic counterparts (Crump and Baross, 1996, 2000; Crump et al., 1998; Grossart and Simon, 2007; Martinez et al., 1996). This may, at least partly be attributed to the difference in the chemical and biochemical composition of particulate matter. The present review attempts to consolidate the available physical, chemical and biological information *viz*., biochemical composition of particulate matter, hydrographic

parameters and heavy metal load, influencing the diversity and ecophysiology of associated microorganisms in the estuaries.

2.1 Chemical and biochemical properties of particulate matter

Particulate matter (PM) consist of chemically and biochemically complex particles formed by various physical, chemical and biological processes such as weathering of bulk materials, resuspension of sediment particles, death and decay of large organisms, and biological repackaging of organic matter through feeding and excretion (Simon et al., 2002) In general, particles having size greater than 0.45 µm are considered to be particulate matter (PM). The particulate matter (PM) might consist of inorganic (PIM) or organic materials (POM) or both. Particulate Inorganic Materials (PIM) mainly consists of clay minerals, heavy metals, insoluble salts etc. Particulate Organic Materials include complex matrices of terrigenous and autochthonous organic matter, living cells of planktonic microorganisms, small phytoplanktons, organic debris, nonliving microorganisms, etc (Simon et al., 2014; Suzumura et al., 2004).

Organic matter in aquatic environment can be classified based on their size and composition. The size based classifications include particulate- (POM) and dissolved organic matter (DOM). It is an operational classification and the exact particle size of DOM is still debatable. Different studies classified POM as particles less than 10 µm which are retained in filter paper having pore size of 0.22, 0.45 and 0.7 µm, while the particles which pass through these filter papers are classified as DOM (Jiao et al., 2010; Simon et al., 2014; Simon et al., 2002; Volkman and Tanoue, 2002). The large composites of organic matter (> 500 µm) found in the marine

ecosystems are termed as marine snow (Kirchman, 2010; Ploug and Grossart, 2000; Simon et al., 2002; Volkman and Tanoue, 2002). The POM found in estuaries are mainly composed of microalgal cells, bacteria, fine organic detritus and inorganic particles such as coccoliths, diatom skeletons and clay particles have an organic coat (Volkman and Tanoue, 2002). The marine snow includes larger microalgae (especially diatoms), phytoplanktons, detritus and fecal pellets, and these particles are formed from the biological repackaging of organic matter through feeding and excretion, clay and other inorganic forms.

The POM from limnetic and marine habitats are reported to harbor different group of microorganisms (Bizic-Ionescu et al., 2014). Limnetic particles are more abundant, densely colonized and smaller in size than marine particles. Apart from the biochemical composition of the POM, the interactions between estuarine circulation, resuspension and sinking particles are also reported to influence the microbial diversity of POM by adjusting the micro-niche within the POM (Bano and Hollibaugh, 2000). The microbial exopolysaccharides present in the POM are rich in charged functional moieties, which may further modulate the chemical characteristics of the POM by functioning as binding site for other charged particles including heavy metals (Decho, 2000; Geesey et al., 1988; Mittelman and Geesey, 1985). The exopolysaccharides (EPS) is widely employed in bioremediation of heavy metals including Pb, Ni, Cd, etc.(Brown and Lester, 1982; Dong and Zhang, 2005; Loaec et al., 1997). Both exopolysaccharides (EPS) and transparent exopolysaccharides (TEP) have been shown to play an important role in aggregation, which in turn regulates the flux of organic matter in marine and estuarine environments (Passow et al., 2001).

Studies on the sedimentary particles of Dona Paula bay by Desouza et al (2003) showed that the carbohydrate content of particulate matter are derived from the diatoms. The particles showed a positive correlation with biogenic silica and found that the source of carbohydrates in the bay varies over a period of the study. A comparison study conducted by Sarma et al (2014) on particulate organic carbon and particulate nitrogen content in the northern and southern estuaries in India showed that the high carbon content are present in the northern estuaries, mainly contributed by the phytoplankton productivity and showed a positive correlation with chlorophyll results. In southern estuaries PN content was higher compared to POC and was mainly contributed by terrestrial sources. About 70- 90 % of organic matter in the northern estuaries of India is mainly contributed by the freshwater algae (Sarma et al., 2014). The studies by Kessarkar et al (2010) at Mondovi estuary showed that the suspended matter mainly composed of floccules, faecal pellets and aggregates that consist of clay and biogenic particles. The studies conducted in Godavari estuary reported that there were multiple sources of organic matter in the estuary and are predominantly from the terrigeneous organic matter (Sarma et al., 2012). The further details on the alterations in the chemical composition of POM, influenced by environmental conditions are discussed in the below sessions of the present review.

2.2 Particle associated microorganisms

The microorganisms such as archaea, bacteria, virus and fungus which are attached to the particles are known as Particle associated microorganisms. Microorganisms associated with particulate matter for various reasons extending from getting nutrients for growth to protection

from grazing (Grossart et al., 2001; Ploug and Grossart, 2000). The functions of particle associated bacteria in the estuarine ecosystem include the hydrolysis of high molecular weight organic materials to low molecular weight compounds for facilitating its recirculation into the food web through absorption by microorganisms, remineralization of organic matter and vertical transport of organic matter between sediment and water column and transportation of trace metals (Cavan et al., 2017; Crump et al., 2017; Edwards et al., 2015; Grossart and Simon, 2007; Hart, 1982; Jiao et al., 2010; Lapoussière et al., 2011; Poli et al., 2010; Simon et al., 2014; Turner and Millward, 2002). Particle associated microorganisms can also short circuit the microbial loop by directly becoming the food for larger metazoans bypassing the consumption by the protozoan grazers and it also increases the nutritional quality of food for detritivores (Baross et al., 1994; Gonsalves et al., 2017). Attached bacteria can also modify the surrounding environment through the production of secondary metabolites and horizontal gene transfer through plasmids results in the evolution of new strains (Fuqua and Greenberg, 2002; Kiørboe et al., 2003). The particle attached microorganisms also involve in various biogeochemical transformations in the estuary and other aquatic systems (Acinas et al., 1997; Amalfitano et al., 2017; Crump and Baross, 2000). The intense hydrolytic enzyme activity has been shown in marine aggregates leading to the rapid dissolution of POM to DOM (Smith et al., 1992). The particle attached bacteria accounted for more than 90 % of the heterotrophic bacterial activity in Columbia estuary, which was 10 – 100 times more active than the planktonic bacteria (Crump and Baross, 1996; Crump et al., 1998). Particle attached bacteria recycles most of the detrital organic matter back

to food web and biogeochemical cycling (Crump and Baross, 1996, 2000), while the remaining organic matter refractive to the bacterial activity sinks down to benthic. The POM are generally colonized by bacteria specialized in hydrolyzing different types of complex polysaccharides (Cottrell and Kirchman, 2000). The major functions of particle associated microorganisms were depicted in Fig 2.1.The diverse particle associated bacteria obtained from different environment at Phylum and Genus level were given in the Table 2.1 and 2.2.

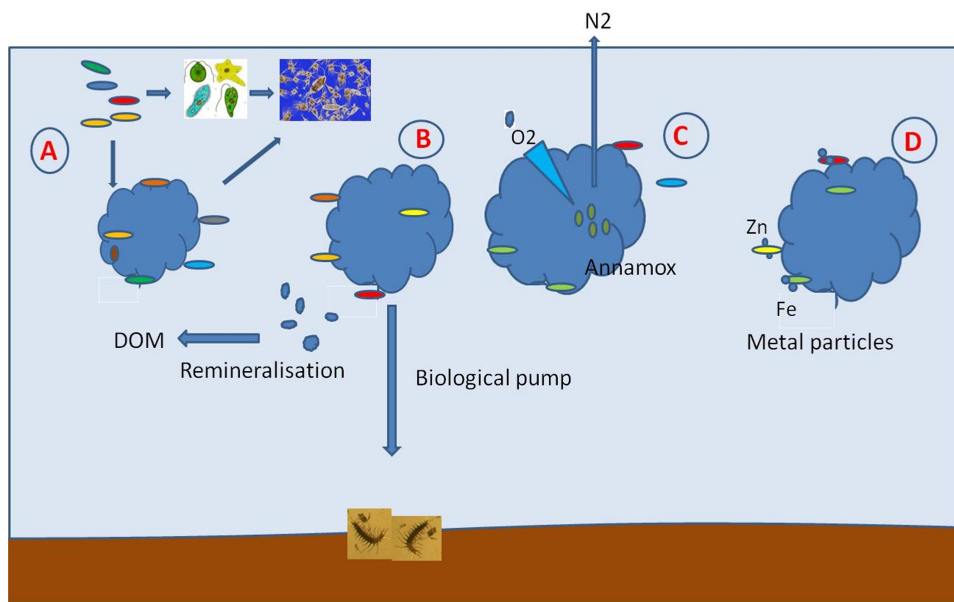

Fig. 2.1. Schematic representation of various role of particle attached bacteria in estuaries **A.** Microbial foodweb **B**. Organic matter remineralization and vertical transport as POM **C**. Biogeochemical cycling **D**. Trace metal dynamics.

The various groups of archaea were also reported associated with POM from different environments (Table 2.3). Archaea are well recorded for various types of nutritional characteristics such as heterotrophic (Teira et al., 2004), photoautotrophic (Finn and Tabita, 2003) and chemoautotrophic (Wuchter et al., 2003) in nature. Some groups of archaea can adapt to different nutritional conditions, which are mixotrophic (Qin et al., 2014). Although heterotrophic archaea has been reported from POM, very little reports are available on their role in organic matter degradation capacity in estuaries (Crump and Baross, 2000a). Although many studies indicated that the total archaeal diversity is more in estuaries compared to open ocean (Crump and Baross, 2000a), there is difference of opinion about the diversity of particle associated and free living archaea. Some studies show significant difference in the diversity of POM associated and free living archaea (Acinas et al., 1997; Galand et al., 2008), while clear difference were reported by some other studies (Orsi et al., 2016; Orsi et al., 2015; Tarn et al., 2016). Studies on particle attached archaea in Guanabara bay showed higher diversity than freeliving ones (Vieira et al., 2007).

Table 2.1: Diversity of Particle associated bacteria from various environments

Sl No	Particle associated bacteria – Diversity	Source of particles Collection	Reference
1	*Cytophaga*, *Planctomyces*, γ- *Proteobacteria*	Santa Barbara Channel - Estuary	DeLong et al. (1993)
2	β-*proteobacteria* , γ- *proteobacteria* *Alteromonas* α- *proteobacteria*	Uberlinger See Lake - Germany	Grossart and Simon (1998)
3	*Cytophaga*, α, β,δ-subclass *proteobacteria*, order *Verrucomicrobiales*	Columbia river estuary	Crump et al. (1999)
4	Ammonia oxidizing β- *proteobacteria* – *Nitrosomonas eutropha*	North western Mediterranean sea	Phillips et al. (1999)
5	α- *proteobacteria*, β- *proteobacteria*, ε- *proteobacteria, Cytophagales*, Oxygenic phototrophs	Laboratory Seawater Mesocosms	Riemann et al. (2000); Riemann and Winding (2001)
6	*Cyanobacteria*, *Bacteriodetes*, *Verrucomicrobia*, *Planctomycetes*	Northeastern Germany – lake water	Allgaier and Grossart (2006)
7	α- *proteobacteria, Bacteriodetes,* γ- *proteobacteria*	Wadden sea water	Rink et al (2007)
8	*Annamox (Candidatus Scalindua* sp), γ- *Proteobacteria, Alphaproteobacteria* and *Bacteroidetes*	Namibian OMZ	Woebken et al. (2007)
9	*Planctomycetes*, *Cyanobacteria*, *Rhodobacterales*, *Flavobacteriaceae*	Coastal ecosystem, Columbia river	Smith et al. (2013)
10	α- *proteobacteria*, γ- *Proteobacteria* (*Oceanospirillales*, *Chromatiales*, *Enterobacteriales*),*Bacteriodetes*	Coastal lagoon, Canada	Mohit et al. (2014)
11	α- β- , γ- *proteobacteria,* and *Cyanobacteria, Planctomycetes, Bacteroidetes, Betaproteobacteria*	Baltic sea	Rieck et al. (2015)
12	α- *proteobacteria*, γ- *proteobacteria, δ-proteobacteria* and *Flavobacteriaceae*	Mediterranean Sea	López-Pérez et al. (2016)
13	γ- *proteobacteria* ,*Bacteroidetes*, *Planctomycetes*, *δ-proteobacteria*, β *proteobacteria*, and *Verrucomicrobia*	Southern Ocean	Milici et al (2017)
14	α- *proteobacteria* , γ- *proteobacteria*, *δ-proteobacteria, Sphingobacteriia, Flavobacteriia, Spirochaetes*, and *Fibrobacteres* (*Fibrobacteria*)	Cariaco Basin's OMZ	Suter et al. (2017)
15	γ- *proteobacteria,* α- *proteobacteria* and *Flavobacteria*	Tropical and Subtropical Pacific Ocean	Suzuki et al. (2017)
16	*Actinobacteria*, β- *proteobacteria*, α - *proteobacteria*	Taihu lake , China	Zhao et al. (2017)
17	γ- *proteobacteria,* α- *proteobacteria* , *Firmicutes, Actinobacteria*,	Urban recreational water, Beijing	Fang et al. (2018)

Table 2.2. Phylum level classification of Particle associated bacteria reported from various studies

Sl No	Phylum		Genus	Reference
1	*Proteobacteria*	α- *proteobacteria*	*Rhodobacter capsulatus, Rhodobacter sphaeroides, Paracoccus aminophilus, Blastobacter natatorius, Erythrobactr longus, Rhizobium Agrobacterium, Roseobacter spp., Sagittula stellata, Antarctobacter Heliotherms, Ruegeria atlantica, Silicibacter sp, Roscivivax sp. SAR 11 cluster,Pelagibacter sp, Sulfitobacter sp, Brevundimonas sp, Sphingobacterium sp, Loktanella sp, Pseudophaeobacter sp, Rhodospirillaceae , SAR 116 clade, Sphingomonas sp, Thalasospira sp.*	(Allgaier and Grossart, 2006; Crump et al., 1999; Fang et al., 2018; López-Pérez et al., 2016; Milici et al., 2017; Mohit et al., 2014; Riemann et al., 2000; Rink et al., 2007; Suter et al., 2017; Suzuki et al., 2017; Woebken et al., 2007)
		β- *proteobacteria*	*Ralstonia pickettii, Leptothrix sp, Rubrivivax subgroup, Alcaligens Faecalis, Nitrosomonas eutropha, Nitrospira sp, Rhodopherax sp,Janthinobacterium sp, Delfia sp.*	(Crump et al., 1999; Milici et al., 2017; Phillips et al., 1999; Woebken et al., 2007)
		γ- *proteobacteria,*	*Oceanospirillum group (Marinobacter sp , Marinomonas sp), Methylobacter sp, Thiothrix nivea, Xanthomonas campestris, Xanthomonas vesictatoria, Alteromonas sp, Alkalilimnicola sp, Nitrosococcus sp, Vibrio sp, Acinetobacter sp,Chromatials, Enterobacterales, Colwellia sp, Kangiella sp, SAR 86, Salinisphaeraceae , Ectothirhodospiraceae, Pseudomonades, Aeromonas sp.*	(Crump et al., 1999; Fang et al., 2018; López-Pérez et al., 2016; Milici et al., 2017; Mohit et al., 2014; Riemann et al., 2000; Rink et al., 2007; Suter et al., 2017; Suzuki et al., 2017; Woebken et al., 2007)
		δ-proteobacteria	*Desulfohopalus vacuolatus, Desulfovibrio sp, Geobacter metallidurans, Bdellovibrio bacteriovorus SAR 324 Cluster, Nannocystaceae, Desulfobulbaceae*	(Crump et al., 1999; López-Pérez et al., 2016; Milici et al., 2017; Suter et al., 2017; Woebken et al., 2007)
		ε- *proteobacteria*	*Acrobacter nitrofigilis*	(Riemann et al., 2000; Woebken et al., 2007)

Table 2.2 Continued…..

2	*Bacteriodetes*	*Cytophaga marinoflava Flavobacterium aquatile, Flavobacterium ferrugineum, Flavobacterium ameridics, Flexibacter maritimus, Ulvibacter sp, Winogradskyella sp, Porphyromonas sp, Lacitrix sp, NS9- Bacteriodetes, Fulvicola sp, Lewinella sp, Crocintomixsp,Reichenbachiella sp, Sphingobacteriia sp.*	Allgaier and Grossart, 2006; Crump et al., 1999; López-Pérez et al., 2016; Milici et al., 2017; Mohit et al., 2014; Riemann et al., 2000; Rink et al., 2007; Suter et al., 2017; Suzuki et al., 2017; Woebken et al., 2007
3	*Verrucomicrobia*	*Prosthecoacter fusiformis, Verrucomicrobium spinosum, Arctic97B-4*	Allgaier and Grossart, 2006; Crump et al., 1999; Milici et al., 2017; Mohit et al., 2014; Suter et al., 2017
4	*Planctomycetes*	*Isosphaera sp,Pirellula sp,, Candidatus Scalinduasp, Planctomyces limnophilus, Rhodopirellula, Physcisphaera, OM 190*	Allgaier and Grossart, 2006; Crump, 1999; Milici et al., 2017; Suter et al., 2017; Woebken et al., 2007
5	*Cyanobacteria*	*Synecococcus sp, Prochlorococcus sp*	Crump et al., 1999; Riemann et al., 2000; Suzuki et al., 2017
6	*Actinobacteria*	*Rhodococcus sp, Mycobacterium sp*	Fang et al., 2018; Woebken et al., 2007
7	*Fibrobacteres*	*Fibrobacteria sp*	Suter et al (2017)
8	*Firmicutes*	*Clostridium sp*	Fang et al (2018)
9	*Spirochaetae*	*MSBL-8*	Suter et al (2017)

Table 2.3. Diversity of Particle associated archaea from various environment.

Sl No	Particle associated Archaea-Diversity	Source of particles Collected	Reference
1	*Euryarchaea*, *Crearchaea* relatives, *Korarchaea*	Columbia river estuary – ETM	Crump and Baross, (2000a)
2	*Thermoplasmatales*, *Methenogenic archaeon group*, *Euryarchaeon*	Guanabara bay-estuary	Vieira et al (2007)
3	*Crenarchaeota*	Namibian OMZ	Woebken et al (2007)
4	*Euryarchaeota* LDS and RC-V Cluster	Mackennzie river-Arctic	Galand et al (2008)
5	*Euryarchaeota Group* II.a.	Coastal waters of Arctic	Galand et al (2008)
6	*Crenarchaeota Marine Group* I.1a	Maine waters Beaufort Sea	Galand et al (2008)
7	*Marine Group* II *euryarchaea* (MGII)	Sea water near California	Orsi et al (2015)
8	*Marine* Group II and *Marine Group* III (*Euryarchaeota*) and *Marine Benthic Group* A *archaea*, Anaerobic methane-oxidizing taxa, ANME 2-D s	Hadal regions of Mariana Trench	Tarn et al (2016)
9	*Arc6* clade, THSCG, clade CCA47, MBG-D and *Methanomicrobia*	Cariaco Basin's OMZ	Suter et al (2017)

2.2.1 Environmental influence on particulate associated microorganisms

The environmental conditions have profound influence on the abiotic and biotic factors affecting the microbial diversity within the micro-niche of POM. The PM are considered as the hot spot of microbial processes in an estuary, the diversity and activities of these microorganisms in turn are modulated by environmental conditions such as salinity, temperature,

nutrient load etc. (Bidle and Fletcher, 1995; Garneau et al., 2009; Rieck et al., 2015; Yung et al., 2016; Zhang et al., 2007). It is not a single parameter, but a combination of parameters which influence the distribution of bacteria in any environment (Dong and Zhang, 2005; Sneha et al., 2016). For example, the concentrations of nitrogen (ammonium, nitrite and nitrate) along with the oxygen influence the diversity of nitrifiers and denitrifier. Archaea and bacteria are reported to involve in the nitrification under aerobic conditions, while it shifts to anaerobic ammonium oxidizing bacteria (anammox) under hypoxic conditions. A gradation in oxygen concentration have been reported from outside to inner core of a POM, which can create a hypoxic environment. The hypoxic environment could be created by physical conditions such as less mixing of water, pollution or due to high rate of microbial activity in the PM. The α- and γ-proteobacteria present in the PM remineralize carbohydrate aerobically which reduces the oxygen concentration within PM (Woebken et al., 2007). The hypoxic environment promotes the growth of anaerobic ammonium oxidizing bacteria inside POM, which converts ammonium directly to nitrogen using nitrite as electron acceptor (Woebken et al., 2007). The hypoxic microniche inside the core of PM also promotes the growth of facultative or obligate anaerobes. The variation in salinity can also induce a strong physiological stress on the activities of microorganisms in the estuaries (Bouvier and del Giorgio, 2002; Bouvier and Giorgio, 2002; Zhang et al., 2007) Suspended solids and turbidity is an important factor which determines the dynamics of particle associated microorganisms (Zhang et al., 2007). The influence of biochemical characteristics of particulate matter on the diversity and activity of associated microorganisms

were less studied. Some of the recent studies connecting the components (polyunsaturated aldehydes) of particulate matter have significant role in the activity and composition of attached particles (Edwards et al., 2015) and the anaerobic environment in particulate matter influence strongly the community modulation of particle associated bacteria (Zhang et al., 2016). The environmental designing of the micro-niche of POM is further done by varied types of pollutants introduced through various routes. Studies by Tang et al (2010) states that water temperature is a significant factor for the organic aggregate associated bacterial community because it would increase the plankton growth, these living and dead organisms are the main substrates for the generation of the organic aggregates. The particle attached community is also influenced by the grazing nature of the metazoans in the environment hence the dynamics of metazoans is a significant factor which determine the diversity of particle attached microorganisms (Crump and Baross, 1996, 2000; Tang et al., 2010; Wörner et al., 2000). The studies from Zuari estuary and Dona Paula Bay in Goa, India showed that the diversity and activities of PAB are influenced by the availability of substrates and resuspension of the sedimented materials (Bhaskar and Bhosle, 2008).

2.3 Heavy metals

Metals having density greater than $5g/Cm^3$ are referred to as heavy metals (Nies, 1999). There are 53 heavy metals (including As) identified so far (Weast and Astle, 1984). Many of the heavy metals at lower concentration (e.g., Zn, Cu, Mn, Co, Ni) are essential micronutrients, constituting the integral part of many structural proteins, pigments and metalloenzymes, especially those enzymes involved in various redox

processes, regulation of the osmotic pressure etc. On the other hand the heavy metals Cd, Pb, Hg, As etc are toxic even at lower concentrations. This can be explained with the example of the interaction of bacteria with some heavy metals available in the estuaries as contaminants. Zinc is an essential component of metalloenzymes and are involved in more than 300 enzymatic functions (Coleman, 1992) and bacteria are predicted to incorporate zinc into 5–6 % of all proteins (Andreini et al., 2006). Nickel is a transition metal required as a cofactor for several bacterial enzymes, like urease (Remy et al., 2013) and structural component of several metalloenzymes involved in energy and nitrogen metabolisms (Banci et al., 2013). Similarly, Copper is also an essential micro nutrient, being an important factor for electron carrier proteins (Yang et al., 1998). But they can also cause lipid peroxidation and protein damage (Dupont et al., 2011) at higher concentrations through a Fenton-like reaction. On the other side, the metals like Cd and Pb cause toxicity even at lower concentrations by inducing oxidative damage to nucleic acids (Jacobson and Turner, 1980) and by displacing Ca and Zn in proteins (Bouton et al., 2001; Stohs and Bagchi, 1995).

2.4 Heavy metal pollution in estuaries

Estuaries are productive ecosystems and considered as a bioreactors because of the immense microbial activity. It receives all type of pollutants including heavy metals from various industrial activities along the banks of the estuary. The toxic metals will affect the ecosystem functioning of the estuary. There are a lot of studies regarding status, effect of heavy metals in each components of estuary, were conducted across the world. Some of the relevant studies were discussed below.

2.4.1 Heavy metal pollution studies in world estuaries

Heavy metal pollution is a serious environmental concern of aquatic systems across the globe especially in estuaries, which receive all types of domestic and industrial wastes. The non-biodegradable nature of heavy metals increases its toxic effect which seeks special attention. Ria De Huvela estuary in Spain is reported as one of the most polluted estuary in the world which receives 895.1 kg/h of metals with major contributions from Tinto Odiel river (Nelson and Lamothe, 1993). The metal toxicity, persistence and composition in this estuary are incomparable with other metal contaminated estuaries (Sainz et al., 2004). The major hazardous heavy metals, Cd and Pb were reported between 200 to 3000 µg/g and 10-1000 µg/g respectively in this estuary. The Pearl river estuary is one of the most heavy metal polluted estuaries in China (Zhou et al., 2004). Although industrially developed, the heavy metals accumulated in suspended sediments of estuaries in China (Yalujiang, Shuangtaizihe, Luanhe, Jiaojiang and Zhujiang) were reported to be low compared with the industrial input to the estuaries of Europe and North America (Zhang and Liu, 2002). The high levels of heavy metal pollution have been reported from various estuaries in UK, which include Mersey estuary (Cd, Pb) (Hurley et al., 2017), Loughr estuary (Cr, Sn) (Bryan and Langston, 1992).The reduction in the concentration of heavy metals such as Ag and Cd has been reported from Severn estuary, UK by Duquesne et al (2006) compared to the values reported by Bryan and Langston (1992). Most of the previous studies focused on reporting heavy metals in the sediment as it is considered to be the major sink of heavy metals in estuaries. Later studies identified suspended matter as a carrier and chelator of heavy

metals (Turner and Millward, 2002). A report on the previous studies related to heavy metal accumulation in particulate matter from world estuaries are consolidated in Table 2.4. The toxicity of the heavy metals accumulated in particulate matter is also a serious environmental concern. The toxicity of metals mainly depends on the bioavailabilty, which is controlled by various factors extending from the physical and hydrographic variables of the environment (salinity, pH, redox process, bioturbation etc), the composition of particulate matter, speciation of metals, the affinity of heavy metals to the organic matter and transformation susceptibility such as methylation (Bryan and Langston, 1992).

Table 2.4. Heavy metals accumulated in the Particulate matter from different world estuaries.

World Estuaries	Author Year	Zn (µg/g)	Cd (µg/g)	Fe (µg/g)	Ni (µg/g)	Mn (µg/g)	Cu (µg/g)	Co (µg/g)	Pb (µg/g)
Changjiang (Yangtze River) estuary, China	Zhang, (1999)		0.47		35.5	871	49.4		35.5
Changjiang (Yangtze River) estuary, China	Yao et al (2016)	238	0.75				70		38
Guanabara Estary (Brazil)	Melo et al (2015)	1039.5	11.7						1.9
Tagus estuary (Portugal)	Duarte and Caçador, (2012)	8689	26	-	50	-	16	41	157
Hudson river estuary (New York)	Feng et al (2002)	292	1.46	6.08	-	-	223	-	168
Scheldt estuary (France)	Van Ael et al (2017)	820	4.8	-	-	-	-	-	145
Daliao river estuary (North- East China)	Ma et al (2015)	884.69	10.21	22.5	228.35	7505	228.35	93.35	671.34
Rio Tinto and Rio Odiel river estuary (Spain)	Achterberg et al (2003)	800	4.1	11.6			440		
Mersay estuary (UK)	Martino et al (2002)	430.20	9.21	30.4	22.30	29100	26.05	1.12	93.24

2.4.2 Heavy metal pollution studies in Indian estuaries

It is found that 13 out of 88 industrial clusters are situated near the coastal waters of India, constantly polluting our coastal waters and estuaries (CPCB, 2009). Out of these 13 industrial clusters, five, Chembur (Maharashtra), Cochin greater (Kerala), Tarapur (Maharashtra), Vapi (Gujarat) and Visakhapattanam (Andhra Pradesh) are identified as the critically polluted ones. The industries of the greater Cochin industrial area are mainly located along the banks of river Periyar and produce ~260 million litres of industrial effluents per day, significant fraction of which, ultimately reach the estuary. The heavy metal pollution in Indian estuaries have previously been reviewed by Chakraborty et al (2014). The heavy metal pollution was well studied in the estuarine sediments of east (Ganges, Godavari estuary, Krishna and Cauvery and Narmada) and west coast (Tapi, Ulhas and Cochin) of India. The intensity of metal pollution in the estuaries is in the order: Godavari > Cochin > Tapi = Cauvery > Ulhas =Narmada >Krishna > Ganges, based on the metal Pollution load index (Balachandran et al., 2006; Banerjee et al., 2012; Chakraborty et al., 2012; Ramesh et al., 1999; Rokade, 2009; Seralathan, 1987; Sharma and Subramanian, 2010). The types and concentrations of different heavy metals vary between estuaries in India, though most of them are contaminated with Cd in sediments (Chakraborty et al., 2014). In Cochin estuary the contamination factor for Pb, Zn and Cd in the sediments were found to be high compared to other estuaries (Balachandran et al., 2005; Martin et al., 2012). The metal pollution in most of the estuaries in India are reported from the water and sediments, while only a limited number of studies were conducted on the particulate matter (Kessarkar et al., 2013; Mukherjee,

2014; Suja et al., 2017). According to available data, Zn and Pb are found to be very high in the particulate matter of Indian estuaries (Table 2.5). The significance of particulate matter in complexing heavy metals was reported from Godavari estuary by Ray et al (2006). Similarly the enrichment of the heavy metal, Mn was reported from the suspended sediments of Mandovi – Zuari estuary in Goa (Kessarkar et al., 2013; Shynu et al., 2012). The study of trace metal levels in Hoogly estuary reveals large accumulation of trace metals in sediments (0.41 $gcm^{-2} year^{-1}$). It is found that Fe and Mn oxides and not organic carbon, are the main controlling factors, contributing to the accumulation of heavy metals in PM (Banerjee et al., 2012). Cochin estuary, one of the heavy metal polluted estuary in the south west coast of India, is perhaps also one of the well-studied for these aspects (Details were given in the following section).

Table 2.5. Heavy metals accumulated in the Particulate matter from different Indian estuaries

Indian estuaries	**Author Year**	**Zn (µg/g)**	**Cd (µg/g)**	**Fe (%)**	**Ni (µg/g)**	**Mn µg/g)**	**Cu (µg/g)**	**Co (µg/g)**	**Pb (µg/g)**
Kali river estuary	Suja et al (2017)	309.1	-	6.4	182.7	-	112.2	38.7	54.8
Hugli estuary	Mukherjee (2014)	115	0.14	4.8	58	717	36	17	25
Mondovi estuary	Kessarkar et al (2013)	245.22	0.55	7.6	147.3		101.07	36.89	30.06
Zuari estuary	Kessarkar et al (2013)	281..97	0.85	8.3	120.62		101.91	33.08	61.03

2.4.3 Heavy metal pollution in Cochin estuary

The level of metal pollution in Cochin estuary has been monitored continuously since 1970s, with particular focus in the sediments and water column (Table 2.6 and Table 2.7). These studies reported that the Cd and Zn are the major pollutants in the estuary (Anju et al., 2011; Mohan et al., 2012a; Ouseph, 1992). The studies by Bindu et al (2015) reported that the heavy metals Cd, Zn, Pb and Ni were very high in Cochin estuary. The accumulation of Zn and Cd in the sediments of Cochin estuary since 1980 was depicted in Fig 2.2 from the upstream and downstream regions of the estuary (Balachandran et al., 2006; Jayasree and Nair, 1995; Martin et al., 2012; Nair et al., 1990; Salas et al., 2017; Venugopal et al., 1982). It indicates that the anthropogenic impact is consistently increasing in the last few years. The concentration of Zinc and Cd were very high in the upstream sediments compared to the downstream of the estuary (Fig 2.2). Jiya et al (2011) reported high concentration of zinc in the water column of the estuary. A study by Manju et al (2014) reported very high values for the metals, Cu, Cd, Co, Cr and Pb near the industrial out let in the upstream of the estuary (Table 2.6). Different studies reported spatio-temporal variation in the distribution of heavy metals in CE starting from the river end to bar mouth. The distribution of heavy metals in the estuary is influenced by the freshwater runoff and seawater influx. The estuary freshens approximately 40 times a year mostly during the monsoon and nearly stagnant during the post monsoon (Vinita et al., 2015). The studies by Anju et al (2011) reported the accumulation of heavy metals such as Cd, Pb, Zn, in the waters of upstream estuary during monsoon season, while studies by Nair et al. (1990), Paul and Pillai (1983) reported higher

concentration of these metals during the post monsoon. The higher concentration heavy metals during monsoon season were attributed to the heavy fresh water runoff. Normally, the heavy metals carried by freshwater into the estuary will chelate to the sediment before reaching to the barmouth. However, the rapid freshening during monsoon season can also carry the sediment and particulate matter carrying the heavy metals to the barmouth. A study by Vinita et al (2017) reported that the suspended sediment concentration were maximum at the main inlet of the estuary while it decreased towards the upstream for both the seasons. The study also reported that the sediment flux in the estuary is mainly controlled by the advective factors such as fresh water flow during monsoon season while during dry season it is sometimes controlled by tidal pumping to the estuary. The study substantiates the hypothesis that the metals accumulated in the suspended particulate matter in the upstream of the estuary can be transported to the downstream of the estuary.

There are only few studies on accumulation of heavy metals in particulate matter (Table 2.8). The effect of metal pollution on chemical characterization of particulate matter and its effect on diversity and metabolic activities of associated bacteria are hitherto remains unreported. Table 2.6, 2.7, 2.8 showed the major studies and reported concentration (irrespective of seasons) of heavy metals from Sediment, Water, and Particulate matter in Cochin estuary respectively.

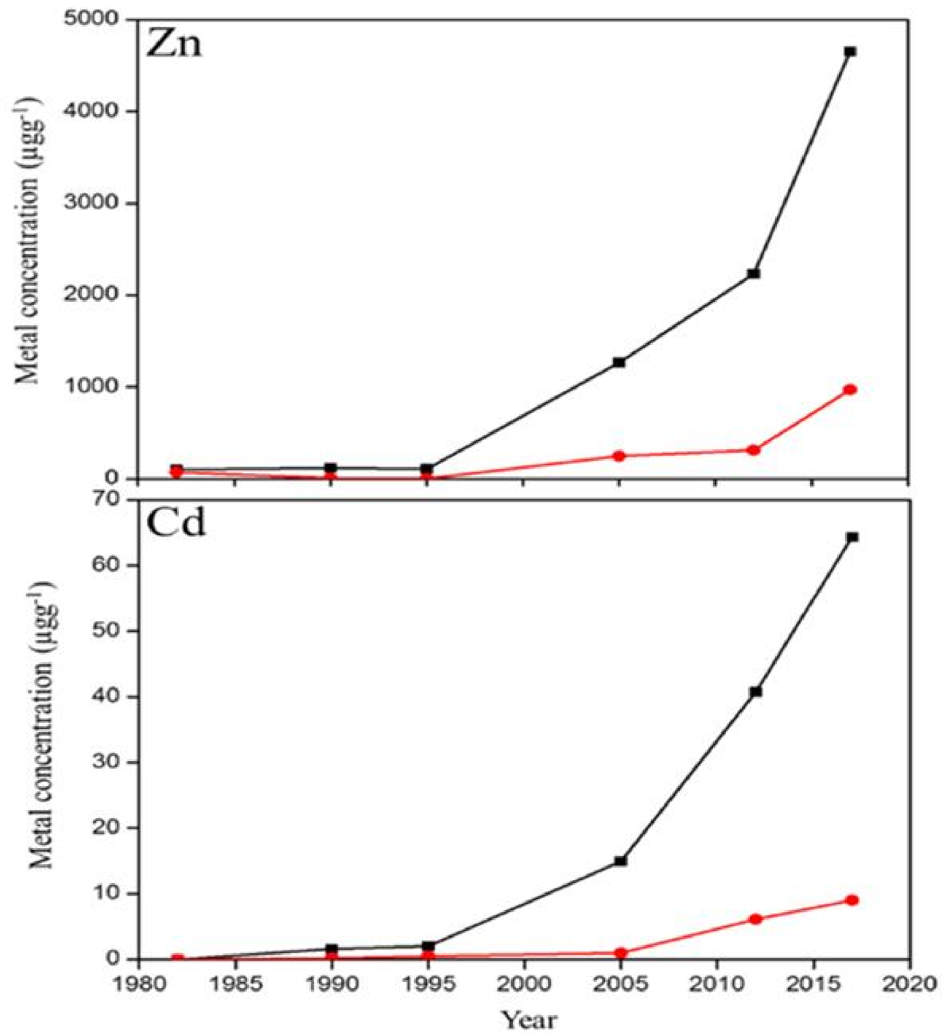

Fig. 2.2. Zn and Cd concentrations reported in the Cochin estuary during the past years. Black line - the metal concentrations from upstream, Red line –metal concentrations from downstream.

Table 2.6. Highest value of heavy metals reported from the sediment of CE irrespective of seasons.

Reference	Sediment								
	Fe	Mn	Cu (µg/g)	Cd (µg/g)	Zn (µg/g)	Co (µg/g)	Ni (µg/g)	Cr (µg/g)	Pb (µg/g)
Venugopal et al (1982)	-	77.1	39.8	-	347.9	38.1	82.9	-	-
Ouseph (1992)	-	-	-	8.4	780	-	-	145	190
Nair et al (1990)	-	-	20	1.8	118	12	68	65	18
Balachandran et al (2006)	6.2	337	53.2	14.9	1266	26.1	66.5	20.5	15.5
Ratheesh et al., (2010)	5.2	252.9	41.8	11	741.9	21.6	58.2	89.4	34.5
Deepulal et al (2012)	8.3	657.5	46.9	5.96	433.1	24.6	72.5	79.2	433.1
Martin et al (2012)	4.9	1946	123.5	40.7	2233	58.4	103.7	379.6	99.6
Manju et al (2014)	-	-	72510*	5290*	--	11990*	-	32980*	45680*
George et al (2016)	8.3	445.4	48.3	10.1	791	18.3	53.4	-	37.4
Salas et al (2017)	7.4	921.25	146.6	64.4	4655	30.18	74.26	921.25	95.64

* sediment from industrial outlet area

Table 2.7. Highest value of heavy metals reported in dissolved form from CE irrespective of seasons.

Reference	Water								
	Fe (µg/L)	Mn (µg/L)	Cu (µg/L)	Cd (µg/L)	Zn (µg/L)	Co (µg/L)	Ni (µg/L)	Cr (µg/L)	Pb (µg/L)
Paul and Pillai (1983)		100	50	5	55	-	<1	-	-
Ouseph (1992)	0.8*	-	22.2*	3.4*	385*	-	0.6*	-	14*
Nair et al (1990)	190	84	36	24	17	-	36	-	82
Anju et al 2011)	662.8	-	25	2.2	40	-	-	-	64
Kaladharan et al (2011)	-	-	2.03	0.47	29.48	-	-	-	2.69
Nair et al (2013)	500	900	5	2.5	180	-	35	-	80

*values are in mg/L

Table 2.8. Highest values of heavy metals reported in a Particulate matter of CE irrespective of seasons.

	Particulate matter								
Reference	**Fe %**	**Mn (μg/g)**	**Cu (μg/g)**	**Cd (μg/g)**	**Zn (μg/g)**	**Co (μg/g)**	**Ni (μg/g)**	**Cr (μg/g)**	**Pb (μg/g)**
Paul and Pillai (1983)	10.7	3397	318	95	2313	-	286	-	-
Nair et al (1990)	6.3	220	370	0.02	0.16	-	370	-	0.095
Shibu et al (1990)	-	-	40	30	1590	-	-	-	-
Ouseph (1992)	9.8	-	298	180	3100	-	80	-	260
Nair et al (2013)	6	160	270	35	1390	-	180	-	900

2.5 Impact of metal: microbe interactions on environment and human health

2.5.1 Microbial community structure

The effect of heavy metal on the community structure of microorganisms were extensively studied in various ecosystems worldwide, such as soil (Kozdrój et al., 2007; Wang et al., 2007),fresh water (Dean-Ross and Mills, 1989), marine (De Souza et al., 2006) , mangrove (Cao et al., 2011), estuaries (Oger et al., 2001), sediments (Gough and Stahl, 2011; Yao et al., 2017), arctic region (Kumar et al., 2012) etc. Chen et al (2014b) opined that the diversity, abundance and activity of microorganisms in an ecosystem changes in response to long term exposure to heavy metal pollution. Heavy metal pollution exerts a selective pressure on microbial community, leading to the emergence of resistant strains with apparent reduction in the extracellular enzyme activity in that particular ecosystem (Asadishad et al., 2018; Kandeler et al., 1996; Kuperman and Carreiro, 1997; Lasat, 2002;

Li et al., 2006). The functional gene array-based studies from Lake DePue showed that the metal contamination influences sediment microbial community structure and function by increasing the abundance of relevant metal-resistant and sulfate-reducing populations (Kang et al., 2013). Heavy metals can also influence the community structure of archaea, evident from the studies conducted in metal contaminated soils of Braunschweig, Germany (Sandaa et al., 1999). Sediment samples collected from one of the most heavy metal polluted river in China, Xiangjiang showed a significant variation in the community structure of archaea and bacteria especially on proteobacteria and actinobacteria and found the adaptation of certain groups of bacteria and archaea to the metal stressed environment (Yin et al., 2015). A shift in the community structure of archaea and bacteria in response to high concentrations of Zn and Cu has been reported from soil contaminated with heavy metal-containing sewage sludge (Macdonald et al., 2011). Recent studies also reported changes in the species richness and diversity of eubacteria and ammonium oxidizing archaea and bacteria in environments contaminated with the micronutrients such as Ni and Cu (Remenár et al., 2017; Wang et al., 2018). There were not many reports available on the effect of metal pollution on community structure of particulate matter associated bacteria.

2.5.2 Enzymes and Biogeochemical cycles

The heavy metal pollution may impact the biogeochemical cycling either by altering the microbial diversity or by inhibiting the enzyme activities. An alarming example of this is the inhibition of bacterial nitrous oxide reductase enzyme by Cd, Zn, Ni, and Co leading to the accumulation of potent greenhouse gas N_2O and its indirect contribution to global

warming (Dickinson and Cicerone, 1986; Minagawa and Zumft, 1988; Sobolev and Begonia, 2008). Other important examples include the replacement of Zn by Cd in the carbonic anhydrase enzyme, leading to impairment of carbon sequestration process in marine algae (Pinter and Stillman, 2015) and inhibition of methane oxidation process by Zn and Cr (Mohanty et al., 2000). The heavy metals like Cu, Zn, Mg, Co etc are part of metalloenzymes and some functionally important biomolecules at lower concentrations. Examples are the presence of Cu and Zn in the enzymes and molecules involved in the photosynthetic electron transport (Raven and Falkowski, 1999) of carbon cycling. Since the particles inhabit a diverse group of microbial flora these are functionally important part of the microbial loop, as it can remineralize the fixed carbon to dissolved forms (Turley et al., 1995). Hence it can contribute to the carbon cycling in the estuarine system. The higher enzyme activity of particle-associated microorganisms compared to their free-living counter parts has been established in several studies (Crump et al., 1998; Kellogg and Deming, 2014), It was supported by the studies of Grossart et al (2003) in Baltic sea which showed 100 to 400 times higher cell-specific protease and β-glucosidase activities respectively in PM-associated bacteria compared to planktonic. The metals like zinc (Zn) and Lead (Pb) can inhibit the remineralization process of organic matter in the environment (Hu et al., 2015; John and Conway, 2014), which would adversely affect the biogeochemical cycling of carbon, nitrogen, sulphur and other trace elements. Metals like Zinc and Mercury would also affect the phosphate solubilization capacity of some of the bacterial species isolated from mangrove sediments contaminated with these metals. The phosphatase

activities of most of the isolates were inhibited by these metals at 1.00 ppm concentrations (Ravikumar et al., 2007; Wahsha et al., 2017). Studies also revealed that the particle associated bacteria can produce dimethyl sulphide (DMS) which can lead to the production of cloud condensation nuclei and have a major role in atmospheric reactions, climatic regulation (Garneau et al., 2009; Stefels et al., 2007) and also in sulphur cycling. Studies on *Ruegeria sp*ecies indicates that the heavy metals Fe, Mn, Zn enhances the cleavage of dimethyl sulfoniopropionate, which is a precursor for the DMS, at lower concentration, while excess Co and Mn enhance the process (Brummett and Dey, 2016; Brummett et al., 2015). Studies in fungus showed that cadmium stress can induce the sulfur assimilation process by the production of Glutathione (Mendoza-Cózatl et al., 2005).

2.5.3 Metal – Microbe interaction, Microbial resistance and Bioremediation

Microbes interact with different kinds of metals and metalloids in the environment, some of these interactions are healthy and others affect the survival of them. The biochemical role of the metals involves as enzyme co-factor, catalysts, in redox processes and stabilizes protein structure (Bruins et al., 2000; Nies, 1999). Microorganisms are widely distributed in the environment and can quickly response or adapt to the varying environment. Many anthropogenic activities impart an excess amount of metals to the environment and microorganisms which respond or adapt to this condition by various processes such as biosorption/bioaccumulation/ intracellular sequestration, bioprecipitation, extracellular sequestration, active transport of metal ions (efflux), chelation or reduction of metal ions. These adaptation mechanisms of the microorganisms can also make use in

bioremediation processes (Caruso, 2013; Gillan et al., 2005). Microorganisms are able to accumulate metals in the cell cytoplasm in the form of minerals such as magnetite (Gillan, 2016) or with the help of complexing agent like glutathione (for metals Cd and Pb) (Helbig et al., 2008), metalloproteins (Zn) (Blindauer, 2011) and polyphosphate inclusions (Pb) (Burgos et al., 2013). Many bacterial extracellular polysaccharides can bind/absorb metals which can make use of removal of metal pollutants from the activated sludge processes (Rudd et al., 1984). Some of the sulphur reducing bacteria can make use of metal bioremediation via bioprecipitation by altering the environment with suitable pH (Han and Gu, 2010). The extracellular sequestration of metals via exopolysaccharides, extracellular molecules like siderophore of microorganisms were reported earlier (Gomathy and Sabarinathan, 2010). Efflux systems are the largest group of metal resistance systems. Expulsion of toxic metals from the cytoplasm of bacteria happens by means of Non- ATPase linked or ATPase linked active transport mechanisms. Three main type of proteins are involved in these process ie, Resistance-nodulation-cell division proteins (belonging to RND super family), Cation diffusion facilitators (CDF family) and P-type ATPases (Nies, 1999; Nies, 2003; Silver and Phung, 2005). RND protein super family members in bacteria and archaea are involved in the transport of heavymetals, hydrophobic compounds, amphiphiles, nodulation factors and proteins (Długoński, 2016). The schematic representation of heavy metal resistance mechanisms through efflux pump were given in the Fig 2.3 (Długoński, 2016).The metal resistance genes merA (Hg), *chrB* (Cr), *czcD*, *czcA, czcB (Cd,Zn,Co)*, ncc (Ni), *pcoR* and *CuS (Cu), , pbrA* (pb), *lltb* (efflux mechanism) of microorganisms can be used as bioindicators of metal pollution in

ecotoxicological studies (Abou-Shanab et al., 2007; Besaury et al., 2013; Coombs and Barkay, 2004; Misra et al., 1984; Nies et al., 1990).

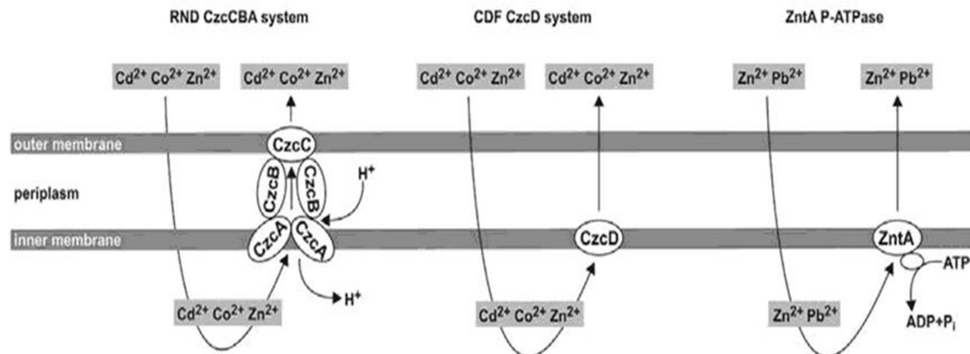

Fig. 2.3. Heavy metal efflux systems (Długoński, 2016)

2.5.4 Metal pollution in estuaries and its impact on food chain and human health

The metals accumulated in the estuarine sediments, particulate matter and other organisms could be transported to higher members of the food chain including humans (Chen and Folt, 2000). The suspended particles composed of small phytoplanktons, zooplanktons, microorganisms and other detritus are the important source of food for higher trophic level organisms and, whatever metals accumulated in these fractions will lead to biomagnification. The primary way of heavy metal transfer to the aquatic foodweb is the dietary exposure (Croteau et al., 2005; Mendoza-Carranza et al., 2016). For example a study by Altındağ and Yiğit (2005), on the effect of metal pollution on the food web in the lake Beysehir, Turkey showed that the heavy metals Cd, Pb, Hg and Cr exchange between water, sediment, plankton, and fish were high. Also the accumulation heavy metals in the foodweb was found to be in the order of water>plankton>sediment>fish tissues, except for Cr. Studies

conducted on the Carnivores, herbivores and their diets near to a lead and zinc mine area in Wales, Europe showed that cadmium has a greater accumulation potential compared to Zn and Pb, and also found that cadmium is preferably accumulated in the soft tissues while Pb in skeletal components (Roberts and Johnson, 1978). The distribution and bioconcentration studies of heavy metals in a tropical estuary, Mexico reported Zn as the most abundant and frequent heavy metal in the environment and biota also. The levels of Cr, Pb, Cd were greater than the threshold values in the commercial species of the estuary (Mendoza-Carranza et al., 2016). Methyl mercury is a notorious organometallic compound which causes cerebral palsy in human beings, were found in particulate material and fish in the estuarine pelagic food web of East coast of United States (Chen et al., 2014a). Minamata incident, one of the best examples due to the consumption of fish contaminated with methyl mercury was reported from Japan. The heavy metals Pb and Cd are potential neurotoxins (De Castro et al., 2010). The studies by Griboff et al (2018) in Argentina freshwater environment found a high accumulation of As and Se in human food through fresh water food web. The particulate associated microorganisms can produce exopolysaccharides, which can chelate the heavy metals. The PAB is important component of microbial food web as it is consumed by the metazoans, the metals accumulated or chelated by these microbes may get biomagnified to higher trophic level organisms.

In Cochin estuary, the metal toxicity and bioaccumulation studies were conducted in bivalves (*Villorita sp) and* mussels *(Perna viridis sp)* and observed high levels of metals in their tissue (Babukutty and Chacko, 1995; Lakshmanan and Nambisan, 1989; Raveenderan, 2011). The Zn was

reported as high in these species along with Hg, Cu, and Pb and the studies by Raveenderan (2011), observed that the zinc values were in the range 1.916 - 255.16 mg/kg in bivalves. The toxic effect and bio accumulation of Hg, Cu, Zn, Cd in fish species of CE have also been reported by earlier workers (Mohan et al., 2012b; Rejomon et al., 2010). Interestingly, some of these bivalves were also reported as good bioindicators of the metal pollution in the estuary (Pillai and Valsala, 1995). However, studies pertaining to the metal-microbe interaction are very rare in CE. The effect of heavy metals on the enzyme expression profile of bacteria isolated from the sediment, water, and cyanobacteria (Anas et al., 2016; Jiya et al., 2011) are few note-worthy examples in this regard. In view of the above facts, it is hoped that the topic of this study, viz., 'the effect of metal pollution on the community structure and activity of particle associated microorganisms' in Cochin estuary is highly relevant and contribute to a better understanding of the functional role of these metals in our backyard.

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Chapter 3

CHEMICAL AND BIOCHEMICAL CHARACTERIZATION AND MICROBIAL COMMUNITY STRUCTURE OF PM IN CE

Contents

3.1 Introduction
3.2 Materials and Methods
3.3 Results
3.4 Discussion

3.1 Introduction

Particulate matter (PM) consists of diverse and complex particles, both living and non-living, formed by various physical, chemical and biological processes such as weathering of bulk materials, resuspension of sediment particles, death and decay of large organisms and biological repackaging of organic matter through feeding and excretion (Simon et al., 2002). The living part of PM is made up of phytoplankton, zooplankton, bacteria, and archaea. PM plays a key role in the functioning of estuarine ecosystems, viz., serving as a micro-niche for the growth and activities of microorganisms and as food for organisms of higher trophic besides facilitating vertical transport of organic matter in the water column. (Garneau et al., 2009). The microorganisms mediate the remineralization of PM through secretion of a variety of hydrolytic enzymes which digest the high-molecular-weight polymeric substances in PM into dissolved

organic matter (Bong et al., 2010; Chróst and Rai, 1994). PM is also known for their ability to chelate heavy metals, which may alter the biochemical properties of the micro-niche and the diversity and functions of associated microorganisms. All microorganisms require certain heavy metals at optimum concentrations for the synthesis of structural proteins and pigments of metabolic importance in the redox processes, regulation of the osmotic pressure, maintaining the ionic balance and enzyme component of the cells (Bong et al., 2010; Kosolapov et al., 2004). On the other side, higher concentrations of heavy metals can interfere with the structural conformation of proteins and nucleic acids, leading to their malfunction (Bong et al., 2010). For example, microorganisms require 10^{-5}–10^{-7} M concentrations of Zn ions in the environment for their optimal growth (Wilson and Reisenauer, 1970), whereas, at higher concentrations, Zn binds with the cell membrane and inhibits cell division (Nies, 1999; Silver and Phung, 2005). Although previous studies have reported on the toxic effects of heavy metals on planktonic microorganisms (Anas et al., 2015; Jiya et al., 2011), their influence on the diversity of PM-associated microorganisms is far less understood. It is possible that pollutants such as heavy metals could impinge on the diversity of PM-associated bacteria, which in turn could influence the digestion of particulate organic matter (POM) into dissolved organic matter (DOM).

3.2 Materials and Methods

3.2.1 Sample collection and preservation

Subsurface (~1 m below surface) water samples were collected across a pollution gradient in CE, southwest coast of India (Fig. 3.1). The

stations S1 (9°58” 8’N & 76°13” 42’E) and S2 (9°58” 48’ N &76°15” 46’E) are located near the bar mouth and are considered as the least polluted (Jiya et al., 2011). The station S3 (10° 2” 24’N & 76°15”29’E) is at the midpoint where pollution levels are medium and S4 (10° 4” 22’ N & 76°16” 53’E), located near the discharge point of river Periyar, is considered to be in a highly polluted region (Aneesh and Sujatha, 2012).

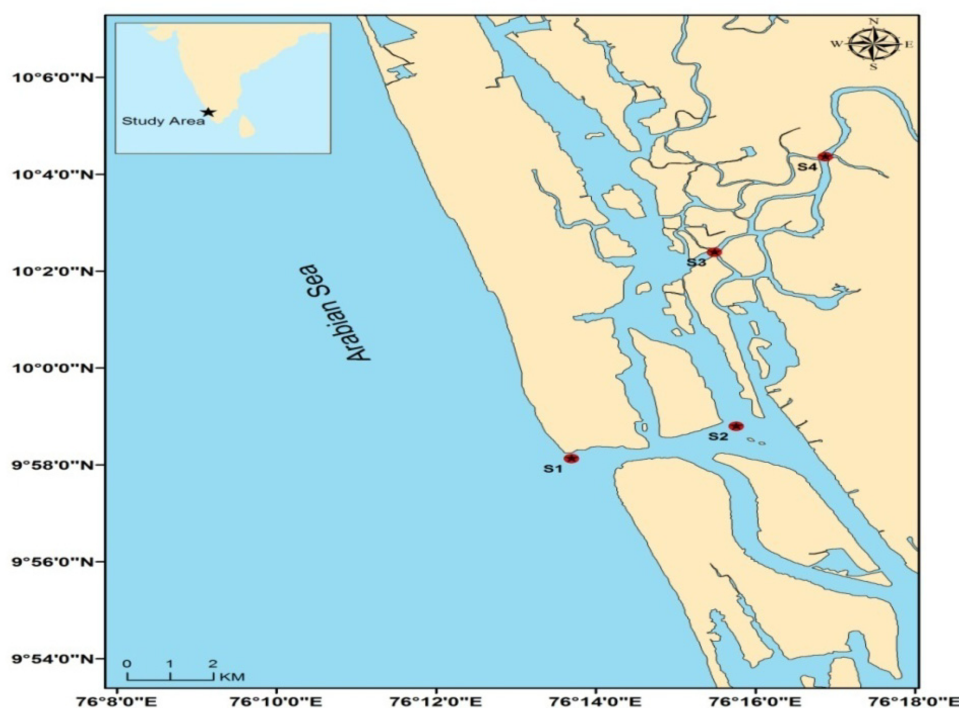

Fig. 3.1. Study Area of Cochin Estuary and sampling stations

Water samples were collected during low tide on 20th September 2012 (wet season) and 26th February 2013 (dry season) using a Niskin water sampler (10 L capacity). Water samples (1 L) for chemical analyses were collected in plastic bottles, avoiding contamination from all possible sources.

Sample (100ml) for Fluorescent *in situ* hybridization (FISH) analysis were collected in sterile polypropylene bottles and preserved in 2 % buffered formalin. One litre of the sample was collected for DNA extraction. PM was separated from the water sample, by passing through 10 µm nylon membrane followed by pre-combusted (450 °C 4 hr) GF/F filters of 0.7 µm pore size for chemical analysis. The filtrates after separating PM were used for analyzing dissolved fractions. The filter papers were stored at -20 °C until further analysis. Schematic representation of analysis strategies was given in Fig. 3.2.

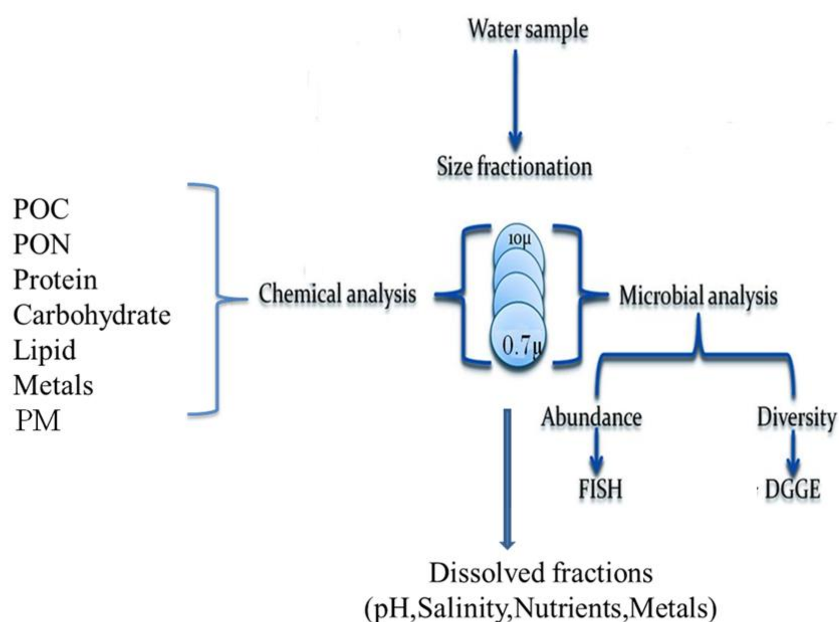

Fig. 3.2. Schematic representation of analysis strategies

3.2.2 Analysis of environmental variables

Environmental variables of water samples were measured in triplicate following the standard protocols. Salinity was determined using a

Digi Auto Salinometer (Model TSK, accuracy ± 0.001) and pH was measured using an ELICO LI 610 pH meter. Samples for nutrients (Ammonium, nitrite, nitrate, phosphate, and silicate) were filtered through Whatman No 1 filter paper and estimated spectrophotometrically Grasshoff et al (1983) within six hours of sampling. Ammonium was determined following indophenol blue method and the absorbance was measured at 630 nm. Nitrite was determined as the formation of highly colored azo dye (Abs 543 nm) in a reaction mixture containing N-(1-naphthyl)-ethylene diamine and a diazo compound formed through the reaction of nitrite in water samples with sulphanilamide in acidic condition. Nitrate in the water samples was measured after reducing it to nitrite by passing through the cadmium-copper column. Phosphate was measured spectrophotometrically (Abs 882 nm) following the reduction of phosphomolybdate complex with ascorbic acid. The phosphomolybdate complex was formed through the reaction of phosphate in the water sample with ammonium molybdate. Silicate was also measured in the same way, where the silicomolybdate complex was reduced with oxalic acid, and the optical density was measured at 810 nm. PM was collected on a pre-combusted 0.7 µm GF/F filter paper (Whatman, USA) and measured gravimetrically after achieving constant weight at 60 °C.

3.2.3 Biochemical characterization of PM

Particulate organic carbon (POC), Particulate organic nitrogen (PON), total carbohydrate, protein and lipids in the PM sample were analyzed following standard procedures. The filter paper maintained at -20 °C was thawed and used for the analysis. For the analysis of POC and PON, the filter paper was placed in scintillation vials and allowed to dry

overnight at 65 °C. Subsequently, it was transferred into a desiccator saturated with HCl fumes to remove carbonates and air-dried in a clean fume hood. These filters were packed tightly in tin cups for the analysis using CHN analyzer (ElementarVario EL III) following standard protocols (Strickland and Parson, 1972).

The carbohydrate was extracted from PM using 5 % trichloroacetic acid and analyzed spectrophotometrically using the phenol-sulphuric acid method. The reducing sugars were measured after converting them into yellow colored furfural and measuring their absorptions at 490 nm with different concentrations of glucose, is used as standard (Kochart, 1978). The proteins were extracted from samples using 1N NaOH and analyzed spectrophotometrically using Lowry's method. The blue-to-purple-colored complex formed through the reaction of phenolic group in the amino acid residue of protein with Folin–Ciocalteau reagent at pH 9 to 10.5 was measured at 750 nm using bovine serum albumin as standard (Lowry et al., 1951).

Lipids were extracted using a solvent mixture of methanol: chloroform: water (5:10:4) following Bligh and Dyer's method (Bligh and Dyer, 1959). Further, the lipids were oxidized using acid dichromate solution (Strickland and Parson, 1972) and the reduction of yellow color was measured spectrophotometrically at 440 nm using stearic acid as standard. The concentrations of protein, carbohydrate and lipids were converted into carbon equivalents by multiplying with standard conversion factors 0.49, 0.40, 0.75 respectively (Fabiano and Danovaro, 1994). The

sum total of the carbon equivalents of protein, carbohydrate, and lipids was referred as biopolymeric carbon (BPC) (Pusceddu et al., 2000).

3.2.4 Heavy metal analysis of water and PM samples

For metal analysis, PM collected in GF/F filters were digested with HF-$HClO_4$- HNO_3 and residue dissolved in 0.5 M HCl (20ml). The metals, Fe, Mn, Cr, Pb, Cu, Cd, Zn, Co, and Ni, were analyzed using an inductively coupled plasma optical emission spectrometer (ICP OES, Perkin Elmer) following the standard protocol (Loring and Rantala, 1992). Blank filters were also analyzed using the same method. For estimation of dissolved metals, known volumes of water samples were filtered through pre-weighed GF/F filter paper (0.7µm) and the filtrate was acidified using concentrated hydrochloric acid. The dissolved metals were concentrated from one-litre water samples using Ammonium Pyrrolidine Dithio Carbamate (APDC) and Methyl Isobutyl Ketone (MIBK) at pH 4.5 and brought back to aqueous layer by back-extraction with concentrated nitric acid and made up to 20 ml with sterile de-ionized water (Smith and Windom, 1972). The extracts were analyzed in the ICP OES (Perkin Elmer) for dissolved trace metals. The accuracy of the heavy metal analyses was checked using standard reference material PACS 2 (Table 3.1). The results of Fe, Cr, and Cd were found to be accurate at 96 % compared to the standard, while it was 87, 89, 82, 97 and 98 % respectively for Co, Cu, Mn, Ni, Pb, and Zn. Most of the results were within the uncertainty values certified by National Research Council of Canada.

Table 3.1. Trace metal extracted from the standard reference material PACS-2 (n=4)

	Fe (%)	Co (μgg^{-1})	Cr (μgg^{-1})	Cu (μgg^{-1})	Mn (μgg^{-1})	Ni (μgg^{-1})	Zn (μgg^{-1})	Pb (μgg^{-1})	Cd (μgg^{-1})
Certified value	4.09	11.5	90.7	310	440	39.5	364	183	2.11
Extracted value	3.94	13.00	94.05	277.20	358.93	40.66	293.48	180	2.02
Accuracy (percentage error) (±%)	4.0	13.0	4.0	11.0	18.0	3.0	19.0	2.0	4.0

3.2.5 Abundance of archaea and bacteria using Fluorescent in situ hybridization (FISH)

The abundance of total Planktonic and PM associated archaea and bacteria were analyzed using FISH technique following the protocol of Glockner et al (1999). The Cy3 labeled oligonucleotide probes ARCH 915 (GTGCTCCCCCGCCAATTCCT) (Stahl and Amann, 1991) and EUB 338 (GCTGCCTCCCGTAGGAGT) (Amann et al., 1990) were used for the enumeration of archaea and bacteria respectively. The buffered formalin-preserved water samples were prefiltered through 10 µm Nylon membranes and used for the enumeration of planktonic and PM associated archaea and bacteria. The PM-archaea and PM-bacteria collected on 0.8 µm (Millipore; ATTP02500) and planktonic ones collected on 0.2 µm polycarbonate filters (Millipore; GTTP02500) were analyzed separately. The archaea and bacteria collected on filters were hybridized with buffer containing respective probes (50 ng) and a mixture of 0.9 M NaCl, 20 mM Tris HCl (pH 8) and 0.01 % of sodium dodecyl sulfate (SDS), formamide (50% for eubacteria and 55 % for archaea).The filters were washed with buffer

containing 20 mM Tris HCl, 5 mM EDTA, 0.01% SDS, 5 M NaCl depending upon the formamide concentration (See the Appendix table No. 1) to remove impurities and unlabelled probes. Fluorescent signals from the cells were counted using an epifluorescent microscope (Olympus BX41) with specific filter sets for Cy3. A blank filter was subjected to the same procedure without probe to remove error due to the background fluorescence.

3.2.6 Community structure of archaea and bacteria using DGGE

Nested PCR and Denaturing Gradient Gel Electrophoresis (DGGE) techniques were used for analyzing the community structure of archaea and bacteria in water and PM samples. Genomic DNA from water samples was extracted following the protocol of Bostrom et al (2004) with slight modification. Briefly, 1 to 2 L of water sample (pre-filtered through 10 µm Nylon membrane) was passed through 0.8 µm (Millipore; ATTP04700) and 0.2 µm polycarbonate membrane (Millipore; GTTP04700) filters sequentially to separate PM-attached and Planktonic microbial fractions, followed by incubation at 37 °C for 1 hr in lysis buffer (NaCl 400 mM, Sucrose 750 mM, EDTA 20 mM and Tris HCl 50 mM), containing 1 mg ml^{-1} lysozyme. Subsequently, SDS (1 %) and proteinase K (100 µg ml^{-1}) were added to the solution and continued incubation for 5 hr at 55 °C. Further 0.6 volume of isopropanol was added and DNA was precipitated by keeping at -20 °C for 60 min. DNA pellet was washed copiously with 70 % ethanol, dissolved in TE buffer and stored at -20 °C until used. The sample filters from station 4 (collected from the Eloor region) were pre-treated with 1 ml of 10 mM Tris HCl and

40 mM Na_2EDTA (pH 7.0) to remove divalent metal inhibitors like Zn before cell lysis, because high concentrations of divalent cations, might contribute to premature precipitation of DNA (Kejnovsky and Kypr, 1997).

The DNA extracted from all samples were subjected to two-step PCR reaction with gene-specific primers for studying the community structure of archaea and bacteria. In the first step, archaeal 16S rRNA genes were amplified using two primer sets separately {A8F and A1492R (Casamayor et al., 2000), Arch 21F and Arch 958R (DeLong, 1992)} and the PCR products were pooled. Bacterial 16S rRNA genes were amplified using a primer set of 27F and 1492R (Lane, 1991) (Table 3. 2). The products of first step PCR reaction was used as template for the second step PCR using forward primers with GC clamp and reverse primers specific for bacteria (Muyzer F and Muyzer R) and archaea (Equimolar concentration of forward primers SAF (1) and SAF(2) and Parch 519r) (Muyzer et al., 1993; Nicol et al., 2003; Overeas et al., 1997) (Table 3.2). Reactions were conducted in triplicate and PCR products were pooled during DGGE. The details of the gene-specific primers and PCR conditions are illustrated in Table 3. 2. In the PCR, 5 µl DNA sample was used as template for 50 µl reaction mixture containing 2 µl each of primers (10 picomoles μl^{-1}), 5 µl of 10X Taq polymerase buffer (NEB, Canada), 1 U Taq DNA polymerase (NEB, Canada) and 200 µM dNTPs (NEB, Canada).

Table 3.2. Details of PCR primers used in the study

Domain	Primer combinations	Tm °C
Archaea	A8F (cggttgatcctgccgga) A1492R (ggctaccttgttacgactt)	51.5 °C
	Arch 21 F (ttccggttgatccygccgga) Arch 958 R(yccggcgttgamtccaatt)	55.0 °C
	Parch 519R (ttaccgcggckgctg) SAF(1)(ctayggggcgcagcagg) SAF (2)(ctacggggcgcagaggg)	53.5 °C
Bacteria	27 F (agagtttgatcctggctcag) 1492 R(tacggctaccttgttacgactt)	55.0 °C
	MuyzerF (cctacgggaggcagcag) Muyzer R (attaccgcggctgctgg)	60.0 °C

Denaturing Gradient Gel Electrophoresis (DGGE) of amplified PCR products of archaea (180 bp) and bacteria (200 bp) were performed using a D-code universal mutation detection system (Bio-Rad Laboratories Inc., USA). DNA concentration in the PCR products was quantified using Nanodrop (Thermo-Fisher, USA) and an equal concentration of DNA per sample (~400 ng) was loaded on the DGGE gel. The PCR products were run on an 8 % polyacrylamide gel prepared with denaturing gradients of urea and formamide 30 – 70 % and 40 – 65 % respectively for archaea and bacteria, for 4.5 h at a constant voltage of 220 V in 1× TAE buffer (40 mM Tris HCl, 20 mM acetic acid, 1 mM EDTA). The bands separated were stained with ethidium bromide and observed in a gel documentation system (BioRAD, USA).

3.2.7 Statistical Analysis

DGGE gel images were analyzed with Bionumerics software version 4.6 (Applied Maths USA). The software carries out a density profile analysis, detects the bands from each lane and calculates the relative intensity of each band. Numbers of operational taxonomic units (OTUs) in each sample were counted as a number of DGGE bands. Gels were cross-checked visually as well as for the number of bands per lane. An intensity matrix was constructed based on the relative contribution of the intensity of the band. The relative intensity of each band was used to calculate the Shannon-Wiener diversity index (H′). Cluster analysis of DGGE bands, based on square root transformed community data matrix through Bray-Curtis similarity was performed with PRIMER v.6 software package (Plymouth Marine Laboratory, UK). The multivariate relationship between community structure of archaea and bacteria with environmental variables and heavy metal concentrations of water and PM were analyzed separately using redundancy analysis (RDA) using Canoco (version 4.5, Centre for Biometry, Wageningen, Netherlands).

3.3 Results

3.3.1 Environmental variables and dissolved heavy metals

Salinity variation between stations during wet and dry seasons were observed. CE was more limnetic during the wet season with the salinity being restricted only up to the stations S1 (30 psu) and S2 (9 psu) in the downstream of the estuary. Seawater influx was more evident during the dry season and it reached up to station S4 (15 psu) in the upstream. The

pH of the water column remained neutral to slightly alkaline during both wet and dry season. The dissolved inorganic nitrogen (sum of ammonium, nitrite, and nitrate) load was higher in CE during the wet season, especially in S3 (30 µM) followed by S4 (24.3 µM), S1 (16.2 µM) and S2 (14.2 µM) in that order. It decreased to 2.9, 2.8, 4.7 and 5.9 µM respectively in S1, S2, S3 and S4 during the dry season. Silicate level also was higher during the wet season, with a maximum of 56.7 µM in S2 followed by S4 (36.1 µM), S3 (35.4 µM) and S1 (31 µM). During the dry season, higher levels of silicate were observed at S3 (36.25 µM), followed by S4 (22.63 µM), S1 (10. 25 µM) and S2 (8.5 µM). Phosphate levels did not show much variation between the wet and dry seasons, being in the range 1- 4 µM (Table 3.3) most of the time. Zn was recorded as the major dissolved heavy metal in the water column of CE. The concentration of Zn was higher in S4 during dry (130.5 μgL^{-1}) and wet (105.9 μgL^{-1}) seasons. Similarly, higher concentrations of Zn were also found in the water column of S3, i.e., 48.3 and 73.7 μgL^{-1} during dry and wet season respectively. The level of Zn was less than 10 μgL^{-1} in other stations during both dry and wet seasons. High concentration of Fe was observed in S1 during the wet season (799 μgL^{-1}). At the same time, concentrations of Cr, Cu, and Ni were found lower during both wet and dry seasons, while Cd, Co, and Pb were not detected at all in the water column of CE (Table 3.3).

Table 3.3. Environmental variables and heavy metal of water column in study area during wet and dry season

	Stations							
	Wet Season				**Dry Season**			
	S1	S2	S3	S4	S1	S2	S3	S4
PM (mgL^{-1})	36	18	6.8	2.8	32.4	26.8	23.2	17.6
Salinity (psu)	30	9	0	0	34.5	32	8	15
pH	7.8	7.8	7.6	7.1	7.83	7.82	7.92	7.84
Nutrients (µM)								
NH_4^+	11.3	7.9	16.1	4.8	1.68	2.23	3.20	5.24
NO_2^-	0.5	0.3	0.3	0.2	0.2	0.1	0.14	0.16
NO_3^-	4.4	6	13.6	19.3	0.996	0.45	1.40	0.54
SiO_4^-	31	56.7	35.4	36.1	10.25	8.5	36.25	22.63
PO_4^-	3.3	2.1	2.4	1.3	1.95	1.85	1.05	3.90
Dissolved Heavy Metals (μgL^{-1})								
Cd	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Co	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cr	0.47	0.07	0.09	0.09	0.22	0.08	0.34	0.20
Cu	0.68	0.27	0.44	n.d.	1.28	0.38	1.74	0.81
Fe	799	45.37	68.05	6.62	262.25	274.20	161.70	55.65
Mn	0.63	0.13	n.d.	n.d.	0.24	0.21	0.39	0.12
Ni	4.56	4.57	5.51	n.d.	3.55	2.97	4.21	2.02
Pb	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Zn	4.36	6.36	73.65	105.85	8.84	6.55	48.28	130.50

3.3.2 Biochemical and chemical characterization of PM

The concentration of PM present in the water column of each station was calculated gravimetrically. The PM was higher in S1 during both wet (36 mgL^{-1}) and dry (32.4 mgL^{-1}) season, followed by S2, S3, and S4

(Table 3.3). Although the overall concentration of PM was higher in CE during the dry season, it contained low levels of POC, PON, proteins, carbohydrates, and lipids. The PM collected from S4 during wet season was rich in POC (17.86 %), PON (10.71 %), protein (7.55 %) and lipids (2.39 %). Similarly, the PM collected from S3 during the wet season also showed high levels of POC (10.29 %), PON (4.41 %), Proteins (7.81 %), Carbohydrate (1.33 %) and lipids (5.83 %) (Table 3.5). The comparatively low level of POC and PON were observed in PM collected from stations S1 and S2 near the bar mouth. Seasonal variation in the biochemical characteristics of PM collected during wet and dry seasons were also observed. The POC levels in all the stations during the dry season were approximately half of that observed during the wet season. A similar trend was observed in PON levels in PM collected from all stations except S1. Such difference was more prominent in the levels of proteins in PM collected during the dry and wet season. The protein content in the PM collected during wet season ranged from 5.12 to 7.81 %, while it reduced to 0.38 to 2.91 % during the dry season. The carbohydrate and lipid content also showed the same trend in stations S1, S2 and S3 during dry and wet seasons. The carbohydrate concentration in PM collected from these stations ranged from 0.19 – 1.33 % and 0.07 – 0.18 % respectively during the wet and dry season. The carbohydrate concentration in PM collected from S4 during wet and dry seasons were 0.56 and 0.52 % respectively. The lipid concentration in PM collected from S4 during wet (2.39 %) and dry (3.25 %) seasons remained similar, while it ranged from 0. 26 to 5.83 % and 0.12 to 0.84 % respectively in other stations.

The protein (PRT) to carbohydrate (CHO) and lipid (LPD) to carbohydrate (CHO) ratio were used as an indicator to assess the quality of particulate organic matter. The PRT: CHO were observed low during the dry season in the estuary compared to the wet season. It ranges from 6.37 to 8.65 during dry season whereas it ranges from 7.6 to 32.21 during the wet season (Table 3.4). The LPD: CHO ratio was considered as an indicator of the energetic quality of particulate matter. The values range between 0.40 and 10.8 during wet season while it varies between 6.84 and 61.99 during the dry season. The biopolymeric carbon (BPC), i.e., the sum of carbon equivalents of protein, carbohydrate, and lipid, was higher in the upstream region of the estuary. The relative contribution of protein was higher to BPC during wet season whereas it turns to lipid during the dry season.

Table 3.4. Biopolymeric carbon (BPC) content of particulate matter

Stations	CHO (µg/mg)	PRT (µg/mg)	LPD (µg/mg)	PRT:CHO	LPD:CHO	BPC
S1W	0.93	25.08	0.88	27.03	0.94	26.89
S2W	0.79	25.49	6.29	32.21	7.94	32.58
S3W	5.34	38.25	2.14	7.16	0.40	45.74
S4W	2.25	36.98	24.39	16.38	10.80	63.63
S1D	0.29	1.87	2.02	6.37	6.84	4.19
S2D	0.23	2.00	3.70	8.65	15.95	5.93
S3D	0.70	5.63	43.72	8.00	61.99	50.06
S4D	2.06	14.27	17.47	6.90	8.45	33.81

Table 3.5. Biochemical and chemical characterization of particulate matter

	Stations							
	Wet Season				**Dry Season**			
	S1	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**
POC (%)	8.61	6.11	10.29	17.86	4.32	2.24	6.03	7.95
PON (%)	1.11	2.78	4.41	10.71	1.23	1.49	2.16	4.55
Protein (%)	5.12	5.20	7.81	7.55	0.38	0.41	1.12	2.91
Carbohydrate (%)	0.23	0.19	1.33	0.56	0.07	0.06	0.18	0.52
Lipid (%)	0.26	0.49	5.83	2.39	0.12	0.84	0.29	3.25
Cd (μgg^{-1})	1.44	4.01	4.64	7.57	1.22	5.25	4.28	22.69
Co (μgg^{-1})	0.43	n.d.	n.d.	n.d.	0.87	3.49	n.d.	5.90
Cr (μgg^{-1})	69.43	14.14	114.34	34.46	5.80	n.d.	35.14	n.d.
Cu (μgg^{-1})	8.57	2.26	24.00	17.85	3.05	4.83	2.12	1.15
Fe (μgg^{-1})	7827.14	3990.48	10662.00	10923.08	2130.55	3561.93	1210.79	4116.67
Mn (μgg^{-1})	65.43	109.67	384.55	188.72	21.27	47.56	22.09	2439.49
Ni (μgg^{-1})	n.d.	n.d.	5.26	n.d.	n.d.	n.d.	n.d.	n.d.
Pb (μgg^{-1})	27.29	5.06	15.69	31.79	2.53	3.38	6.26	13.59
Zn (μgg^{-1})	1600.00	2561.40	3998.07	8451.28	765.45	788.20	2086.33	2294.87

The level of heavy metals was also higher in PM collected from all the stations compared with the dissolved levels of respective metals (Table 3.5). The levels of heavy metals were higher during the wet compared with the dry season, and maximum levels were observed in PM collected from stations S3 and S4. The level of Fe remained the same in PM collected from S2 during the dry and wet seasons, while it showed a 2.5 to 10 times increase in other stations during the wet season. The maximum level of Fe was observed in PM (PM-Fe) collected from S3 (10622 μgg^{-1}) and S4 (10923.1μgg^{-1}) during the wet season. The level of PM-Fe collected from S3 and S4 during dry season were 1210.8 and 4116.7 μgg^{-1} respectively.

The level of PM-Fe in S1 was 7827.1 μgg^{-1} during the wet season, while it decreased to 2130.6 μgg^{-1} during the dry season. The level of Cd, Mn, Pb, and Zn showed higher accumulation in PM collected from the northern part of the estuary compared with those from the bar mouth (station S1) during both wet and dry seasons. The level of PM-Cd during wet and dry seasons ranged from 1.44 to 7.57 and from 1.22 to 22.69 μgg^{-1} respectively, with a maximum concentration of 22.69 μgg^{-1} recorded at S4 during the dry season. The lowest concentration of PM-Cd was observed near the bar mouth (station S1) during both wet and dry seasons. The highest (2439.49 μgg^{-1}) and lowest (21.27 μgg^{-1}) concentrations of PM-Mn were observed at S4 and S1 stations respectively during the dry season. The level of PM-Mn towards bar mouth stations (S1 and S2) was higher during the wet season (i.e. 65.43 and 109.67 μgg^{-1} respectively) compared with those of the dry season (i.e. 21.27 and 47.56 μgg^{-1} respectively). The concentrations of PM-Pb in the riverine end of the estuary, S3 and S4, were higher during the wet season (15.69 and 31. 79 μgg^{-1} respectively) compared with those of the dry season (6.26 and 13.59 μgg^{-1} respectively). The lowest levels of PM-Pb was observed at S1 (2.53 μgg^{-1}) and S2 (3.38 μgg^{-1}) during the dry season. The level of PM-Zn was higher during wet season compared with the dry season, with particularly higher levels in the riverine end of the estuary (stations S3 and S4) during the wet season (3998.07 and 8451.28 μgg^{-1} respectively). The lowest level of PM-Zn was observed near the bar mouth stations (S1 and S2) during the dry season (765.45 and 788.20 μgg^{-1} respectively). Comparatively lower levels of PM-Cu were observed at all the stations. The level of PM-Cu ranged from 8.57 to 24.00 and 2.12 to 4.83 μgg^{-1} respectively during the wet and dry seasons.

Highest levels of PM-Cu was observed in S3 and S4 (24.0 and 17.85 μgg^{-1} respectively) during the wet season. The PM-Cr was observed at all the stations during the wet season but was not detectable at S2 and S4 during the dry season. The highest level of PM-Cr was observed at S3 (114. 34 μgg^{-1}), followed by S1 (69.43 μgg^{-1}) and S4 (34.46 μgg^{-1}) during the wet season. Low levels of PM-Co were detected at S1, S2, and S4 during the dry season and at S1 during the wet season. The level of PM-Co ranged from 0.43 to 5.9 μgg^{-1}. PM-Ni was detected only at S3 (5.26 μgg^{-1}) during the wet season (Table 3.5).

3.3.3 Microbial abundance and community structure

The abundance of archaea and bacteria as plankton in the water and as components of PM were measured using the FISH technique. The microscopic image of Planktonic archaea and PM- associated archaea were shown in Fig. 3.3. The abundance of planktonic archaea was ten times higher compared with the PM-associated ones (PM-archaea) as evident from FISH counts (Fig. 3.4).

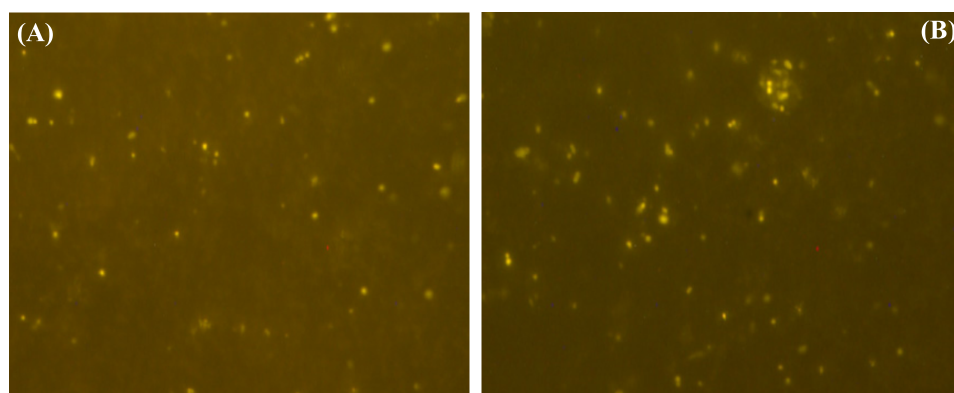

Fig. 3.3. Representative image of Planktonic Archaea (A) and PM- associated Archaea (B) using FISH

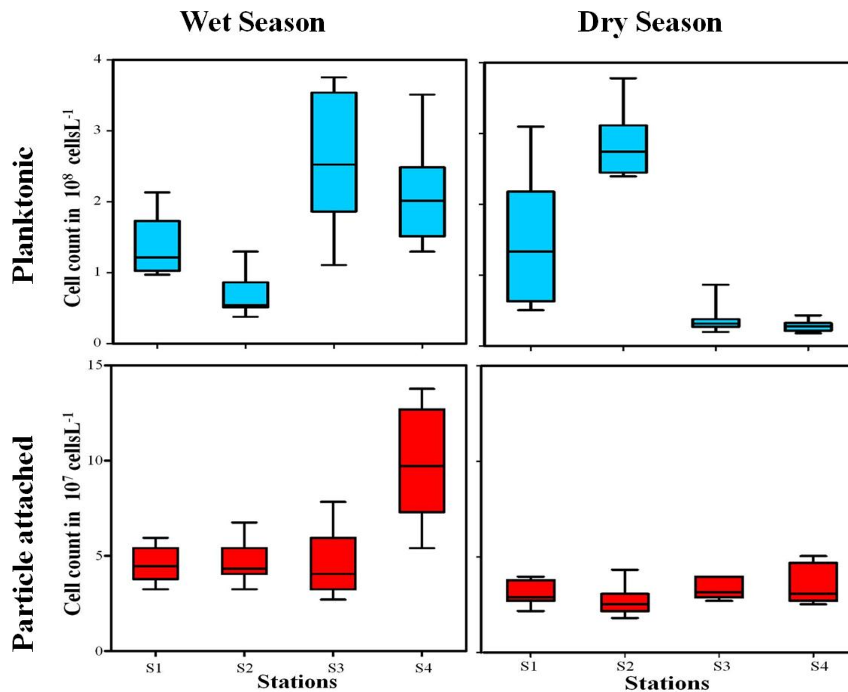

Fig. 3.4. Whisker box plot showing the abundance of Archaea

There was no significant difference in the abundance of planktonic archaea between dry and wet seasons, and it ranged between 0.28 – 2.83 $\times 10^8$ cells L^{-1}, with the higher archaeal abundance being observed at station S4 during the wet season. The abundance of PM-archaea ranged between 4.27 to 9.5 $\times$ 10^7 cells L^{-1} and 2.73 to 3.85 $\times$ 10^7 cells L^{-1} respectively in samples collected during the wet and dry seasons. The abundances of Planktonic and PM-bacteria were tenfold higher compared with the archaeal abundance of their respective counterparts (Fig. 3.5). The abundances of planktonic bacteria in both wet and dry seasons ranged from 0.55 to 3.4 $\times 10^9$ cells L^{-1}, while that of PM-bacteria ranged from 1.14 to 6.72 $\times$ 10^8 cells L^{-1}. The microscopic image of Planktonic bacteria and PM associated bacteria were shown in Fig. 3.6 using FISH analysis.

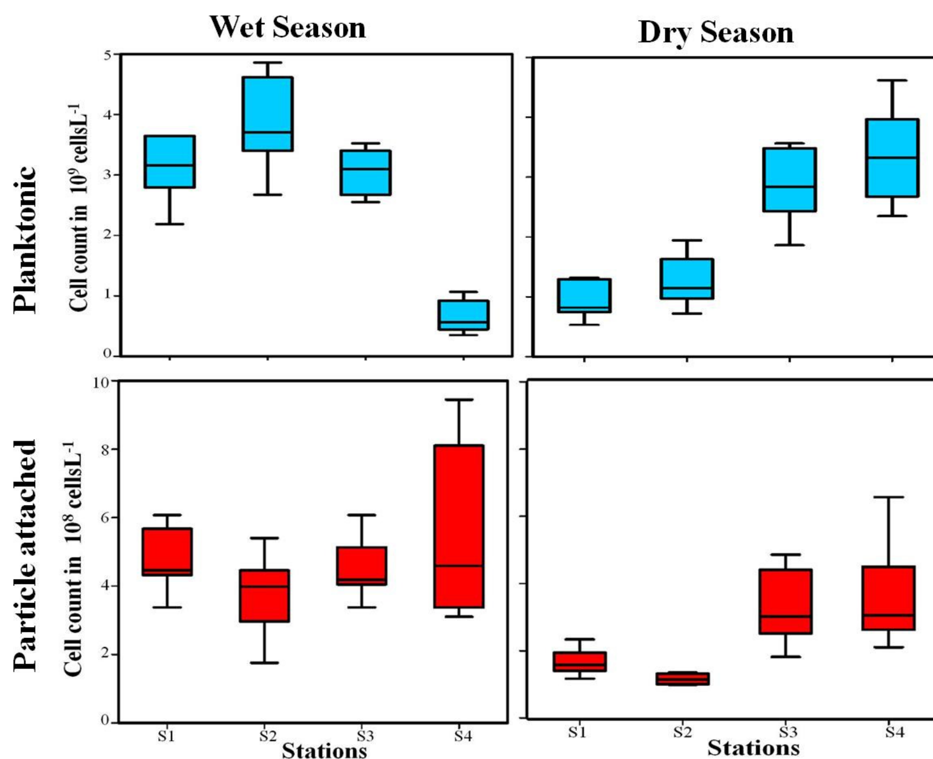

Fig. 3.5. Whisker box plot showing the abundance of Bacteria

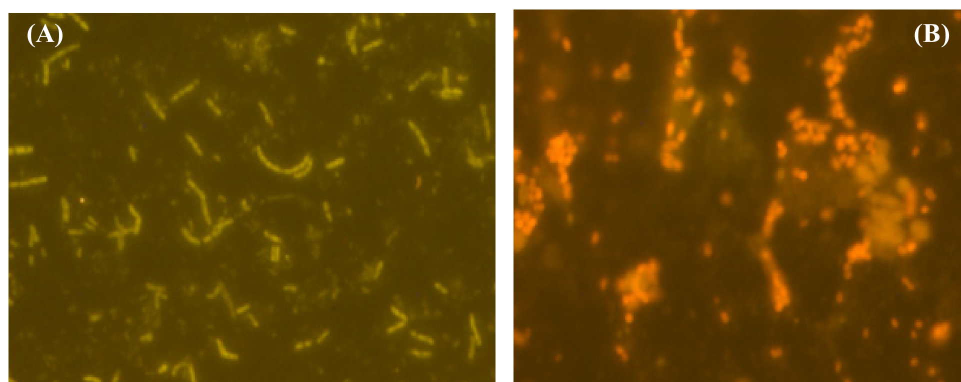

Fig. 3.6. Representative image of Planktonic Bacteria (A) and PM- associated Bacteria (B) using FISH

The community structure of archaea and bacteria was studied using the PCR-DGGE technique (Fig. 3.7 & 3.8). Here each band in the DGGE was considered to have originated from one group of archaea/ bacteria, and accordingly, each of them was considered as an independent operational taxonomic unit (OTU). The community structure of PM-archaea was more diverse (27 and 21 OTUs respectively during the wet and dry seasons) compared with planktonic ones (23 and 19 OTUs respectively during the wet and dry seasons) (Table 3.6).

Table 3.6. Number of Operational Taxonomic Units (OTUs) obtained from DGGE profile

Number of Operational Taxonomic Units(OTU)	Wet season					Dry season				
	S1	S2	S3	S4	Total	S1	S2	S3	S4	Total
Planktonic archaea	11	15	13	11	23	6	7	10	15	19
PM- archaea	11	9	13	17	27	14	12	11	9	21
Planktonic bacteria	12	10	7	5	16	15	18	16	12	24
PM- bacteria	18	16	13	13	26	10	11	9	8	18

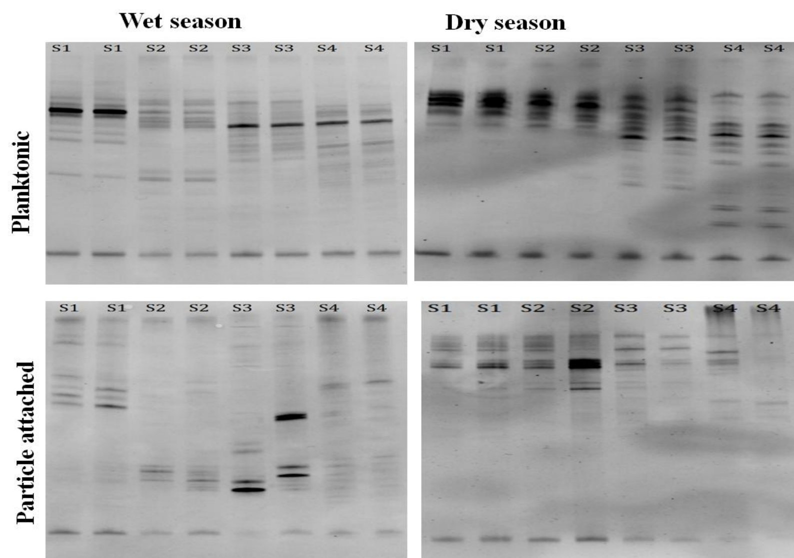

Fig. 3.7. DGGE profile of Particle attached archaea and Planktonic archaea during wet and dry season

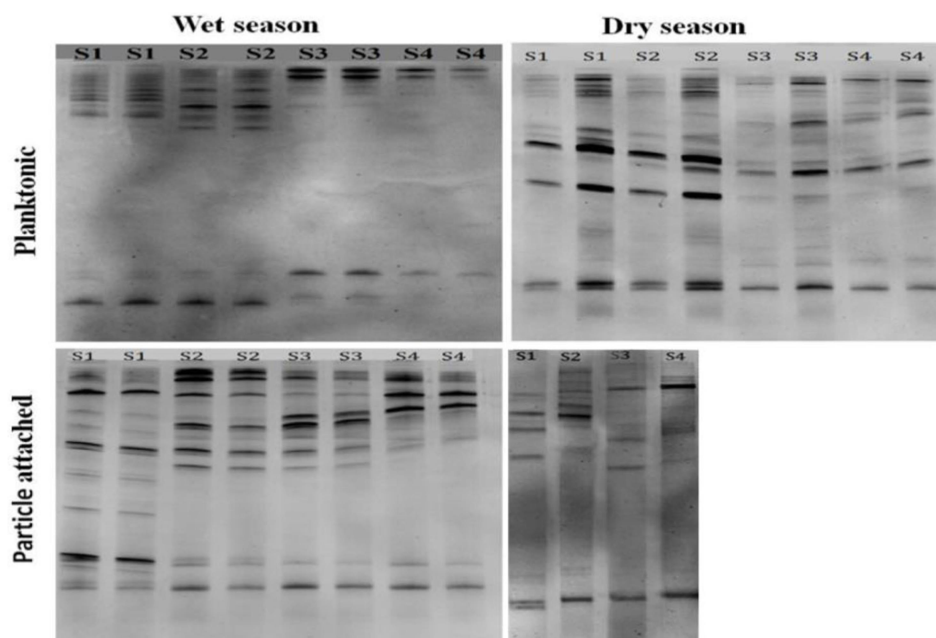

Fig. 3.8. DGGE profile of Particle-attached bacteria and Planktonic bacteria during wet and dry season

Shannon-Wiener diversity (H') index of planktonic archaea was higher during the wet season, while that of PM-archaea was higher during the dry season (Table 3.7). The cluster analysis of DGGE band pattern of planktonic and PM-archaea showed the spatial difference in their community structure (Fig. 3.9). The community structure of planktonic archaea of the near the riverine-end stations S3 and S4 showed nearly 80 % similarity during the wet season, while such a clustering was observed between the bar-mouth stations S1 and S2 during the dry season. In the case of PM-archaea, similarities were observed between S4 and S2 (~60 %) during the wet season and between S1 and S2 (~ 60 %) during the dry season.

Table 3.7. Shannon-Weiner Diversity Index of Planktonic and PM attached bacteria and archaea

Diversity index	Wet season				Dry season			
	S1	S2	S3	S4	S1	S2	S3	S4
Planktonic bacteria	1.54	2.04	2.02	1.67	2.28	2.19	2.3	2.16
Planktonic archaea	1.74	2.49	1.88	1.87	1.56	1.55	1.97	2.37
PM- archaea	2.08	1.95	1.78	2.23	2.08	2.03	2.01	1.78
PM- bacteria	2.44	2.26	2.32	2.07	2.12	1.97	1.92	1.65

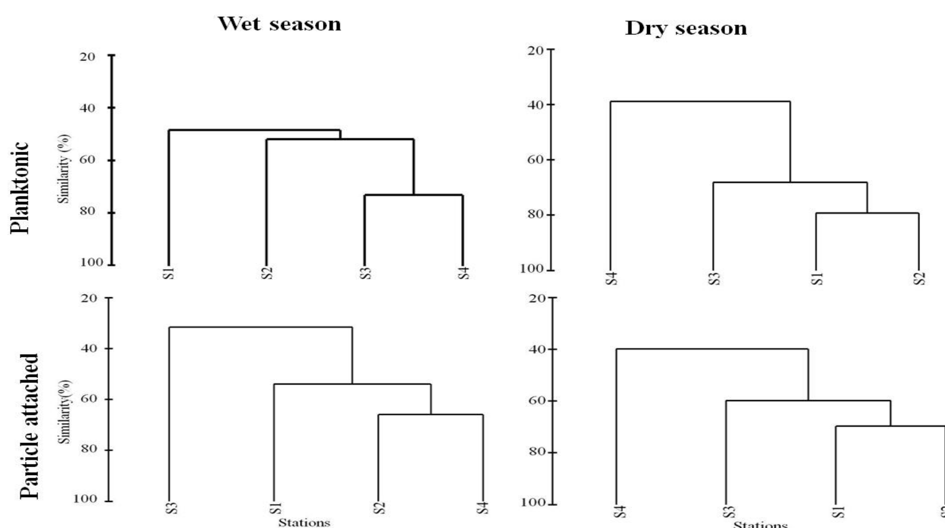

Fig. 3.9. Cluster analysis of Planktonic and PM-attached archaea during wet and dry season

Significant seasonal differences in the community structure between Planktonic and PM-bacteria were observed. The community structure of Planktonic bacteria was less diverse (16 OTUs) compared with that of PM-bacteria (26 OTUs) during the wet season. On the contrary, the community structure of Planktonic bacteria was more diverse (24 OTUs) compared with that of PM-bacteria (18 OTUs) during the dry season. During the wet season, the highest number of OTUs corresponding with Planktonic (12 OTUs) and PM-bacteria (18 OTUs) were found in station S1.

During the dry season, highest numbers of OTUs corresponding to planktonic (18 OTUs) and PM-bacteria (11 OTUs) were found in station S2 (Table 3.6). Cluster analysis of DGGE profile of planktonic and PM-bacteria showed clear demarcation between the bar mouth and riverine end stations in the estuary during wet and dry seasons (Fig. 3.10). The community structure of planktonic bacteria in bar mouth stations (S1 and S2) formed a separate cluster of ~80 % similarity during wet and dry seasons. In the case of PM-bacteria, more than 80 % similarity was observed between S2 and S3 during the wet season and more than 60 % similarity between S3 and S4 during the dry season. Differences were also evident in the Shannon-Wiener diversity index between planktonic and PM-bacteria. Higher diversity index of planktonic bacteria was found during the dry season, while that of PM-associated bacteria were higher during the wet season (Table 3.7).

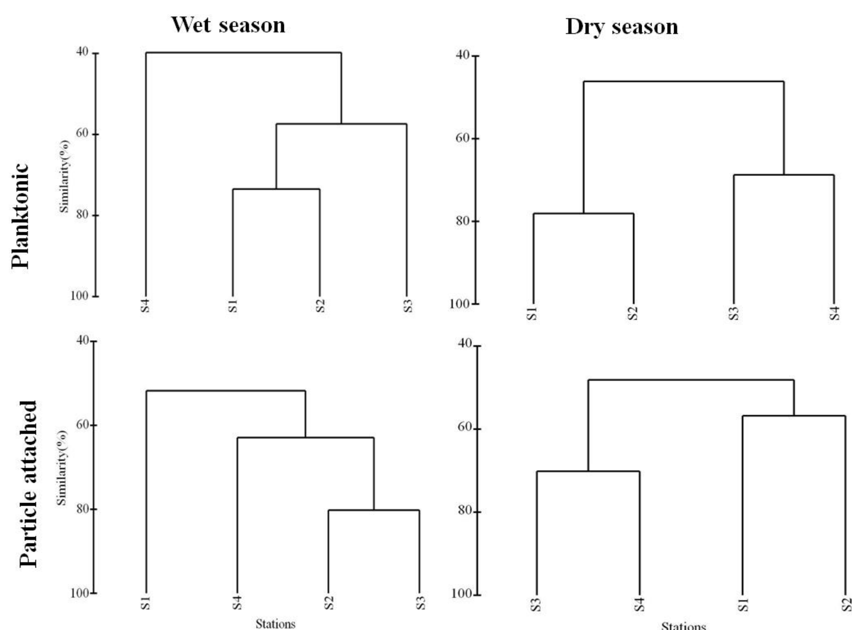

Fig. 3.10. Cluster analysis of planktonic and PM-attached bacteria during the wet and dry season.

3.3.4 Environmental influence on microbial distribution

Two-tailed correlation analysis was done to understand the influence of environmental characteristics on the abundance and community structure of Planktonic and PM-associated archaea and bacteria. The levels of PM-Pb and PM-Zn were correlated with protein concentrations of PM (r-value for Pb=0.73 and Zn=0.75) while the levels of PM-Ni (r=0.90) and PM-Cr (r=0.76) were well correlated with carbohydrates. The levels of PM-Cu and PM-Fe were correlated with both protein and carbohydrates. There is no significant influence of soluble metals on the abundance and community structure of planktonic archaea and bacteria were observed. But the abundance and community structure of PM-archaea and PM-bacteria were strongly influenced ($p<0.05$) by biochemical properties and heavy metal concentrations of PM (Fig. 3.11). The abundance of PM-archaea and PM-bacteria showed a positive correlation with POC, PON, PM-Pb, PM-Zn, and PM-Fe (Fig. 3.11). The biochemical characteristics and heavy metal concentration do not show any significant correlation with the diversity of PM-archaea while that of PM-bacteria were negatively correlated with PM-Co and PM-Cd (r-value for Co=-0.75 and Cd=-0.77). The RDA indicates that heavy metal variables have a pronounced impact on the abundance and diversity of archaea and bacteria associated with PM (Fig. 3.12).

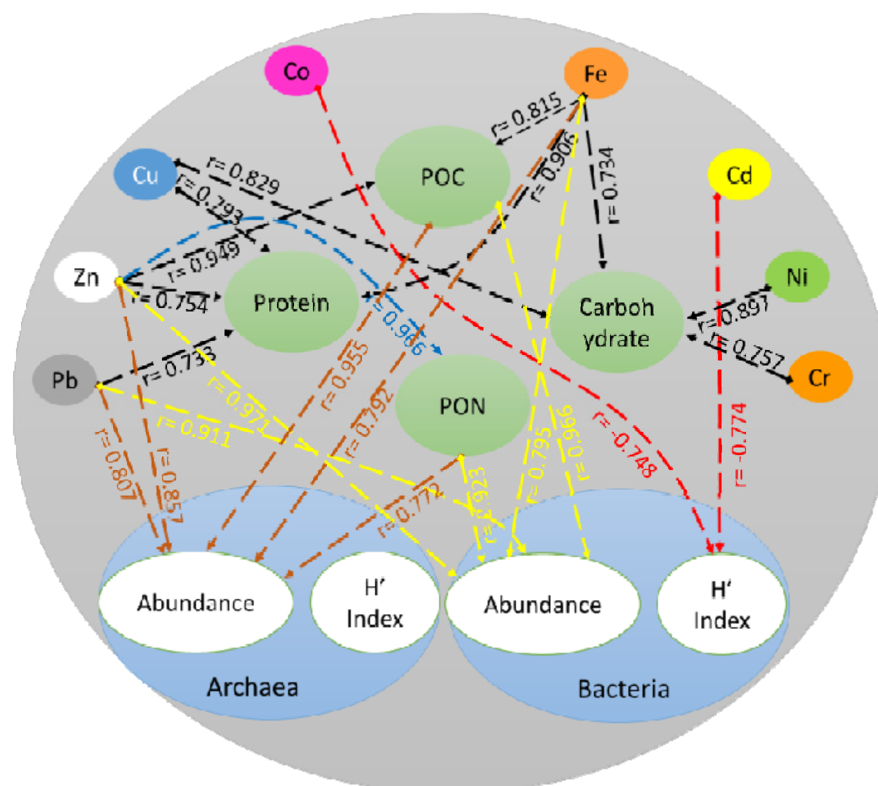

Fig. 3.11. Schematic diagram showing the two-tailed correlation of the abundance and diversity index of archaea and bacteria with heavy metal concentration and biochemical characteristics of PM. The parameters with significant correlation are connected through dotted lines and coefficient of correlation (r value) are given along the line.

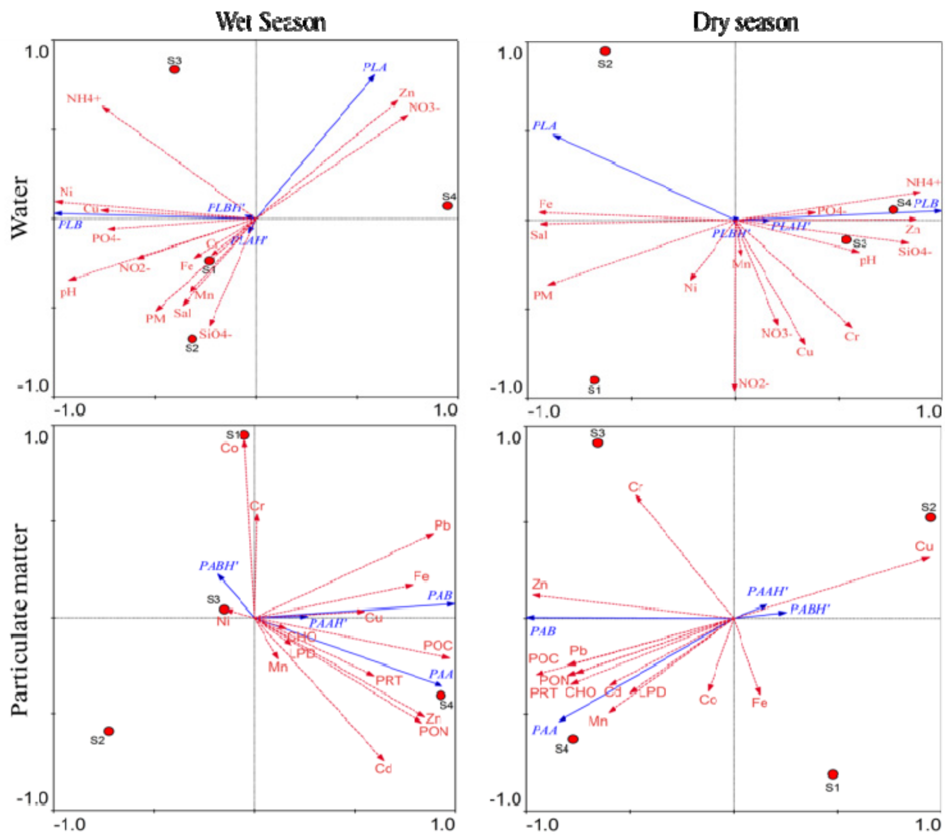

Fig. 3.12. Redundancy analysis showing the influence of hydrographic variables and heavy metal concentrations on the abundance (PLA, PLB, PAA, PAB) and diversity (PLAH', PLBH', PAAH', PABH') of planktonic (PLA, PLB, PLAH', PLBH') and PM (PAA, PAB, PAAH', PABH') associated archaea (PLA,PLAH',PAA,PAAH') and bacteria (PLB, PAB,PLBH',PABH'). (Protein PRT; Carbohydrate CHO; Lipid LPD; Particulate organic carbon POC; particulate organic nitrogen PON; particulate matter PM; salinity sal)

3.4 Discussion

The present study reports the influence of heavy metal pollution on the abundance and community structure of PM-archaea and PM-bacteria in CE, a tropical estuary along the southwest coast of India. Samples were collected along a pollution gradient from the riverine end to the bar mouth of the estuary. CE is a monsoon-driven estuary in which the hydrographic variables and the level of pollution may vary depending upon the seasonal flushing (Vinita et al., 2015). The estuary flushes multiple times during the wet season, because of high river-water influx as evident from salinity measurements. The fresh-water influx was reduced during the dry season and hence we could record the intrusion of saline water up to station S4. The concentrations of nutrients, viz. ammonia, nitrite, and nitrate, were also high in the CE during the wet season. The high nutrient load and associated phytoplankton productivity in CE during wet seasons were attributed to high terrigenous input and effluent discharge (Madhu et al., 2010). The present and previous studies confirm that the level of Zn in the CE has been increasing over the past decade (Jiya et al., 2011; Martin et al., 2012). The zinc values were reported as enhancing over the years in the estuary for example in 1982, it was reported as 102.5 mgL^{-1}, while in subsequent years the values in the upstream regions of the estuary were piled up to 120 mgL^{-1} in 1995, 1266 mgL^{-1}in 2005, 2233.2 mgL^{-1} in 2012, 4655 mgL^{-1} in 2017 (Balachandran et al., 2006; Martin et al., 2012; Nair et al., 1990; Salas et al., 2017; Venugopal et al., 1982).

High levels of all heavy metals tested, except Ni, in Particulate Matter were observed. The major heavy metal pollutant in the CE, PM-Zn,

varied from 1600 to 8451 and 766 to 2295 $\mu g g^{-1}$ during wet and dry seasons, respectively. The observed concentration of PM-Zn was 2.5 times higher compared with that reported from the CE in 1992 (Ouseph, 1992). Accumulation of heavy metals in PM have been reported from the world estuaries such as the Seine estuary in France (Ouddane et al., 1992), Texas estuary in USA (Benoit et al., 1994), Humber estuary in UK (Comber et al., 1995), Scheldt Estuary in Belgium (Baeyens et al., 1998), Tagus estuary in Portugal (Duarte and Caçador, 2012) and Jade Bay in Germany (Beck et al., 2013). Long et al (1995) depicted three types of impact of pollution on sediment biota based on the level of pollution calculated using two guideline values such as ER-L (Effective range –limit) and ER-M (Effective range –medium). The effect of pollution on biota would be rare (good sediment quality) when the concentration of heavy metals are less than ER-L, while the effect on biota increase to occasional (intermediate quality) and frequent (poor quality) when the concentration increases from ≥ER-L but <ER-M and ≥ER-M respectively (Table 3.8). Based on this calculations, the levels of PM-Zn in CE are highly toxic, and PM-Cd is toxic at the intermediate level at certain locations.

Table 3.8. Sediment quality guidelines by Long (1995)

Metal	Zn	Cd	Cu	Pb	Cr	Ni
ER-L(mg kg^{-1})	150	1.2	34	46.7	81	20.9
ER-M(mg kg^{-1})	410	9.6	270	218	370	51.6

The presence of Cd, Co, and Pb was observed in PM, although they were below detectable limits in the water column. Generally, heavy metals show a tendency to accumulate in PM, with the level of accumulation

varying for different metals. This may be attributed to the differential affinity of heavy metals towards different biochemical components of PM. Statistical analysis showed higher affinity of Zn and Pb to protein, Ni, and Cr to carbohydrate and Cu and Fe towards both protein and carbohydrate. The concentrations of heavy metals accumulated in PM were in the order Fe>Zn>Mn>Cr>Pb>Cu>Cd>Co>Ni. Previously, Fang and Lin (2002) have also reported the differential binding of heavy metals with PM, and they observed a decrease in affinity of heavy metals to PM in the order Fe>Zn=Cu>Co>Mn>Ni. Heavy metals have a tendency to form metal complexes with organic matter, which is mediated through the interaction of metal cations with anionic ligands, such as hydroxyl groups (OH), carboxyl groups (COOH), phosphates (PO_4^{2-}), sulphates (SO_4^{2-}), sulfonates, ketal-linked pyruvates, acetyl groups, amine groups etc. of proteins, lipids and carbohydrates of PM (Abou-Shanab et al., 2007; Decho, 2000; Diop et al., 2014; Hernandez and Jimenez, 2012; Lenoble et al., 2013). The high plankton biomass and nutrient levels (Gireeshkumar et al., 2013; Madhu et al., 2010) could contribute to the production of organic-rich PM in CE and subsequent attachment of heavy metals.

The high protein-to-carbohydrate ratio in PM collected was observed during the wet season, which indicates the presence of newly generated organic matter. Since the proteins are more amenable to bacterial digestion compared with carbohydrates, the protein-to-carbohydrate ratio decreases with the age of PM. This is considered as an indication of seasonal transition of the ecosystem from autotrophic to heterotrophic nature (Fabiano and Danovaro, 1994). In a previous study, Shoji et al (2008) showed the influence of freshwater influx during wet season on bacteria-

mediated carbon flux and an autotrophic-to-heterotrophic shift in CE. The protein contributes more to the biopolymeric carbon composition (BPC) during the wet season, while the lipid levels dominated the BPC of PM during the dry season. A possible reason for higher BPC in stations near the riverine end (S3 and S4) during both wet and dry seasons is the low microbial remineralization due to heavy metal pollution. A reduction in hydrolytic enzymes, involved in remineralization of organic matter, has been reported in planktonic bacteria isolated earlier from these locations (Jiya et al., 2011). The heavy metal pollution may impinge microbial remineralization of PM (Zampieri et al., 2016) and also become toxic to humans through bioaccumulation in higher trophic levels of the food web. Many organisms cannot tolerate heavy metals beyond certain concentrations, which may force them to reside in a viable but non-cultivable (VBNC) status or to die (Hobman and Crossman, 2015; Nies, 1999). Those organisms which can tolerate the pollutants at higher concentrations may flourish under such conditions and a reduction or adaptation in the diversity and activities of microorganisms have been reported (Jiya et al., 2011).

The present study monitored the impact of heavy metal pollution on the distribution and community structure of Planktonic and PM-associated archaea and bacteria. Among the different metals observed in the particulate matter, Fe, Zn, and Mn are reported to function as electron acceptors during anaerobic respiration and as a cofactor for metalloenzymes in bacteria and archaea (Lovley, 2013; Voica et al., 2016). On the contrary, the metals such as Pb and Cd do not have any significant biological role and can become toxic to the organism by entering the cytoplasm by replacing the

essential metals from their natural binding sites or by interacting with other specific ligands (Voica et al., 2016). There are different mechanisms through which archaea and bacteria acclimatize with the heavy metal pollution, which includes extracellular sequestration by biopolymers, regulating intracellular concentration by efflux pump mechanisms and enzymatic detoxifications (Nies, 1999; Voica et al., 2016). The results showed that the Planktonic and PM-archaea were less abundant than bacteria in CE. This is in agreement with previous reports from various aquatic ecosystems across the world (Bouvier and Giorgio, 2002; Garneau et al., 2009). A possible explanation is that archaeal metabolism slows down at eutrophic conditions (Vipindas et al., 2015). Among archaea, the abundance of planktonic ones was higher than that associated with a PM. A positive correlation of PM-archaeal abundance with biochemical (PON and POC) and heavy metal (Pb, Zn, and Fe) concentrations of PM was observed. However, there is no significant correlation observed between archaeal diversity and biochemical or chemical characteristics of water column and PM. The difference in the abundance of archaea observed between dry and wet seasons in S4 may be due to the difference in the level of heavy metals. The influence of heavy metal pollution on the community structure of archaea was earlier reported from sewage-sludge contaminated soil (Sandaa et al., 1999), marine sediments (Besaury et al., 2014), wetland soil (Wu et al., 2016) and mine tailings (Tan et al., 2008). More detailed studies are required to understand the influence of heavy metal accumulated in PM on the ecophysiology of archaea in marine and estuarine environments.

The abundance and community structure of planktonic bacteria were not influenced by heavy metal concentration, while that of PM-bacteria were significantly influenced by the chemical and biochemical characteristics of PM. The abundance of PM-bacteria varied between estuaries depending on the particle size and chemical composition of PM. The abundance of PM-bacteria observed in CE was comparable with Ria de Aveiro estuary in Portugal (Santos et al., 2014) and less than that reported from Columbia River estuary in the USA (Crump et al., 1998) and Zuari estuary in India (Gonsalves et al., 2017). The metabolic activities of PM-bacteria were reported to be higher in many studies, although their abundance was less compared to the planktonic counterparts (Ghiglione et al., 2009; Grossart and Simon, 1998). The level of POC, PON, PM-Zn, PM-Pb, and PM-Fe had a positive influence on the abundance of PM-bacteria and the PM-Co and PM-Cd had a negative influence on the diversity index of PM-bacteria in CE. Interestingly the differences in the abundance of PM-bacteria between different stations were not drastic. At the same time, the DGGE band pattern showed differences in the community structure of PM-bacteria between stations. There was a smaller number of OTUs in stations near the industrial zone (S3 and S4), during the wet and dry season compared with other stations. Station S4 is the point where the river Periyar carrying the industrial effluents joints with the estuary. The redundancy analyses showed that the heavy metal pollution impinges the community structure of PM-bacteria in CE. The reduction in the community structure of bacteria in response to heavy metal pollution has been reported earlier from different marine and estuarine environments (Bååth et al., 1998; Bezverbnaya et al., 2005;

Gillan et al., 2005; Naik et al., 2012; Shi et al., 2002). These studies also reported an increase in abundance and a decrease of community structure in sediments contaminated/ amended with heavy metals such as Cd, Cu, Pb, Cr, and Zn. It is possible that heavy metals may function as a selective factor on bacterial community structure in polluted environments and may lead to the proliferation of metal-resistant bacteria.

The chemical, biochemical and microbiological characteristics of particulate matter collected from different stations along a pollution gradient in a nutrient-rich tropical estuary were discussed in this chapter. The study showed the accumulation of heavy metals in particulate matter in the Cochin Estuary, which may invoke serious threats to the ecosystem functioning, as PM is considered as hotspots of microbial processes. The low diversity index of PM-bacteria in polluted regions of CE indicates the role of heavy metals in structuring the bacterial community associated with a PM.

Chapter 4

ISOLATION AND IDENTIFICATION OF PARTICLE-ASSOCIATED BACTERIA

Contents
4.1 Introduction
4.2 Methodology
4.3 Results
4.4 Discussion

4.1 Introduction

Particulate matters (PM) are the hot spots of microbial activity in the marine and estuarine environment, where the remineralization of organic matter takes place. The PM play significant role in the global biological pump (Giering et al., 2014), linking chemical constituents between sediment bed, water column and food chain (Cavan et al., 2017), transportation of chemical pollutants (Jiao et al., 2010; Volkman and Tanoue, 2002) and pathogens (Zheng et al., 2014) and carbon sequestration (Jiao et al., 2014). They are chemically and biologically complex particles within the operational size range of 0.7 to 10 µm and are formed by the continuous aggregation and disaggregation of both biotic and abiotic components (Crump and Baross, 2000). The composition of the particulate matter includes microalgae, bacteria, fine organic detritus and inorganic particles such as coccoliths, diatom skeletons and clay particles which often have an organic coating (Volkman and Tanoue, 2002). The metal ions, chemical pollutants and sediment particles attached to the biotic component makes

the PM further complicated. Although opinions differ on the dominance of free-living and PM associated bacteria in different estuaries worldwide, most of the studies have recorded PM associated bacteria to be metabolically more active than their free-living counterparts (Crump, 1999; Riemann and Winding, 2001). The biochemical characteristics of PM have a significant role in modulating the diversity and activity of microorganisms associated with them (Crump and Baross 1999, Sheeba et al 2017). Also, a different group of bacterial phyla such as Proteobacteria (α, β, γ, δ – classes), Firmicutes, Bacteroides etc, have been found to inhabit in these suspended particles as detailed in chapter 2 –Review of the literature.

The chemical characterization of particulate matter in CE indicates a higher accumulation of heavy metals in the PM, many of them are much beyond the acceptable levels (Chapter 3 results). Further, it is plausible that the heavy metals in PM might play a significant role in the selection of bacteria associated with it. Accordingly, the heterotrophic bacteria associated with PM collected from the polluted and nonpolluted regions of the CE were isolated, purified and identified to species levels using the 16S rRNA gene sequencing method.

4.2 Methodology

4.2.1 Sample collection and preservation

The particulate matter were retrieved from water samples collected from two stations each from less polluted downstream (S1 and S2) and polluted upstream (S3 and S4) (Fig. 4.1). PM was separated from the water sample by passing through 10 µm nylon membrane followed by sterile 0.8 µm (Millipore; ATTP04700) filter. The filter papers were stored at

4 °C until further analysis. Further details of sample collection, preservation, chemical and biochemical characterization of PM in the study area are discussed in Chapter 3.

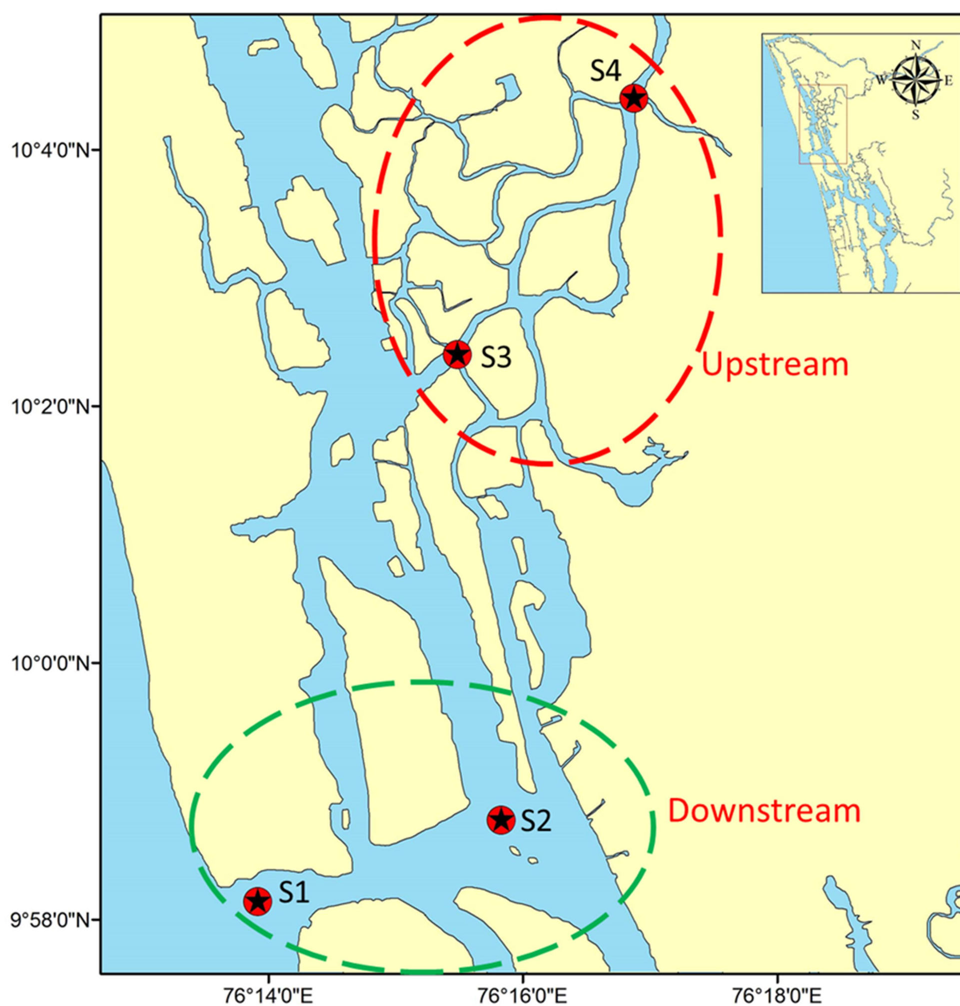

Fig. 4.1. Map of northern part Cochin estuary showing the upstream and downstream, with details of station locations

4.2.2 Isolation and identification of particle-associated bacteria

Particle-associated bacteria (PAB) were isolated from PM collected from the downstream and upstream of the CE. The PM collected on 0.8 µm polycarbonate membrane filter (Millipore ATTP02500) were re-suspended in saline (1 ml), and subjected for three cycles of vortexing (5 min) followed by sonication (5 cycles of 20s pulse followed by 50s pause with amplitude 20%) for detaching bacteria from PM. Further, the samples were serially diluted up to eight times, and one hundred microlitres from each dilution were spread over the surface of ZoBell's marine agar medium (Himedia, India). The plates were incubated at 28 ± 2 °C up to 72 h to form distinct and representative colonies of each morphological characteristics, which were purified and maintained in ZoBell's marine agar slants with sub-culturing at every 2-3 weeks. The purified isolates were cryo-preserved in media containing 20 % glycerol and maintained at -80 °C.

The PAB were identified based on similarity of 16S rRNA gene sequences (>99%) with their cultured representatives in Eztaxon (http://eztaxon-e.ezbiocloud.net) (Kim et al., 2012). The genomic DNA was extracted from overnight grown isolate using standard phenol-chloroform method (Sambrook, 2001) and the quality of DNA was checked in 0.8 % agarose gel (Sambrook and Russell, 2001). Briefly, a loop full of overnight grown bacteria were inoculated into ZoBell's Marine Broth (5 ml) and incubated for 24 h in an incubator shaker, maintained at 28 ±2°C and 100 rpm. Subsequently, the bacterial cells were separated by centrifugation at 8000 rpm and subjected to DNA extraction. The cells were suspended in 565 µl of TE buffer (10mM Tris HCl, 1mM EDTA, pH 8.0) containing 30 µl of 10 % SDS and 3 µl Proteinase K (20µg/ml), mixed by inverting10 times and incubated for 1h at 37^0C. After 1h of

incubation, a solution of CTAB/NaCl (80 µl) and 5M NaCl (100 µl) was added to the reaction mixture and continued incubation for additional 15 min more at 65 °C. To the above, 700 µl of Chloroform: isoamyl alcohol mixture (24:1) was added and the DNA extracted to the aqueous phase by centrifugation at 10000 rpm for 10min. The DNA was precipitated after 1h incubation with 0.6 volume of isopropanol at -20 °C. The precipitated DNA was separated by centrifuging at 10000 rpm for 10min, the residue washed with 500 µl of 70 % alcohol, air dried, dissolved in 50µl of TE buffer (10mM Tris HCl, 1mM EDTA, pH 8.0) and then stored at -20 °C. The quality of DNA was monitored in a gel documentation system, after separating DNA bands in an agarose gel (0.8%) by electrophoresis at 80 V for 1 h using 1X TAE buffer having pH 8. The quantity of DNA was measured spectrophotometrically using a Nanodrop (UV-Mini Model 1240).

The 16S rRNA gene of the bacteria (~1465 bp) was amplified using universal primers 27F(AGAGTTTGATC(AC)TGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Lane, 1991). The PCR reactions were carried out in a 25 µl reaction volume containing 1 µl DNA (50–100 ng), 1 µl each of primers (10 pmol), 2.5 µl 10x Taq polymerase buffer (NEB, Canada), 0.5U Taq DNA polymerase (NEB, Canada), and 200 mM each dNTPs (NEB, Canada). The following PCR cycling conditions were employed : initial denaturation at 95 °C for 2 min, followed by cycle denaturation at 95 °C for 40 s, annealing at 55 °C for 40 s, extension at 72 °C for 1.5 min for a total of 30 cycles and a final extension for 10 min at 72 °C. The PCR products were purified using Nucleo-pore Genetix brand sure extract PCR cleanup/Gel extraction kit (Genetix Biotech, India)

and cloned into a TOPO-PCR cloning vector (Life Technologies, USA), following the instruction manual. The recombinant plasmids were extracted using Nucleo-pore Genetix brand SureSpin plasmid miniprep kit (Genetix Biotech, India) and were used as a template for sequencing PCR using the primer 1090R. Sequencing PCR was done with ABI PRISM BigDye terminator ready reaction mixture (Life Technologies, USA). The cycle extension products were purified following EDTA (125mM)/ Ethanol (70%)/ Sodium acetate (3M) precipitation method. The products were analyzed on Applied Biosystems ABI 3730XL DNA analyzer. Sequence data obtained were analyzed and edited using Sequencher V4.10.1 (GeneCodes, USA). The sequences were submitted to NCBI Gene bank with accession numbers: KX015913 to KX015956 and KX056147 to KX056215.

4.2.3 Data analysis

The 16S rRNA gene sequences were analyzed for the presence of vector sequences using Vecscreen program of NCBI. Chimeric sequences were identified using DECIPHER (http://decipher.cee.wisc.edu/FindChimeras.html) (Wright et al., 2012) before removal from the data set and final sequence classification into different phyla using RDP classifier (http://rdp.cme.msu.edu/index.jsp)(Cole et al., 2008). Sequences were trimmed at 5'end at Bact341 and 3' at Bact534 and grouped using PSI algorithm to calculate operational taxonomic units (OTUs). Sequences from all OTUs were searched in NCBI using BLASTn and the nearest neighbors were selected. Sequences were multiple aligned using ClustalW and neighbor-joining and maximum-likelihood phylogenetic trees were constructed using MEGA 5.2 version (Tamura et al., 2011). Bootstrap tests were performed 1000 times using MEGA 5.2.

4.3 Results

The PAB isolated from downstream and upstream of CE were submitted to the Marine Microbial Reference Facility (MMRF), National Institute of Oceanography, Kochi (Table 4.1 & 4.2). A total of 113 PAB were isolated from the upstream (52 Nos) and downstream (61 Nos) of CE.

Table 4.1. The details of the identity of PAB isolated from the downstream of the CE and MMRF number assigned to them

Sl No	MMRF ID	Culture Identity based on Ez taxon	Similarity Index	NCBI Accession No.
1	MMRF964	*Vibrio campbellii*	99.51	KX015934
2	MMRF965	*Nitratireductor kimnyeongensis*	100	KX015935
3	MMRF966	*Altererythrobacter marinus*	100	KX015936
4	MMRF967	*Vibrio alginolyticus*	99.1	KX015937
5	MMRF968	*Bacillus halosaccharovorans*	99.49	KX015938
6	MMRF969	*Catenococcus thiocycli*	100	KX015939
7	MMRF970	*Stappia indica*	99.88	KX015940
8	MMRF971	*Chromohalobacter israelensis*	99.7	KX015941
9	MMRF972	*Marinobacter hydrocarbonoclasticus*	99.87	KX015942
10	MMRF973	*Sagittula stellata*	99.75	KX015943
11	MMRF974	*Altererythrobacter marinus*	99.86	KX015944
12	MMRF975	*Bacillus haikouensis*	99.73	KX015945
13	MMRF976	*Erythrobacter Flavus*	100	KX015946
14	MMRF977	*Alcanivorax marinus*	99.09	KX015947
15	MMRF978	*Altererythrobacter marinus*	100	KX015948
16	MMRF979	*Chryseomicrobium amylolyticum*	100	KX015949
17	MMRF980	*Acinetobacter junii*	99.33	KX015950
18	MMRF981	*Nitratireductor kimnyeongensis*	100	KX015951
19	MMRF982	*Sagittula stellata*	99.85	KX015952
20	MMRF983	*Altererythrobacter marinus*	100	KX015953
21	MMRF984	*Oceanicola nanhaiensis*	99.87	KX015954
22	MMRF985	*Nitratireductor kimnyeongensis*	99.46	KX015955
23	MMRF986	*Halomonas meridiana*	99.86	KX015956
24	MMRF987	*Bacillus haikouensis*	99.75	KX056186

Table 4.1 Continued…..

25	MMRF988	*Altererythrobacter marinus*	100	KX056148
26	MMRF989	*Paracoccus homiensis*	99.63	KX056155
27	MMRF990	*Paracoccus homiensis*	99.63	KX056158
28	MMRF991	*Pelagibacterium halotolerans*	100	KX056159
29	MMRF992	*Donghicola eburneus*	99.5	KX056162
30	MMRF993	*Catenococcus thiocycli*	100	KX056164
31	MMRF994	*Halomonas aquamarina*	99.7	KX056166
32	MMRF995	*Vibrio antiquarius*	99.48	KX056173
33	MMRF996	*Marinobacter guineae*	96.11	KX056181
34	MMRF997	*Paracoccus homiensis*	99.38	KX056183
35	MMRF998	*Vibrio antiquarius*	99.73	KX056202
36	MMRF999	*Pseudooceanicola nanhaiensis*	99.85	KX056208
37	MMRF1000	*Erythrobacter flavus*	100	KX056213
38	MMRF1001	*Hoeflea suaedae*	99.5	KX056214
39	MMRF1002	*Bacillus oceanisediminis*	99.73	KX056187
40	MMRF1003	*Bacillus haikouensis*	99.86	KX056147
41	MMRF1004	*Halomonas meridiana*	99.75	KX056176
42	MMRF1005	*Sulfitobacter pontiacus*	98.42	KX056150
43	MMRF1006	*Catenococcus thiocycli*	100	KX056153
44	MMRF1007	*Catenococcus thiocycli*	99.88	KX056156
45	MMRF1008	*Vibrio antiquarius*	99.15	KX056161
46	MMRF1009	*Altererythrobacter marinus*	100	KX056163
47	MMRF1010	*Catenococcus thiocycli*	99.24	KX056165
48	MMRF1011	*Halomonas meridiana*	99.62	KX056167
49	MMRF1012	*Bacillus vietnamensis*	100	KX056168
50	MMRF1013	*Bacillus haikouensis*	99.87	KX056169
51	MMRF1014	*Catenococcus thiocycli*	99.76	KX056177
52	MMRF1015	*Erythrobacter flavus*	100	KX056178
53	MMRF1016	*Alteromonas marina*	99.87	KX056180
54	MMRF1017	*Altererythrobacter marinus*	100	KX056171
55	MMRF1018	*Erythrobacter flavus*	100	KX056182
56	MMRF1019	*Bacillus firmus*	99.71	KX056189
57	MMRF1020	*Pseudomonas composti*	99.02	KX056195
58	MMRF1021	*Nitratireductor indicus*	97.93	KX056203
59	MMRF1022	*Bacillus vietnamensis*	99.85	KX056205
60	MMRF1023	*Bacillus haikouensis*	99.83	KX056212
61	MMRF1024	*Altererythrobacter marinus*	100	KX056215

Table 4.2. The details of the identity of PAB isolated from the upstream of the CE and MMRF number assigned to them

Sl No	MMRF ID	Culture Identity based on Ez taxon	Similarity Index	NCBI Accession No.
1	MMRF943	*Rheinheimera aquimaris*	99.1	KX015913
2	MMRF944	*Bacillus Oceanisediminis*	99.76	KX015914
3	MMRF945	*Rheinheimera aquimaris*	98.65	KX015915
4	MMRF946	*Acinetobacter junii*	98.95	KX015916
5	MMRF947	*Pseudomonas mendocina*	98.95	KX015917
6	MMRF948	*Acinetobacter junii*	99.39	KX015918
7	MMRF949	*Erythrobacter citreus*	99.48	KX015919
8	MMRF950	*Bacillus Oceanisediminis*	99.71	KX015920
9	MMRF951	*Aeromicrobium erythreum*	100	KX015921
10	MMRF952	*Bacillus Oceanisediminis*	99.47	KX015922
11	MMRF953	*Sphingopyxis ummariensis*	99.88	KX015923
12	MMRF954	*Vibrio xuii*	99.76	KX015924
13	MMRF955	*Bacillus Oceanisediminis*	99.78	KX015925
14	MMRF956	*Chromohalobacter israelensis*	99.83	KX015926
15	MMRF957	*Acinetobacter junii*	98.92	KX015927
16	MMRF958	*Alteromonas litorea*	99.86	KX015928
17	MMRF959	*Porphyrobacter colymbi*	99.72	KX015929
18	MMRF960	*Erythrobacter nanhaisediminis*	99.88	KX015930
19	MMRF961	*Acinetobacter junii*	99.32	KX015931
20	MMRF962	*Enterobacter cloacae subsp.cloacae*	99.87	KX015932
21	MMRF963	*Pseudomonas composti*	98.84	KX015933
22	MMRF1025	*Stappia indica*	99.87	KX056184
23	MMRF1026	*Hyphomonas jannaschiana*	99.63	KX056185
24	MMRF1027	*Pseudomonas composti*	99.13	KX056149
25	MMRF1028	*Aeromicrobium erythreum*	99.74	KX056152
26	MMRF1029	*Acinetobacter junii*	99.36	KX056154
27	MMRF1030	*Seohaeicola westpacificensis*	98.53	KX056175

Table 4.2 Continued…..

28	MMRF1031	*Alteromonas marina*	99.58	KX056194
29	MMRF1032	*Acinetobacter junii*	99.28	KX056197
30	MMRF1033	*Labrenzia aggregata*	100	KX056199
31	MMRF1034	*Nitratireductor aquibiodomus*	98.94	KX056200
32	MMRF1035	*Labrenzia aggregata*	100	KX056204
33	MMRF1036	*Labrenzia aggregata*	100	KX056206
34	MMRF1037	*Bacillus vietnamensis*	100	KX056209
35	MMRF1038	*Bacillus oceanisediminis*	99.77	KX056157
36	MMRF1039	*Rheinheimera pacifica*	98.85	KX056151
37	MMRF1040	*Halobacillus profundi*	99.87	KX056160
38	MMRF1041	*Brevibacillus agri*	99.43	KX056170
39	MMRF1042	*Pseudomonas mendocina*	98.54	KX056172
40	MMRF1043	*Rheinheimera aquimaris*	99.88	KX056174
41	MMRF1044	*Enterobacter cloacae subsp. cloacae*	99.87	KX056179
42	MMRF1045	*Pseudomonas indoloxydans*	99.34	KX056188
43	MMRF1046	*Pseudomonas mendocina*	99.72	KX056190
44	MMRF1047	*Brevundimonas nasdae*	99.37	KX056191
45	MMRF1048	*Micrococcus lylae*	99.87	KX056192
46	MMRF1049	*Bacillus oceanisediminis*	99.72	KX056193
47	MMRF1050	*Bacillus oceanisediminis*	99.72	KX056196
48	MMRF1051	*Acinetobacter junii*	99.36	KX056198
49	MMRF1052	*Aeromonas caviae*	100	KX056201
50	MMRF1053	*Aeromicrobium erythreum*	99.73	KX056207
51	MMRF1054	*Enterobacter cloacae subsp. cloacae*	99.86	KX056210
52	MMRF1055	*Sphingopyxis ummariensis*	99.57	KX056211

The PAB isolated from the upstream were dominated by γ-proteobacteria (48.1 %) followed by α-proteobacteria (25%), Firmicutes (19.2%) and Actinobacteria (7.7%). The dominant PABs in the downstream was in the order of α-proteobacteria (45.9%), followed by γ-proteobacteria (36.1%) and Firmicutes (18%) (Fig 4.2).

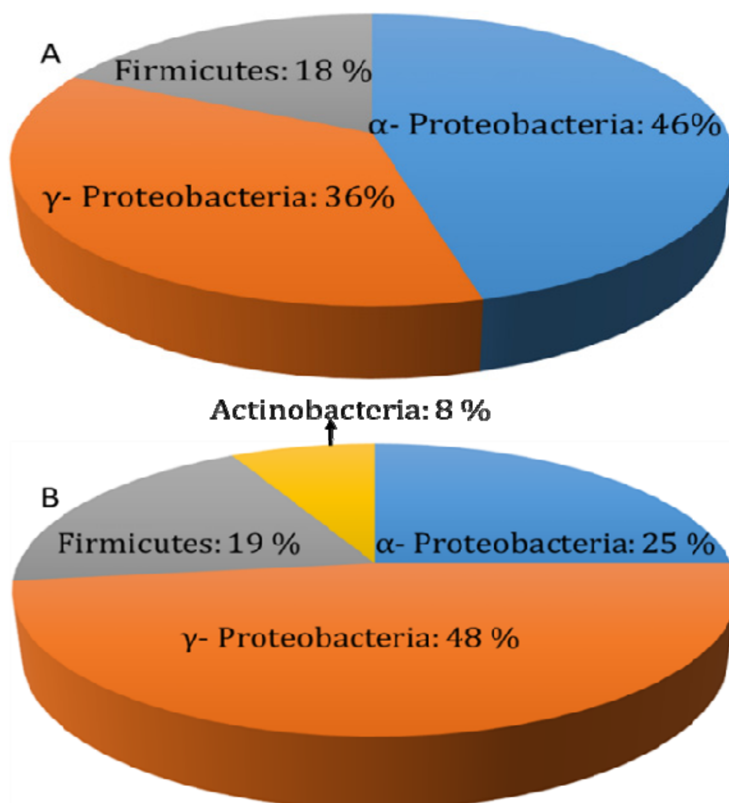

Fig. 4.2. Major group of PAB at phylum level isolated from the downstream (A) and upstream (B) of the CE

The genus level classification of the PAB showed the presence of 22 and 23 genera respectively in the upstream and downstream of the estuary (Fig. 4.4). The Venn diagram showed that 25 % of the PAB isolated from upstream and downstream were identical (Fig. 4.3), while 36.1 and 38.9 % of the isolates were unique to upstream and downstream samples respectively. At the genus level, the diversity of Proteobacteria was high in downstream with a higher diversity of α-Proteobacteria observed.

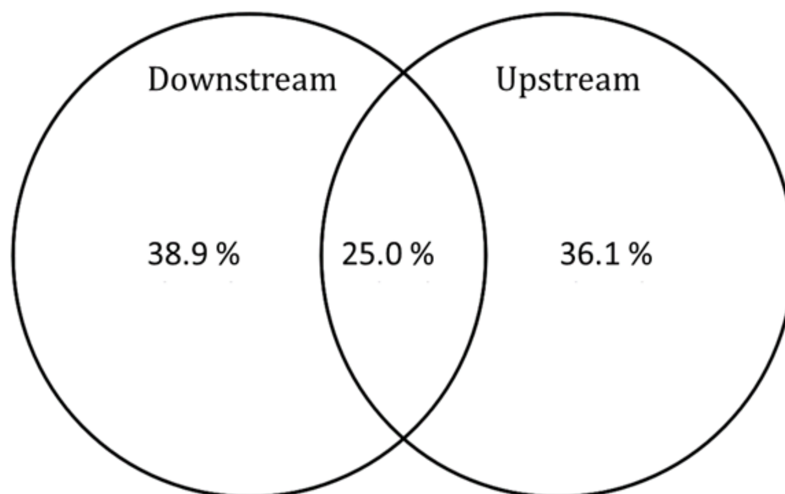

Fig. 4.3. Venn diagram showing the similarity between the diversity of PAB isolated from downstream and upstream

Among α-Proteobacteria, *Erythrobacter* sp., *Nitratireductor* sp. and *Stappia* sp. were isolated from both upstream and downstream, while the diverse population of α-Proteobacteria in the downstream included *Altererythrobacter* sp., *Sagittula* sp., *Oceanicola* sp., *Paracoccus* sp., *Pelagibacter* sp., *Donghicola* sp., *Pseudooceanicola* sp., *Hoeflea* sp. and *Sulfitobacter* sp.The α-Proteobacteria isolated from the upstream also included *Sphingopyxis* sp., *Porphyrobacter* sp., *Labrenzia* sp., *Brevundimonas* sp., *Hyphomonas* sp., and *Seoheicola* sp. The five out of twelve species of γ-Proteobacteria found in upstream and downstream were common, which include *Acinetobacter* sp., *Pseudomonas* sp., *Vibrio* sp., *Chromohalobacter* sp. and *Alteromonas* sp. The γ-Proteobacteria such as *Rheinheimera* sp., *Enterobacter* sp., and *Aeromonas* sp. were isolated only from the upstream, while *Catenococcus* sp., *Marinobacter* sp. *Alcanivorax* sp and *Halomonas* sp.

were unique to downstream. The Actinobacteria, such as *Micrococcus* sp., and *Aeromicrobium* sp., were found only among PAB isolated from the upstream station. The neighborhood joining tree showing the phylogenetic identity of PAB is given in Fig. 4.5.

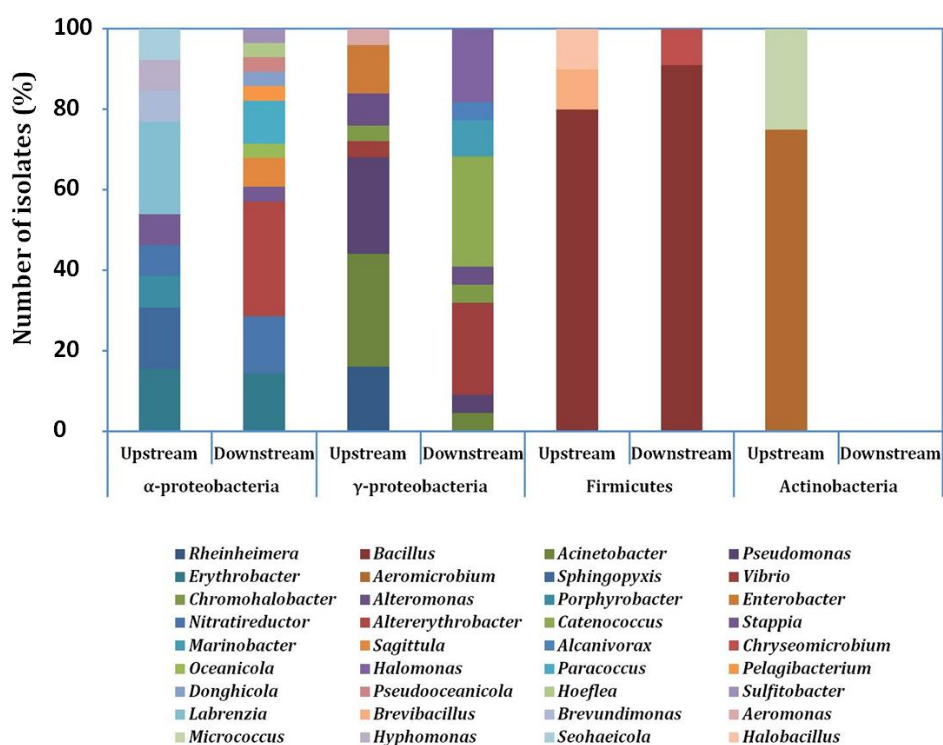

Fig. 4.4. Genus level diversity of PAB isolated from upstream and downstream

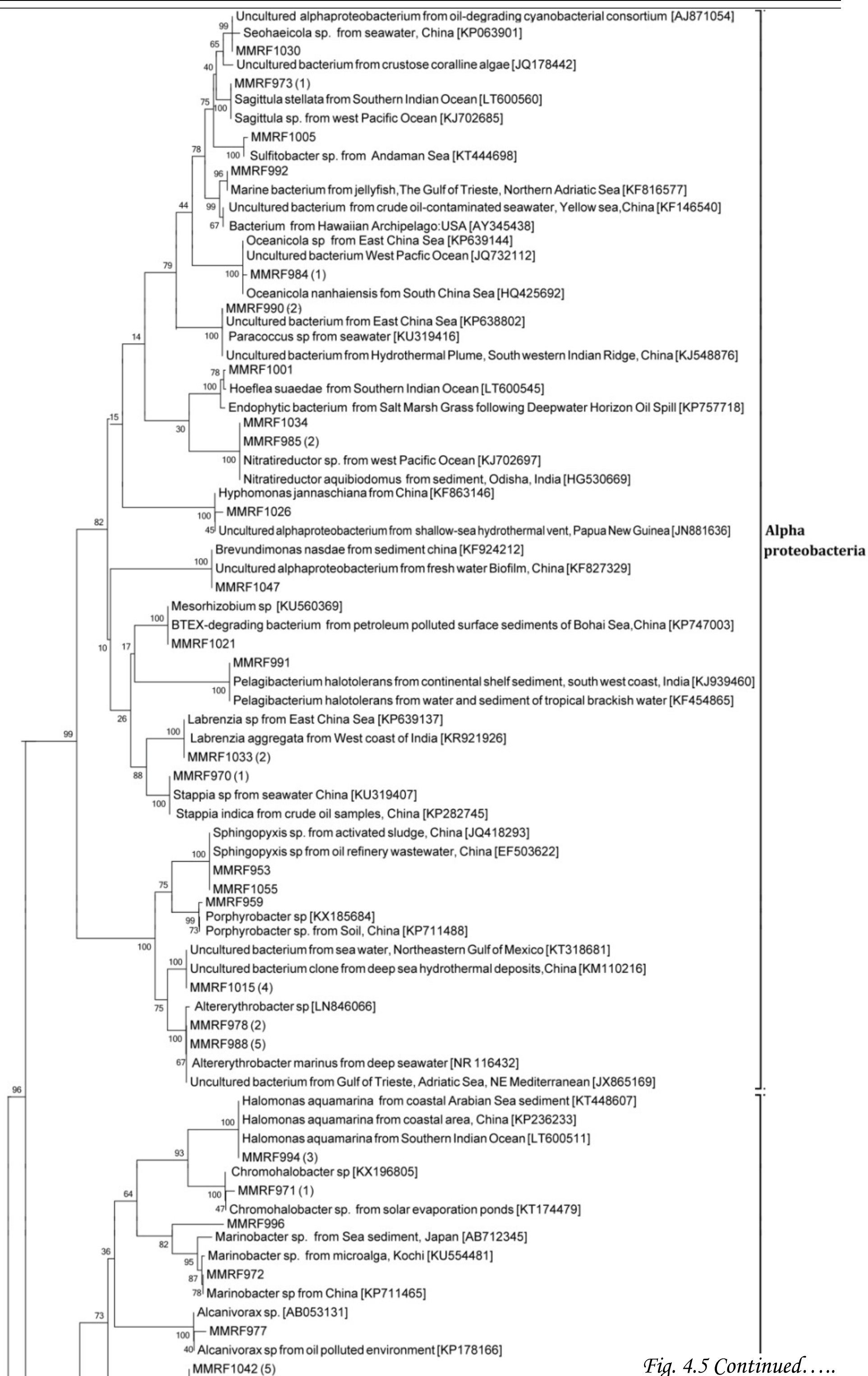

Fig. 4.5 Continued…..

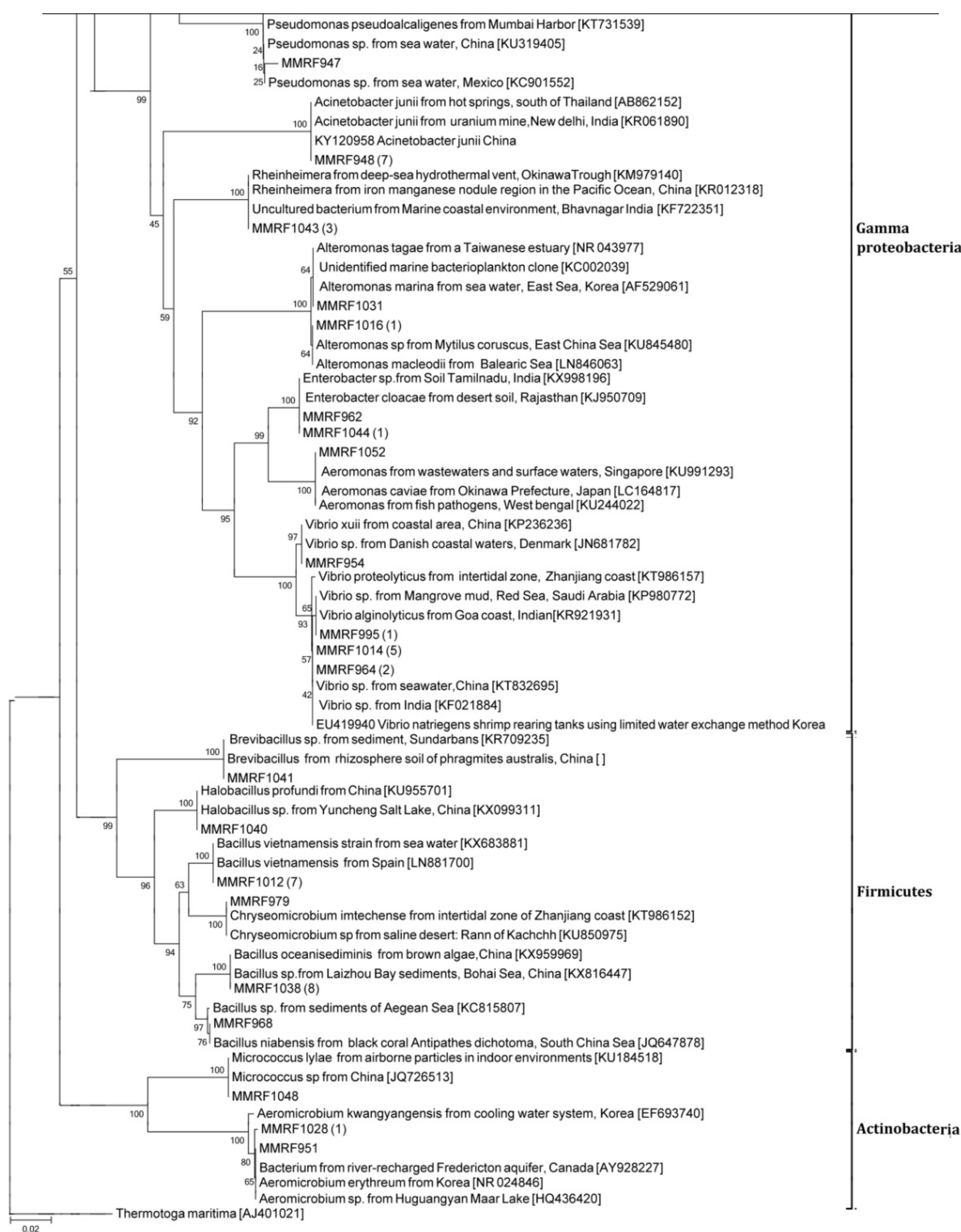

Fig. 4.5. Rooted neighbor-joining phylogenetic tree based on 16S rRNA gene sequences retrieved from the DNA of the isolated cultures from the estuary. The numbers at the nodes are percentages indicating the levels of bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets. Scale bar represents 10% estimated sequence divergence. *Thermotogamaritima* was used as an out group. Numbers in bracket indicates the additional cultures isolated under the same OTU present in the library.

4.4 Discussion

This chapter discusses the difference in the diversity of PAB isolated from two regions of a tropical estuary impacted with different levels of heavy metal pollution (polluted -upstream and non-polluted- down-stream). Increasing trend of heavy metal pollution, Zn, and Cd has been recorded in the upstream of CE since the 1990s and reached a 40 – 45 % increase in 2017 compared to 1995 (Please see Fig. 2.2 in Review of Literature). The upstream of the estuary receives approximately 12000 m^3s of freshwater from river Periyar during monsoon and ~1000 m^3s during non-monsoon. The river also carries a significant fraction of ~260 million litres of industrial effluents, rich in heavy metals, polycyclic aromatic hydrocarbons, and nutrients into the estuary. The heavy metals may flow down to a certain distance with water currents, before getting trapped into the sediment particles, resulting in the generation of a pollution gradient in the estuary (from North to South). Previous studies have shown that the accumulation of heavy metals in CE has directly impacted the diversity and activity of sediment-associated bacteria (Jiya et al., 2011). The results presented in Chapter 3 shows that the heavy metal pollution has a definite role in designing the community structure of bacteria and archaea associated with PM in the CE. The present chapter focuses more on the diversity of cultivable PAB in CE.

Only 25 % of the PAB were found common between particulate matter from upstream and downstream. This is in agreement with the Chapter 3 results based on DGGE and was attributed to the difference in the chemical and biochemical characteristics of particulate matter in upstream

and downstream and previous studies from sediments (Sheeba et al., 2017; Zhang et al., 2016). Although 70 % of the PAB isolated from both upstream and downstream belonged to phylum Proteobacteria, there were clear differences in the distribution at the class level. Majority of the particulate associated Proteobacteria isolated from upstream belonged to γ-proteobacteria (48.1%), while it was α-proteobacteria (45.9 %) in the downstream. The γ-proteobacteria commonly reported from other polluted environments, viz *Pseudomonas* spp., *Rheinheimera* sp. and *Acinetobacter* sp (Patel et al., 2014) were also found dominant among the PAB from the upstream of CE. There were many isolates of α-proteobacteria such as *Sagittulla* sp., *Altererythrobacter* sp., found only in downstream of the CE, which are known for their participation in degradation of complex organic compounds such as lignins and hydrocarbons (Gonzalez et al., 1997; Mcleod et al., 2011; Teramoto et al., 2013). The PM from the upstream were also devoid of *Cytophaga*- *Flavobacteria* and *Bacteriodetes* important in the degradation of high molecular weight dissolved organic matter (Crump, 1999; DeLong et al., 1993; Fandino et al., 2001) which are commonly found in the PM of less polluted waters (Kirchman, 2002). Similarly, the other ecologically significant organisms such as *Catenococcus* sp, *Paracoccus* sp, *Sulfitobacter* sp etc important in the biogeochemical cycling of nitrogen, carbon and sulfur were also found only in the less polluted downstream waters (Roh et al., 2009; Sorokin et al., 1996; Teske et al., 2000). On the other side, several bacteria isolated from the upstream region were found to produce large amounts of exopolysaccharides and other biofilms perhaps, for rendering protection from heavy metal toxicity (Vivas et al., 2006). It may be mentioned that *Acinetobacter* sp found in the upstream were also

reported from the coastal environment polluted with Cd (Mathivanan and Rajaram, 2014). Similarly, the *Sphingopyxis* sp is documented for their ability to degrade xenobiotics such as triclosan in waste treatment plants (Alvarez et al., 2016; Krzmarzick and Novak, 2014).

The above study indicates that distribution of particle-associated heterotrophic bacteria varies in the polluted and nonpolluted regions of the estuary, indicating the role of metal pollution in structuring the microbial community associated with it. The dominance of microbes which are usually found in polluted regions in the upstream area of the estuary also indicates the role of metals in the selection of bacterial community associated with particulate matter. Further, it is significant that the bacterial species, playing important role in the biogeochemical cycling are absent in polluted waters, further supporting the view that metal pollution affects the ecosystem functioning in the estuary in a deleterious manner.

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Chapter 5

METAL TOLERANCE PROFILE OF PARTICLE ASSOCIATED BACTERIA

Contents

5.1 Introduction
5.2 Methodology
5.3 Results
5.4 Discussion

5.1 Introduction

Bacteria play a significant role in the aggregation and disaggregation of particulate matter in the marine and estuarine environment. The negative surface charge of bacterial extracellular polysaccharides attracts cationic trace metals to the particulate matter (Cavan et al., 2017; Giering et al., 2014). Many of the trace metals are double-edged swords, which while supporting cellular processes of microorganisms at lower concentrations may become toxic at higher concentrations (Gadd, 2010; Jiya et al., 2011; Lemire et al., 2013; Nies, 1999). For example, Zn is a cofactor of many enzymes but may inhibit the activities of microbial enzymes at higher concentrations (Jiya et al., 2011; Sheeba et al., 2017; Zheng et al., 2014). The toxic effects of heavy metal pollution on the distribution and activities of free-living bacteria have been reported from riverine, estuarine and marine environments (Nweke et al., 2006; Patel et al., 2014). The studies on the effect of heavy metal pollution on particle associated bacteria in the

marine and estuarine environment are important as it may shed more light on the fate of microbial hotspots in polluted estuaries and may support in the development of newer strategies for preserving the normal biogeochemical processes under such extreme / harsh conditions. The heavy metal tolerance of bacteria can be used as an indicator of potential toxicity of metals to other higher forms of life (Olson and Thornton, 1982). Further, these studies will also help in its application in the bioremediation of metal polluted effluents in treatment plants.

Earlier studies have reported the influence of heavy metal pollution on the diversity and activity of heterotrophic bacteria and cyanobacteria in the water column and sediments of CE (Anas et al., 2015; Jiya et al., 2011). The metal accumulations in the particulate matter were also reported from the estuary (Nair et al., 2013; Ouseph, 1992). In the present study, the difference in the metal tolerance profile of particle associated bacteria isolated from the polluted and non-polluted regions of Cochin estuary are reported.

5.2 Methodology

The Sample collection and Preservation: The isolation and preservation of PAB are as detailed in the methodology section of chapter 4. The cryo-preserved PAB, retrieved using ZoBell's Marine broth were used for the analysis.

5.2.1 Heavy metal tolerance of PAB

The metal tolerance profile of each PAB was determined by well diffusion method described by Hassen et al (1998). Briefly, 1 cm diameter

wells with 4 mm depth were prepared at the center of nutrient agar plates. Subsequently, 500 µl of different concentrations of a metal salt solution of $NiCl_2$ 6 H_2O (30-250 mM), $ZnSO_4$ $7H_2O$ (20-250 mM), $CuSO_45H_2O$ (30-180 mM), $CdSO_4$ $8H_2O$ (20-120 mM) were filled into the wells of separate plates. The metal salt solution was allowed to diffuse into surrounding media for 24 h at room temperature. On each plate, three isolates were streaked radially in quadruplicate.

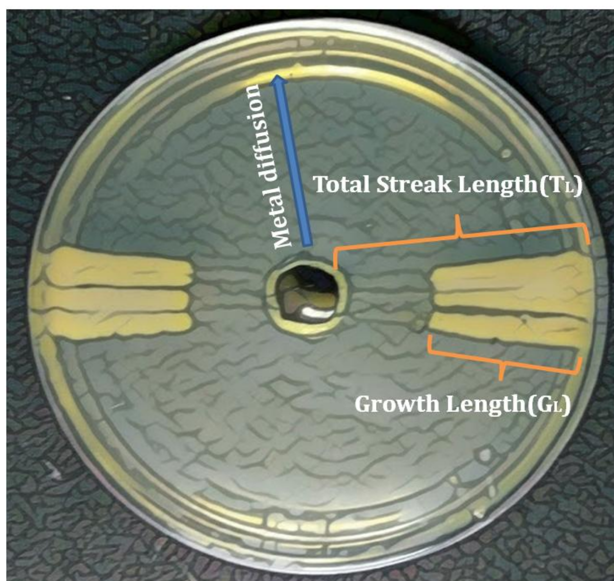

Fig. 5.1. Representative image showing the total length (T_L) of streak and growth length (G_L) of heterotrophic bacteria in well diffusion assay

The plates were then incubated at room temperature for 48 h. The total length of the inoculation streak (T_L) vs the length of growth of the culture (G_L) was measured, from the edge of the plate to the well-edge in mm (Fig.5.1). The concentrations of various metals used for the study were given in Table 5.1.

Table 5.1. Concentration ranges of various metals used for the study

Metals	Concentrations in mM					
Zinc (Zn)	20	50	100	150	200	250
Cadmium (Cd)	20	40	60	80	100	120
Copper(Cu)	30	60	80	120	160	180
Nickel (Ni)	30	70	100	150	200	250

The metal tolerances of the cultures were calculated using the equation, Metal tolerance index = (Tg/Ts). The isolates were classified into low (0-0.25), medium (0.25-0.5), high (0.5-0.75) and Very high (0.75-1) tolerant groups based on their metal tolerance index. The multiple resistance of among the cultures were analysed by drawing Venn diagram using Venny 2.1 software.

5.3 Results

The tolerance of 113 PAB, isolated from polluted upstream (52) and nonpolluted downstream (61) of CE, towards increasing concentrations of Zn (20 - 250 mM), Cd (20 - 120 mM) Cu (30 – 180 mM) and Ni (30 -250 mM) were studied using plate diffusion assay.

5.3.1 Zinc Tolerance

The tolerance index of PAB to increasing concentrations of Zinc is shown in Fig. 5.2. Majority of the PAB isolated from both polluted upstream and non-polluted downstream showed high tolerance index towards lowest concentration (i.e 20 mM) of Zn tested. More than 50% of particle associated bacteria isolated from the upstream showed very high or medium tolerance index up to 200 mM, where such a high tolerance was restricted up to 50mM among isolates from downstream. The isolates

with very high tolerance index for 250mM Zn was distributed among nine genus such as *Enterobacter* sp, *Alteromonas* sp, *Acinetobacter* sp, *Altererythrobacter* sp, *Marinobacter* sp, *Pseudooceanicola* sp, *Bacillus* sp and *Erythrobacter* sp (Table 5.2).

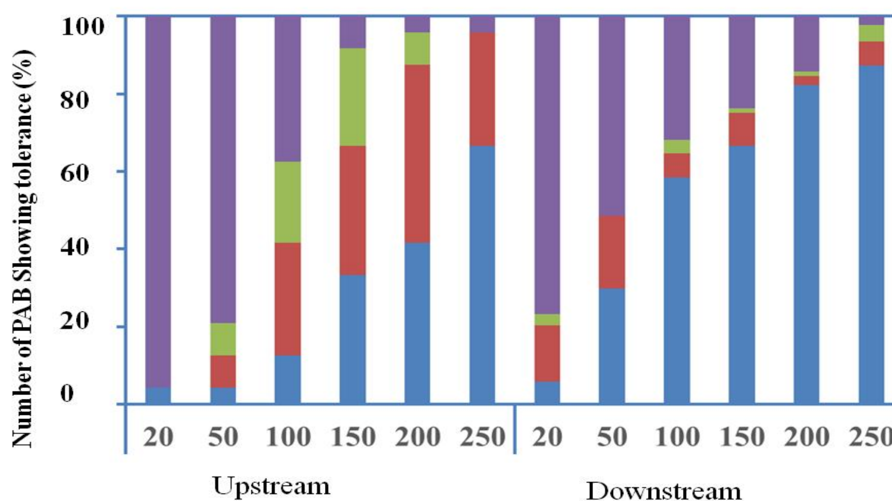

Fig. 5.2. Tolerance of PAB from upstream and downstream towards increasing concentrations of Zn. The isolates were classified into low (blue), medium (red), high (green) and very high (violet) tolerant groups based on the metal tolerance.

5.3.2 Cadmium Tolerance

A difference in tolerance index to Cd was observed among PAB isolated from upstream and downstream (Fig. 5.3). Nearly 50 % of the PAB from upstream showed medium or very high tolerance index towards Cd at 60 mM concentration while it was observed among 10 % of PAB from the downstream. More than 90 % and less than 40 % of PAB isolated respectively from the upstream and downstream showed medium or high tolerance index to lowest concentration i.e. 20 mM of Cd. Interestingly, there were PAB

isolated from upstream which showed high tolerance index to highest concentration (i.e 100 mM) of Cd tested. The *Brevundimonas* sp (MMRF No 1047) and *Acinetobacter* sp (MMRF No. 1051) isolated from the upstream region of the estuary showed the highest tolerance index (Table 5.2).

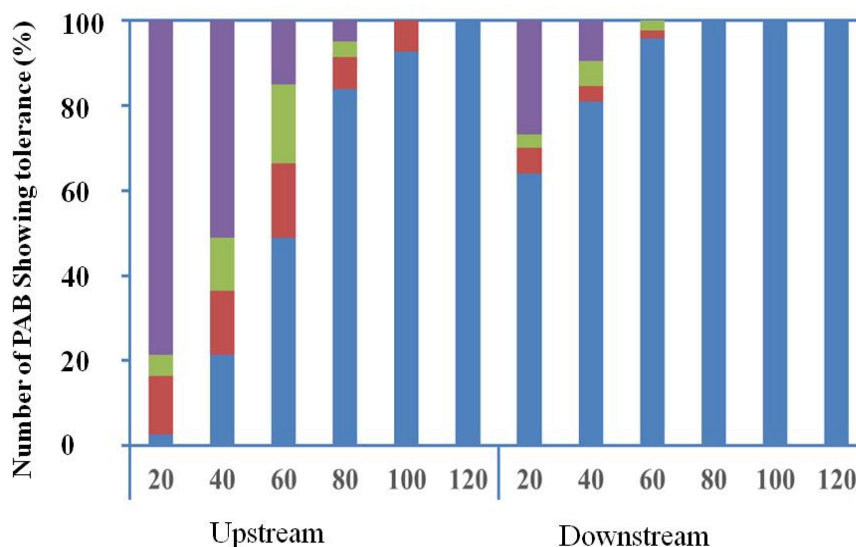

Fig. 5.3. Tolerance of PAB from upstream and downstream towards increasing concentrations of Cd. The isolates were classified into low (blue), medium (red), high (green) and very high (violet) tolerant groups based on the metal tolerance.

5.3.3 Copper Tolerance

The resistance index of PAB towards Cu is depicted in Fig. 5.4. More than 50 % of PAB from the upstream and downstream region of the estuary showed very high tolerance index at 80 mM concentration. All the PAB isolated from both the upstream and downstream showed medium and high tolerance index at 30 mM Cu. Nearly 70 % of PAB from upstream of 40 % of downstream showed medium or high tolerance index towards highest concentration (180 mM) of Cu. These isolates belonged to seven genera

such as *Alteromonas* sp, *Halomonas* sp, *Bacillus* sp, *Catenococcus* spp, *Chromohalobacter* sp, *Erythrobacter* sp, *Altererythrobacter* sp (Table 5.2). The representative image of Cu tolerance study was given in the Fig. 5.5.

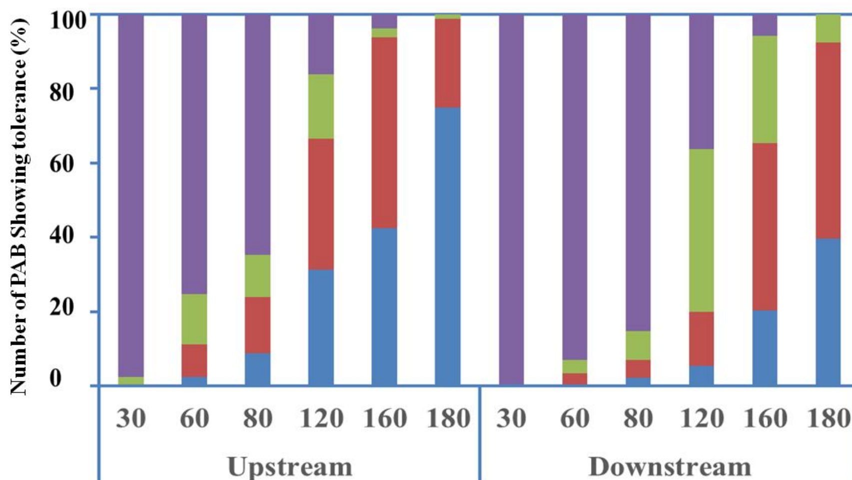

Fig. 5.4. Tolerance of PAB from upstream and downstream towards increasing concentrations of Cu. The isolates were classified into low (blue), medium (red), high (green) and very high (violet) tolerant groups based on the metal tolerance.

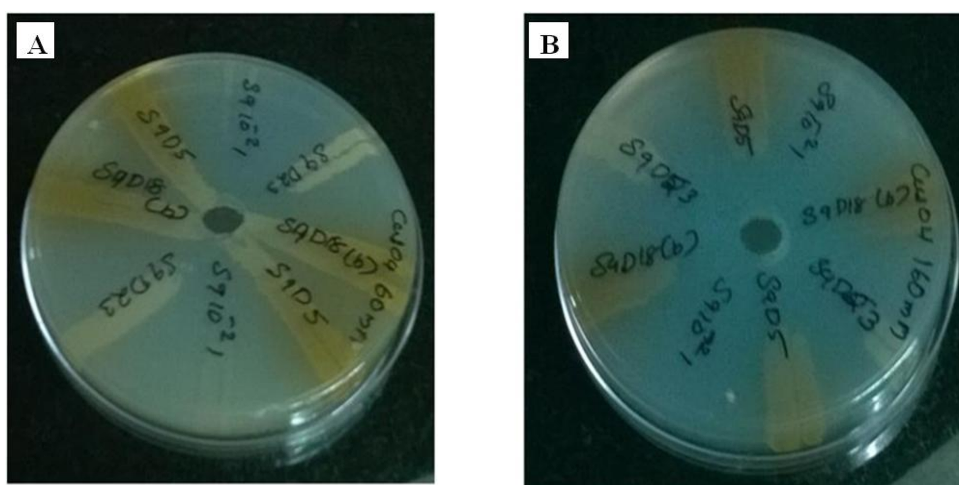

Fig. 5.5. Representative images showing the metal tolerance of PAB to 60 (A) and 120 mM (B) Cu in plate diffusion assay

5.3.4 Nickel Tolerance

The nickel tolerance of the PAB was depicted in Fig. 5.6. Interestingly the tolerance index towards Ni was more prominent among PAB from downstream. More than 50 % of the isolates from downstream and upstream showed high tolerance index to 200 and 150 mM Ni respectively. The PAB of upstream, which showed high tolerance index to highest concentrations of Ni tested (i.e 250 mM) were identified as *Porphyrobacter* sp, *Pseudomonas* sp, *Enterobacter* sp, *Acinetobacter* sp, *Erythrobacter* sp. A total of 30 number of PAB isolated from downstream, belonging to 16 genera, showed maximum tolerance index 250 mM Ni (Table 5.2).

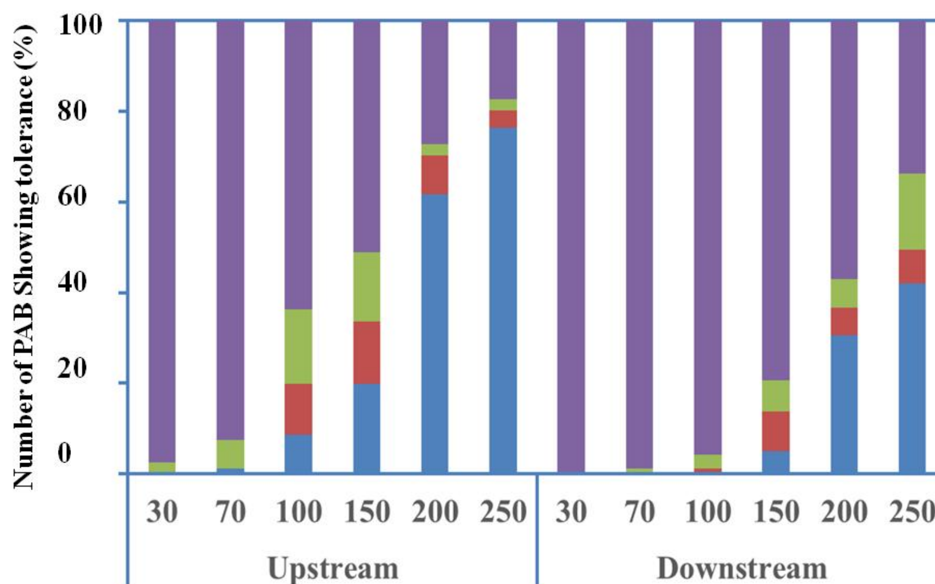

Fig. 5.6. Tolerance of PAB from upstream and downstream towards increasing concentrations of Ni. The isolates were classified into low (blue), medium (red), high (green) and very high (violet) tolerant groups based on the metal tolerance.

Table 5.2. PAB showed maximum tolerance index against Zn, Cd, Cu and Ni in upstream and downstream of the estuary.

Sl No	Metals	PAB tolerant to highest concentration tested	
		Upstream	**Downstream**
1	Zn (250 mM)	*Enterobacter* sp MMRF 962 *Alteromonas* sp MMRF 1031 *Acinetobacter* sp (MMRF 1051, MMRF 948)	*Altererythrobacter* sp (MMRF 983, *MMRF 978)* *Marinobacter* sp MMRF 996 *Pseudooceanicola* sp MMRF 999 *Bacillus* sp MMRF 1002 *Erythrobacter* sp MMRF 1018
2	Cd (100 mM)	*Brevundimonas* sp MMRF 1047 *Acinetobacter* sp MMRF 1051	Nil
3	Cu (180 mM)	*Alteromonas* sp MMRF 1031	*Halomonas* sp MMRF 994 *Bacillus* sp MMRF 968 *Catenococcus* sp (MMRF 969, MMRF 1007) *Chromohalobacter* sp MMRF 971 *Erythrobacter* sp MMRF 1015 *Altererythrobacter* sp (MMRF 1017, MMRF 1024)
4	Ni (250 mM)	*Porphyrobacter* sp MMRF 959 *Pseudomonas* sp (MMRF 1027, MMRF 1045) *Enterobacter* sp (MMRF 1044, MMRF 1054) *Acinetobacter* sp MMRF 1051 *Erythrobacter* sp MMRF 949	*Nitratireductor* sp (MMRF 981, MMRF 985, MMRF 965) *Sagittula* sp (MMRF 982, MMRF 973) *Altererythrobacter* sp *(*MMRF 983, MMRF 966, MMRF 1009) *Paracoccus* sp (MMRF 989, MMRF 990) *Pelagibacterium* sp MMRF 991 *Donghicola* sp MMRF992 *Vibrio* sp (MMRF 995, MMRF 995 MMRF 967 MMRF 1008) *Marinobacter* sp MMRF 996 *Erythrobacter* sp MMRF 1000 *Hoeflea* sp MMRF 1001 *Bacillus* sp (MMRF 1002, MMRF 968, MMRF 975, MMRF 1003, MMRF 1023) *Catenococcus* sp (MMRF 969, MMRF 1007) *Stappia* sp MMRF 970 *Acinetobacter* sp MMRF 980 *Halomonas* sp MMRF 1004 *Sulfitobacter* sp MMRF 1005

Multiple metal resistance of PAB, which were showing metal resistance index >0.5 at mid concentration used for the metal resistance analyses (i.e.Cd 60mM , Cu 80mM, for Zn and Ni 100mM). The result showed that about 55 % of PAB showed multiple resistances (≥3 metals). Among this 14.3 % of organisms showed resistance to all the metals was analyzed (Zn,Cu,Cd,Ni) (Fig 5.7). A 38% of PAB which showed resistance were isolated from downstream while 62 % were isolated from the upstream region of the estuary.

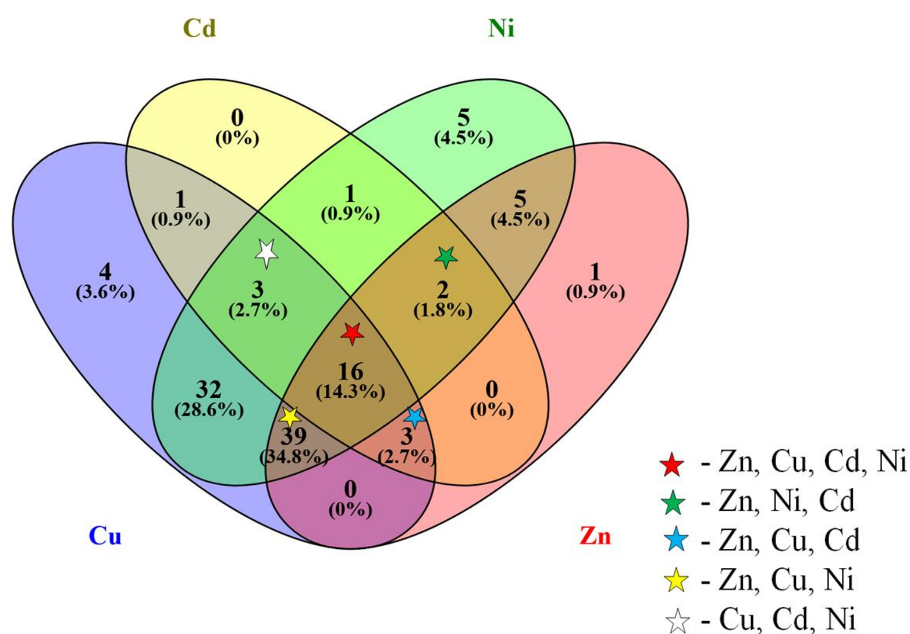

Fig. 5.7. Venn diagram showing the Multiple Metal Resistance (MMR) of PAB

Generally, more number of PAB isolated from upstream were tolerant to Zn and Cd, while Ni and Cu tolerance were more visible among PAB from downstream. The *Acinetobacter sp* MMRF 1051 isolated from the upstream was resistant to even the highest concentrations of Zn, Cd, Cu

and Ni (Table 5.2). The isolates from downstream were also tolerant towards the highest concentrations of Zn, Cu, and Ni. Tolerance towards the highest concentrations of multiple metals (≥3 metal) was not evident among PAB from downstream. Also, none of the downstream isolates showed tolerance to the highest concentration of Cd.

5.4 Discussion

The metal tolerance of particle associated bacteria in the CE was in the order Ni>Cu>Zn>Cd (most tolerant to most sensitive). The present study confirms the broad spectrum metal tolerance of PAB isolated from the upstream. The *Acinetobacter* sp., MMRF1051, isolated from upstream showed resistance up to 250 and 100 mM concentrations respectively of Zn and Cd. A *Virgibacillus* sp., MMRF571, previously isolated from an upstream phytoplankton of upstream of the CE showed resistance up to 180 mM Cd (Anas et al., 2016). Previous studies had shown that Zn and Cd are present up to concentrations of 129 mM and 0.2mM in the particulate matter and 42mM and 0.2 mM in sediment respectively (Jiya et al., 2011; Sheeba et al., 2017).These high concentrations must have functioned as the selective pressure for the development of metal tolerance among the isolates. Although Zn, Cu, and Ni are essential micronutrients for the functioning of many microbial enzymes, higher concentrations of these metals are reported to be toxic. For example, high concentration of zinc is known to inhibit the respiratory electron transport systems of bacteria (Schulte et al., 2014). Excess copper may accelerate the production of reactive oxygen species, which at a higher concentration can cause lipid peroxidation and damage to protein and nucleic acid (Mocquot

et al., 1996; Wang et al., 2014). Similarly, excess Ni also can induce toxicity through various routes such as replacing essential metals in metalloprotein, binding to catalytic residues of non-metalloproteins and indirectly causing oxidative stress (Macomber et al., 2011). On the other hand, Cd is more bioavailable to bacteria due to its high solubility in water and hence can be toxic even at lower concentrations. The intracellular accumulation of Cd ions increases the oxidative stress may induce lipid peroxidation, DNA strand breakages and chromosomal aberrations (Anas et al., 2008; Arshad et al., 2016).The bacteria tolerant up to 15mM for Zn and 0.5.mM for Cd has been reported from the waste treatment plants (Ozdemir et al., 2004). Also, bacteria with tolerance up to 250 mM Zn 180 mM Cd has been reported from the estuarine and marine environment (Anas et al., 2016) and 1.77mM for Cd and 24.47mM for zinc from polluted marine sediments (Zampieri et al., 2016). The Ni tolerance up to 700 mg/L in *Pseudomonas sp* isolated from the industrial effluents was observed in a previous study Raja et al (2006). The high tolerance of PAB isolated from the upstream of the estuary to Zn and Cd indicates that the accumulation of heavy metals in particulate matter favors the colonization of metal tolerant species. Most of the studies on bacterial metal resistance were from sediments (Konstantinidis et al., 2003; Li and Ramakrishna, 2011; Ramaiah and De, 2003; Zampieri et al., 2016) and water (Ivanova et al., 2002; Sabry et al., 1997). The abundance and diversity of metal resistant organisms in the water column and sediment of Cochin estuary were reported previously by Jiya et al (2011). On the other side, the metal tolerance of particle associated bacteria in the estuary is reported for the first time in the present study. In general, More than 55% of the isolates

showed multiple metal resistances in the estuary. Also, the occurrence of metal tolerant species in the downstream may be the indication of the particulate matters act as carrier of these tolerant species from polluted to non polluted regions of the estuary The highest percentage PAB from the upstream showed resistance towards the metals such as Zn (100mM), Cu (80mM), and Ni (100mM), while 90 – 100 % of the isolates from the water column and sediment of Eloor in upstream showed resistance to 5 mM concentrations of Zn, Co, Cu, Ni (Jiya et al 2011). This multiple resistance may be due to the genes located together on the same genetic material such as plasmid, transposon, integron etc. (Baker et al., 2006; Billman et al., 2018; Diels et al., 1995; Foster, 1983). The high metal tolerance observed in the particle associated bacteria belongs to the phylum Proteobacteria in Cochin estuary. The studies by Ivanova et al (2001) and Jiya et al (2011) reported that the mechanism of tolerance attributed to the high concentration of polysaccharides in them.The study indicates that those bacteria which can adapt to higher concentrations of heavy metals are colonizing on the particulate matter in the upstream, while such segregation is not found among PAB from downstream. However further studies are necessary to understand the tolerance mechanisms of these bacteria.

The maximum tolerance for Cd was observed in the genus *Acinetobacter and Brevundimonas* in upstream of the estuary. This was supported by the previous studies on the metal resistance capacity of these group of bacteria attributed from the production of exopolysaccharides (Dhakephalkar and Chopade, 1994; Masoudzadeh et al., 2014; Yadav et al., 2012). Most of the bacteria contain the functional groups like –OH, -SH and –COOH on its surfaces which helps the adsorption of metals (Gadd, 2009;

Lemire et al., 2013), or can bind the metals and this may attribute its tolerance. Such types of bacteria can be further used for studying its application in bioremediation of the heavy metals. The bacteria showed metal tolerance through a variety of mechanisms including metal sorption by intra or extracellular sequestrations by the production of exopolysaccharides, siderophores, magnetosomes etc or by the detoxification through efflux pump systems (Nies, 1999). Detailed studies are required to understand the mechanisms of metal tolerance in particle-associated bacteria.

The high metal tolerance profile of particle associated bacteria observed in Cochin estuary by the plate diffusion method impart the knowledge that the capacity of adsorption and complexation of metals or its mobility in terms of its bioavailability to the bacteria. Also the metal tolerance profile of particle associated bacteria in Cochin Estuary provides an insight into the response and adaptation of them to the heavy metal pollution gradient prevailed in the estuary. Also, the Zn and Cd-tolerant species higher among the particle associated microorganisms isolated from the upstream of the estuary indicates that Zn and Cd had a significant impact on these microorganisms. The high metal tolerance of PAB to the metals like Cu and Ni, which can be used as a micronutrient, were prevailed in the downstream of the estuary while it reduced to the upstream. Even though zinc is a micronutrient, the high concentration of zinc that was observed in the PM in the estuary (Chapter 3 Results) may result in the continuous exposure to the metal. This will lead to the development of resistance to Zn among PAB.

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Chapter 6

ENZYME EXPRESSION PROFILE OF PARTICLE-ASSOCIATED BACTERIA

Contents

6.1 *Introduction*
6.2 *Methodology*
6.3 *Results*
6.4 *Discussion*

6.1 Introduction

The particle associated bacteria (PAB) contribute significantly to the enzymatic remineralization of complex organic matter to dissolved organic matter and nutrients amenable to planktons in the aquatic environment (Ambrosio et al., 2014; Kellogg and Deming, 2014; Weiss et al., 1996). The role of PAB in the microbial loop is already well discussed by previous workers (Azam, 1998; Azam and Long, 2001) and is attributed to their higher enzyme expression profile compared to the planktonic bacteria (Crump et al., 1998; Grossart et al., 2006; Kellogg and Deming, 2014). The PABs are recorded to be responsible for nearly 75% of the total hydrolytic activity and contribute to the major portion of bacterial carbon production in aquatic ecosystems (Lemarchand et al., 2006; Middelboe et al., 1995; Simon, 1987). The major hydrolytic enzymes involved in the degradation of organic matter are β-glucosidase, aminopeptidase, lipase, and phosphatase. The β-glucosidases are responsible for the breakdown of carbohydrates

leading to the release of glucose, while the other fractions of particulate matter are mineralized into their monomers like lipids to fatty acids by lipase, proteins to amino acids and peptides by aminopeptidase and release of soluble phosphate by phosphatase.

Although the biochemical composition of particulate matter (PM) varying between estuarine, fresh water and marine environments, the major ingredients of PM are carbohydrates (5 -25 %), proteins (20 to 50%) and lipids (3- 55%) (Benner and Kaiser, 2003; Biersmith and Benner, 1998; Decho, 1990; Nagata and Kirchman, 1992; Riley and Segar, 1970; Sargent et al., 1977). The enzymatic degradation of PM by the microorganism is an important step in the remineralization. Most of the studies on microbial associates of PM and their enzymes are focused on finding biotechnological applications and optimization of fermentation conditions to enhance enzyme production. On the other side, the importance of microbial enzymes in biogeochemical cycles in the environment and impact of pollution on the enzyme activities are least discussed. The studies on enzyme activities of microorganisms in the water column of Arabian Sea (Mamatha et al., 2015; Martin et al., 2018) and Cochin estuary (Jiya et al., 2011; Nair et al., 2012) has confirmed the significance of the studies on microbial enzymes in the ecosystem functioning. The enzyme activities of PABs are reported to be higher compared to that of planktonic bacteria. For example, the cell-specific protease and β-glucosidase enzyme activity of particle-associated bacteria were observed as 100 to 400 times higher than the surrounding microbial flora (Grossart et al., 2003). It is a general perception that bacteria use most of its energy for the cell survival in a polluted environment, while their enzyme expression and participation in the

process would be minimal. The heavy metals can impact microbial enzyme activities in several ways (Fig.6. 1).

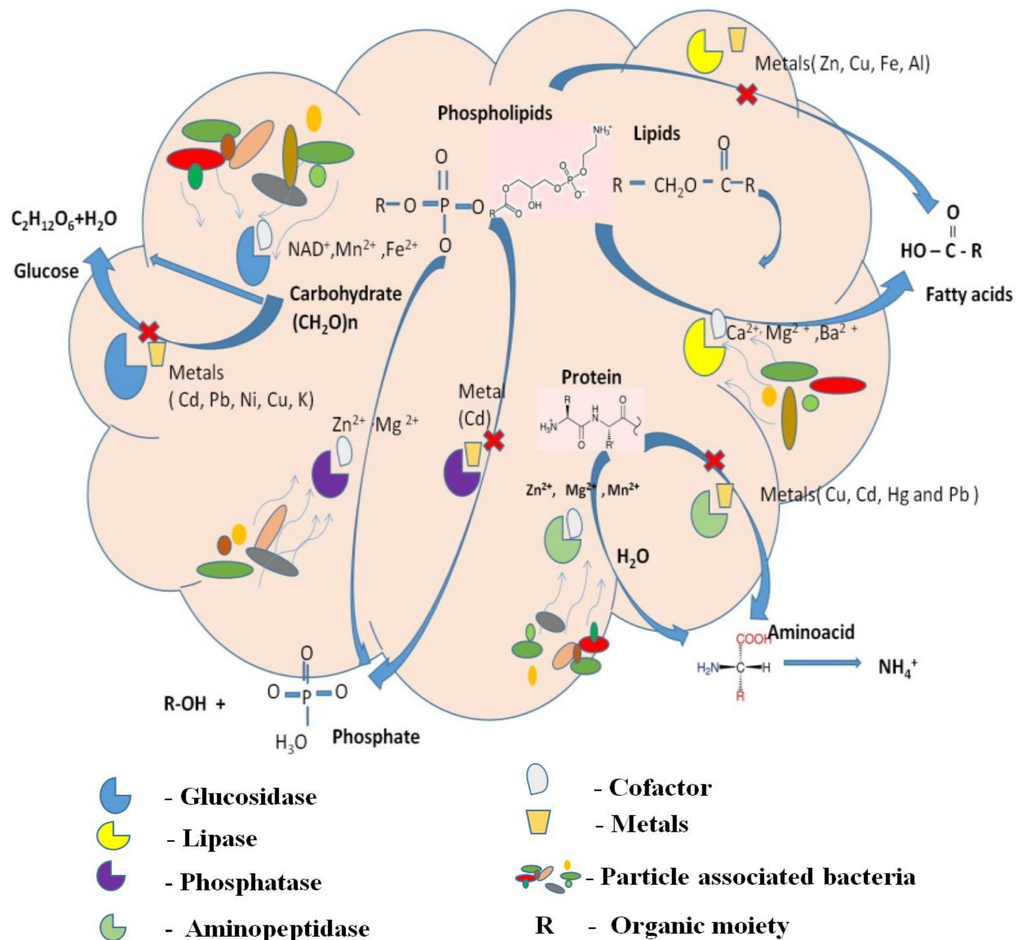

Fig. 6.1. Schematic representation of the interaction of heavy metals with microbial enzymes

For example, certain metal ions such as Fe and Mn (Wang et al., 2018; Zhang et al., 2017) enhances the glucosidase activities of microorganisms while it is inhibited by Pb, Cd, Cu, Ni (Nweke and Okpokwasili, 2011).

Studies also showed the enhancement of lipase activity by Ca, Mg and Ba and its inhibition by Zn, Cu, Fe, Al (Liu et al., 2017; Mobarak et al., 2011). Metal ions like Zn function as the prosthetic group of enzymes like alkaline phosphatase and aminopeptidase. In polluted environments, these prosthetic groups may be replaced competitively by ions of Cd, Cu, Hg, and Pb leading to enzyme inactivation (Chróst, 1991; Crofton, 1982; Hill and Smith, 1960; Linden et al., 1977; Macaskie and Dean, 1984). Such inactivation may lead to slow remineralization process leading to accumulation of organic matter in the system. A schematic representation of the possible interaction of heavy metal pollution on microbial enzyme activities is depicted in Fig. 6.1. The present chapter discusses the enzyme expression profile of particle-associated bacteria isolated from the polluted and non-polluted waters of Cochin estuary.

6.2 Methodology

6.2.1 Bacterial isolates for the study

Bacteria associated with particulate matter collected from upstream (polluted) and downstream (nonpolluted) of Cochin estuary are used for the enzyme activity studies. Details of sample collection, isolation of bacteria and preservation are given in Chapter 4. A total of 113 isolates were used, among which 61 were from downstream and 52 from upstream. The isolates were retrieved from cryopreserved vials and were inoculated in ZoBell's marine broth. The identification of the isolates was given in Chapter 4.

6.2.2 Preparation of crude enzyme for the assay

The bacterial isolates for each enzyme assay were cultivated in separate tubes containing ZoBell's marine broth supplemented with 1 %

enzyme substrates (Potassium bi phosphate for alkaline phosphatase, Casein for Aminopeptidase, Starch for β- glucosidase and Tween 80 for lipase). An inoculum of overnight grown isolates (1 ml) was inoculated into a glass tube containing 10 ml of ZoBell's marine broth supplemented with 1 % substrate and incubated in a shaking incubator maintained at 100 rpm and 28 ± 2 °C for 48 h. The supernatant was separated by centrifugation at 8000 rpm for 10 min at 4 °C and was used as a crude enzyme for assays. The crude enzymes were stored at 4°C until analysis. All the assays were done in triplicate and were completed within 24 hr of crude enzyme preparation.

6.2.3 Enzyme assay

The enzyme activities of the isolates were measured following the standard fluorescent substrate-based assays (Bongiorni et al., 2005; Hoppe, 1983). The enzymes assays were performed in triplicate in a 96 micro-well plate and the activities were measured as a function of the release of the fluorescent dye from the substrate. One hundred microliters of crude enzyme were added to micro-well containing their respective fluorescent dye conjugated substrates (50µl) and buffers (50 µl) (Table 6.1). The reaction mixture was incubated under dark for 1 hour in the microplate reader (Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader) at 28 °C with intermittent shaking. Subsequently, the enzyme expression was measured as a function of fluorescence signals emitted by the dye molecules released from each substrate using a multimode microplate reader. Appropriate blanks were also used to minimize the error due to background fluorescence by media and buffer.

Standard graph was prepared from fluorescence of serially diluted MUF (5, 10, 15,20,25,30 and 35 $\mu M^{-1}ml^{-1}$) and AMC (2,4,6,8,10,12,14 and 16$\mu M^{-1}ml^{-1}$ h^{-1}). The isolates were classified into low (0-5 $\mu M^{-1}ml^{-1}h^{-1}$), medium (5- 10$\mu M^{-1}ml^{-1}h^{-1}$), high (>10 $\mu M^{-1}ml^{-1}h^{-1}$) expression groups.

Table 6.1. Technical details of fluorescent substrates and buffers used in each assay and the excitation and emission maxima of each dye released

Enzyme	Substrate	Buffer	Dye released	Absorption maxima	Emission maxima
Alkaline phosphatase	MUF-Phosphate	Na_2CO_3 (pH 9)	MUF	364nm	445 nm
β- glucosidase	MUF-Glucopyranoside	Na_2PO_4 (pH-7.4)	MUF	364nm	445 nm
Lipase	MUF-Oleate	Na_2PO_4 (pH-7.4)	MUF	364nm	445 nm
Aminopeptidase	AMC-L-leucine	Na_2PO_4 (pH-7.4)	AMC	380nm	450 nm

6.2.4 Statistical analyses

The statistical significance of the difference between the enzyme expression profile of PAB isolated from upstream and downstream were tested by two-way ANOVA using Primer 6 software.

6.3 Results

More than 99 % of the Particle-associated bacteria from CE showed activities of lipase, β-glucosidase, and alkaline phosphatase while aminopeptidase activities were restricted to 75 % of the isolates (Table 6.2). The range of enzyme activity varied between organisms and accordingly they were classified into low, medium and high expression groups.

Table 6.2. The range of enzyme activity of the PAB

Sl No	Enzyme	Range of enzyme activity ($\mu M^{-1}ml^{-1}h^{-1}$)	No. of organisms showing enzyme activity	Percentage (%)
1	Alkaline phosphatase	0.22 - 92.5	113	100
2	Aminopeptidase	0 - 53.88	85	75
3	Beta-glucosidase	0 - 22.59	112	99
4	Lipase	0.23 - 92.5	113	100

6.3.1 Alkaline Phosphatase activity

Nearly 80 % of the isolates from downstream showed high or medium levels of alkaline phosphatase activity while it was restricted to less than 20 % PABs from the polluted upstream (Fig. 6.2). The two–way ANOVA results showed significant variation ($p<0.01$) in alkaline phosphatase activity between PAB from upstream and downstream of the estuary (Table 6.4). Out of the ten PAB which showed a high level of alkaline phosphatase activity, nine were retrieved from the downstream. These isolates were identified as *Vibrio sp* MMRF (995, 998), *Sulfitobacter sp* MMRF 1005, *Catenococcus sp* MMRF 1006, *Altererythrobacter sp* MMRF 1009, *Bacillus sp* MMRF (1012, 1023), *Nitratireductor sp* MMRF 981, *Sagittula sp* MMRF 982. The only one high alkaline phosphatase producer from the upstream was identified as *Bacillus sp* MMRF 1049. (Table 6.3).

6.3.2 β–glucosidase activity

The β–glucosidase *activity* was also very prevalent among PABs isolated from both up and downstream. More than 60 % of the PAB from both upstream and downstream showed medium or higher levels of

β-glucosidase activities (Fig. 6.2). The ANOVA results showed there is no significant variation between the activity of the downstream and upstream cultures. There were only four isolates with very high activities of β–glucosidase, among which one was from the upstream (*Brevibacillus sp* MMRF 1041) while remaining ones (*Sulfitobacter sp* MMRF 1005, *Halomonas sp* MMRF 1011, *Erythrobacter sp* MMRF 1018) were from the downstream (Table 6.3).

6.3.3 Aminopeptidase activity

Aminopeptidase activity observed among the PAB from CE were low compared to other enzymes. Most of the high activity PAB were isolated from the upstream of the estuary, *Acinetobacter* MMRF 1032, *Pseudomonas* MMRF (1042,1045), *Rheinheimera* MMRF 1043, *Brevundimonas* MMRF 1047, *Bacillus* MMRF 1049 while only two species i.e, *Vibrio sp* MMRF 964 and *Altererythrobacter sp* MMRF 966 from downstream showed similar activity (Table 6.3). *Acinetobacter* MMRF 1032, *Brevundimonas* MMRF 1047 were the isolates showed highest aminopeptidase activity. The high aminopeptidase activity was observed among PAB from upstream (>30 %) compared those from downstream (< 20 %) of CE (Fig. 6.2). The ANOVA results showed that there is a significant demarcation between the aminopeptidase activity of PAB ($p<0.01$), from the downstream and upstream region of the estuary (Table 6.4).

6.3.4 Lipase activity

All isolates of PAB showed lipase activity with high expression levels among 35 % of isolates. Numerically, 22 isolates from downstream and 18 from upstream showed high activity (Table 6.3).

Table 6.3. PAB showing high (>10 $\mu M^{-1}ml^{-1}h^{-1}$) range of enzyme activities in CE

Sl No	Enzymes	PAB showing high range (>10 $\mu M^{-1}ml^{-1}h^{-1}$) of enzyme activity	
		Upstream	**Downstream**
1	Alkaline phosphatase	*Bacillus sp* MMRF 1049	*Nitratireductor sp* MMRF 981, *Sagittula sp* MMRF 982, *Vibrio sp* MMRF (995, 998) *Sulfitobacter sp* MMRF 1005, *Catenococcus sp* MMRF 1006, *Altererythrobacter sp* MMRF 1009, *Bacillus sp* MMRF (1012, 1023)
2	β- glucosidase	*Brevibacillus sp* MMRF 1041	*Sulfitobacter sp* MMRF 1005, *Halomonas sp* MMRF 1011, *Erythrobacter sp* MMRF 1018
3	Aminopeptidase	*Acinetobacter* MMRF 1032, *Pseudomonas* MMRF (1042,1045), *Rheinheimera* MMRF 1043, *Brevundimonas* MMRF 1047, *Bacillus* MMRF 1049	*Vibrio sp* MMRF 964, *Altererythrobacter sp* MMRF 966
4	Lipase	*Sphingopyxis sp* MMRF 953, *Chromohalobacter sp* MMRF 956, *Alteromonas sp* MMRF 958, *Pseudomonas sp* MMRF (963, 1027, 1042, 1046) *Acinetobacter sp* MMRF (1029, 1032, 1051, 948) *Bacillus sp* MMRF (1037, 1038, 944) *Brevibacillus sp* MMRF, 1041 *Enterobacter sp* MMRF 1044, *Aeromicrobium sp* MMRF 1053	*Vibrio sp* MMRF (998, 964, 995,967, 1008), *Oceanicola sp* MMRF 984, *Paracoccus sp* MMRF (990, 997), *Donghicola sp* MMRF992, *Bacillus sp* MMRF (975, 1003), *Erythrobacter sp* MMRF 976, *Alcanivorax sp* MMRF 977, *Acinetobacter sp* MMRF 980, *Sulfitobacter sp* MMRF 1005, *Catenococcus sp* MMRF 1014, *Alteromonas sp* MMRF 1016, *Altererythrobacter sp* MMRF (988, 1017, 1024, 1009, 966)

The PAB with high lipase activity in upstream were belonged to *Acinetobacter* spp, *Pseudomonas* spp, and *Bacillus* spp, while those from the down-stream were identified as *Vibrio* spp and *Altererythrobacter* spp (Table 6.3). The lipase activity was high or medium among the majority (i.e. >90 %) of PAB from downstream (Fig. 6.2) while it was restricted to among 60 % in the upstream of the estuary.

Table 6.4. Analysis of variance (ANOVA) between the PAB enzymes from downstream and upstream of CE (* significant *P* <0.01)

Sl No	Enzyme	*P* Value (ANOVA)
1	Alkaline phosphatase	0.008316*
2	β- glucosidase	0.99735
3	Aminopeptidase	0.001868*
4	Lipase	0.246037

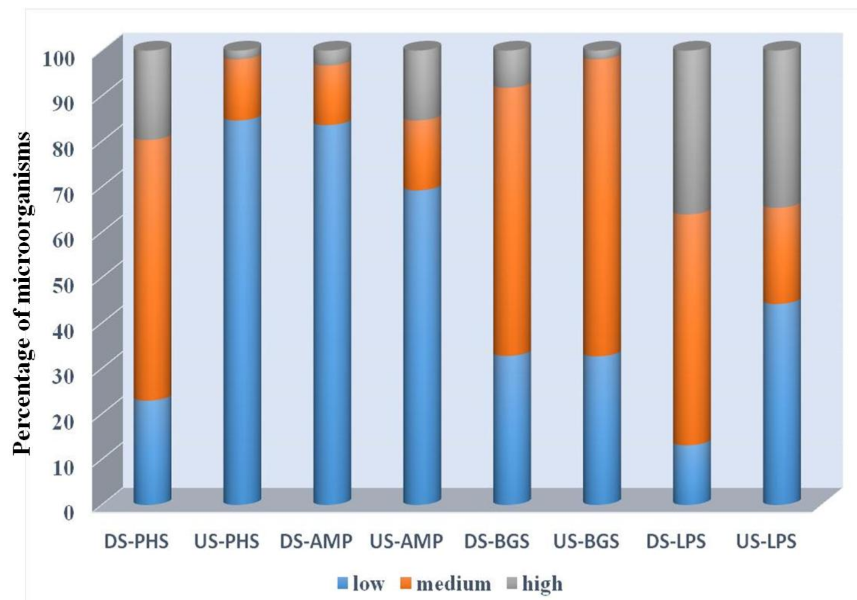

Fig. 6.2. The alkaline phosphatase (-PHS), aminopeptidase (-AMP), β- glucosidase (-BGS) and, lipase (-LPS) activities of PAB isolated from downstream (DS-) and upstream (US-) of CE. The isolates were classified into low (blue), medium (orange), high (grey) expression groups.

6.4 Discussion

The importance of enzyme activities of Particle-associated microorganisms in remineralization process is well documented in marine and estuarine environments (Ambrosio et al., 2014; Crump et al., 1998; Murrell et al., 1999; Riemann et al., 2000; Smith et al., 1992; Unanue et al., 1992; Unanue et al., 1998). However, the influence of anthropogenic factors on the microbial remineralization of particulate matter in general and activities of microorganisms are least studied. Generally, the bacteria spend most of their energy to protect themselves under high concentrations of heavy metals and do not secrete enzymes to participate in the biogeochemical processes. This may halt the remineralization leading to the accumulation of organic matter in polluted waters. The present study, investigate the enzyme expression profile of PAB isolated from the polluted (upstream) and nonpolluted (downstream) regions of CE. The enzyme expression profile of PAB from downstream was in the order of lipase > phosphatase > β-glucosidase >aminopeptidase, while it was in the order of β-glucosidase > lipase > aminopeptidase > phosphatase in upstream of the estuary. The studies by Karrasch et al (2003) showed that the enzyme activities in the estuarine waters of Elbe Germany were the same order of activity observed in PAB of downstream ie, lipase> Phosphatase> β-glucosidase = aminopeptidase. The particle attached aminopeptidase and β-glucosidase activity were observed as 400 picomol^{-1} l^{-1} min^{-1} and 70 picomol^{-1} l^{-1} min^{-1} respectively in Columbia River estuary when compared to these values very high enzyme activity of PAB from CE were observed. Pinhassi et al (2004) observed β-glucosidase, aminopeptidase, alkaline phosphatase activity were 20, 1500,

100 ($\mu M^{-1} l^{-1} h^{-1}$) respectively from the heterotrophic bacteria- phytoplankton bloom experiment, the alkaline phosphatase, and β-glucosidase were 1000 times higher and aminopeptidase activity 50 times higher in PAB from CE. Another study conducted by Riemann et al (2000) showed that the lipase activity of bacteria associated with phytoplankton bloom was less (180 nmol $l^{-1} h^{-1}$) compared to that of PAB in the estuary.

The enzyme activities of PAB with high lipase and phosphatase activity was largely distributed in the downstream of CE. High phosphatase and lipase activity were also observed in other estuaries, lakes, surface micro layer etc. (Grossart and Simon, 1998; Labry et al., 2016). Enhanced lipase activity in CE may be associated with the anthropogenic source such as nearby oil refineries, Fishery related activities or its close proximity to the harbor area. Such correlations were previously reported from Ria de Aveiro estuary in Portugal (Santos et al., 2009; Santos et al., 2013). The PAB with phosphatase and lipase activities were less abundant in the upstream, and it could be due to the impact of cadmium or excess zinc toxicity. Such type of inhibition was earlier reported from the soil system (Lorenz et al., 2006). The substitution of Cd^{2+} and Zn^{2+} at the active site of phosphatase enzyme slows the phosphorylation and dephosphorylation rate of the enzyme by approximately 2 orders of magnitude (Applebury et al., 1970; Chlebowski and Coleman, 1974). The less number of bacteria with phosphatase activity in the upstream may lead to reduced release of phosphate from organic matter resulting in the accumulation of biopolymeric carbon (BPC), which is evident from the earlier and present studies (Lallu et al., 2014; Sheeba et al., 2017).The hydrolysis of organic phosphorus compounds liberates organic moiety

containing carbon and nitrogen, which can be directly utilized by bacteria (Hoppe, 2003; Labry et al., 2016; Steenbergh et al., 2011). The low lipase expression among bacteria isolated from upstream of the estuary may also be due to the high Zn pollution in this region. It is reported in previous studies that the high concentrations of Zn, Cu and Mn ions can inhibit the lipase activity by inhibiting the catalytic site of the lipase enzyme or affecting the solubility and behavior of ionized fatty acid at interface (Lesuisse et al., 1993; Linden et al., 1977; Liu et al., 2017; Masomian et al., 2013).The expression of β-glucosidase, was comparable between the PAB from upstream and downstream, while the PAB from upstream showed marginally higher aminopeptidase activity compared to those from downstream. The decreasing trend of alkaline phosphatase and lipase activity of PAB towards the polluted upstream may be due to the stress imparted by the heavy metal pollution prevailed there while, comparatively high aminopeptidase and β-glucosidase activity observed in the particle associated bacteria from polluted upstream regions may be because of the requirement of high carbon for the repair and maintenance of the cell under the stressed condition or may be the substrate availability (Fliessbach et al., 1994; Kandeler et al., 1996). The substrate availability for lipase activity is high in the upstream of the estuary while it was less in the case of phosphate (Chapter 3 results).

The PAB with the medium or high level of aminopeptidase activity plays a significant role in the biogeochemical cycling of nutrients by initiating the hydrolysis of proteins to aminoacids and further to ammonia. Thus generated ammonium would be taken up by the nitrifying bacteria. Aminopeptidase activity of PAB was high in the upstream of the estuary

compared to the downstream.This may due to the relatively high concentration of zinc in the upstream of the estuary. The Zn induced increase in the aminopeptidase activities has been reported earlier from sewage polluted freshwater environments (Rasmussen and Olapade, 2015). Similarly, Fukuda et al (2000) also found strong positive correlations between cell-specific aminopeptidase activity and concentrations of dissolved zinc in the upper layer of the Pacific ocean. Previous reports on higher levels of ammonium in the upstream indicate the high rate of protein hydrolysis (Madhu et al., 2010; Miranda et al., 2008; Vipindas et al., 2015) in the upstream compared to downstream.

The study gives clear indication that the heavy metal accumulation in PM would affect the alkaline phosphatase, lipase activity of PAB in polluted environment of the estuary. The β- glucosidase and aminopeptidase activity didn't show much variation and it may be because of the microbial requirement of basic elements, carbon, and nitrogen for the maintenance of cellular functions for the survival in the metal stressed environment. The results also give information that the complex organic matter remineralization in the polluted upstream of the estuary will seriously be affected by the heavy metal pollution.

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Chapter 7

SUMMARY AND CONCLUSION

The thesis entitled “Studies on Particle associated microorganisms in Cochin Estuary and their response to metal pollution” reports the impact of heavy metals accumulated in particulate matter on the community structure and activity of particle associated microorganisms in Cochin estuary, Southwest coast of India. To the best of my knowledge, this is the first report across the world focusing on this aspect.

Estuaries are the ecotone area between the ocean and freshwater and one of the most productive ecosystems in the world. Estuaries are also considered as one of the most stressed environment due to the anthropogenic activities. It receives wide range of pollutants including metals from industrial activities, urban runoff etc. Estuaries are characterized by various hydrodynamic processes such as sedimentation, resuspension etc. The suspended particulate matter formation through resuspension of sediment particles and continuous aggregation and disaggregation of organic matter makes them more turbid than the adjoining sea or river. The particulate matter in the estuary is considered as a hot spot of microbial activity, which is a micro-niche where intensive biogeochemical transformations occur.

The hypothesis of the present thesis was that the heavy metal accumulation in the particulate matter may impinge the diversity and functions of microorganisms, leading to the proliferation of metal resistant ones with concomitant reduction of enzyme profile/activities, leading to drastically reduced remineralization in the estuary. Cochin estuary, with its pronounced metal pollution gradation, is an ideal site for studying the effect of heavy metals on PM associated microorganisms. A multidisciplinary approach with four main objectives as mentioned below was designed to study the hypothesis.

The objectives addressed in the thesis were

1) Chemical and biochemical characterization of PM and its effect on community structure of planktonic and particle associated bacteria and archaea in CE
2) Isolation and Identification of particle associated bacteria
3) Metal tolerance profile of particle associated bacteria
4) Enzyme expression profile of particle associated bacteria

Each objective was discussed in separate chapters in the thesis. The particulate matter and water samples for the study were collected during the wet and dry season from four locations along a pollution gradient in the Cochin estuary from Barmouth (least polluted) to Eloor (site of intense industrial pollution). The first and second chapters detail a general introduction and relevant literature related to the work, respectively. Chapters 3-6 are devoted to discussing the four main objectives of the thesis cited above. Thus, the third chapter discusses the spatio-temporal

variation in the chemical and biochemical characterization of PM and the community structure of PM-associated archaea and bacteria. The major observations of chapter 3 are given below.

- The nutrient concentrations in the estuary were high during wet season compared to dry season
- The particulate matter concentrations were observed in the range between 2.8 to 36 mgL^{-1}.
- Zinc was observed as the major pollutant in water (4.36 to 130 mgL^{-1}) and in particulate matter (765.5 to 8451.28 mgL^{-1}).
- The heavy metal accumulation in the PM were in the order Fe > Zn > Mn > Cr > Pb > Cu > Cd > Co > Ni.
- The biopolymeric carbon was higher in the upstream of the estuary (near to Eloor region) during both wet and dry season.
- The abundance of PM- archaea, and bacteria were ten times less in PM compared to Planktonic ones. The abundance of PM-archaea ranged between 4.27 to $9.50 \times 10^7$and 2.73 to 3.85×10^7 cells L^{-1} respectively for the wet and dry season, while that of PM-bacteria was between 1.14 and 6.72×10^8 cells L^{-1} for both seasons.
- The community structure analysis of archaea showed that the diversity of Particle Associated Archaea (PAA) was higher compared to Planktonic archaea (PLA) diversity during both the season (27OTU- wet season, 21OTU-dry season).

- The statistical analysis showed a positive influence of particulate organic carbon, nitrogen, PM-Pb, PM-Zn and PM-Fe on the abundance of PM-archaea and PM-bacteria. All these results showed that community structure of PM-bacteria varied between polluted and non-polluted stations, while their abundance does not show a drastic difference. The results indicate the existence of a selective enrichment of bacteria by heavy metals in PM and have a significant role in selecting the bacterial community associated with it.

The isolation and molecular taxonomy of particulate matter - associated bacteria from the upstream and downstream of the estuary are discussed in Chapter 4. The major observations discussed in this chapter may be summarized as below:

- A total of 113 PAB were isolated from the upstream (52 Nos) and downstream (61 Nos) of CE. Only 25 % of the PAB isolated were common to both upstream and downstream. The Particle-associated bacteria (PAB) were identified using 16S rRNA gene sequencing and the sequences are submitted to NCBI with the accession numbers KX015913 to KX015956 and KX056147 to KX056215.
- The PAB isolated from the upstream were dominated by γ-proteobacteria (48.1 %) followed by α-proteobacteria (25%), Firmicutes (19.2%) and Actinobacteria (7.7%).

- The dominant PABs in the downstream was in the order of α-proteobacteria (45.9%), followed by γ-proteobacteria (36.1%) and Firmicutes (18%). The dominance of γ-proteobacteria was observed in the polluted upstream of the estuary, in polluted regions the dominance of γ-proteobacteria were previously reported.
- The γ-Proteobacteria such as *Rheinheimera* sp., *Enterobacter* sp., *Aeromonas* sp. *Acinetobacter* sp. and *Pseudomonas* sp isolated were well known for their metal tolerance in other environments.
- Most of the PAB isolated from the downstream of the estuary, *Catenococcus* sp, *Paracoccus* sp, *Sulfitobacter* sp., are known species involved in various biogeochemical cycles from other environments.
- There were many isolates of α-proteobacteria such as *Sagittulla* sp*., Altererythrobacter* sp., found only in downstream of the CE, which are known for their participation in degradation of complex organic compounds such as lignins and hydrocarbons which were absent in the upstream.
- The results indicate that the heavy metal pollution has significant role in structuring the community of particle associated bacteria in the estuary.

Chapter 5 explains the study on metal resistance profile of particle associated bacteria. Metal tolerance profile of particle associated heterotrophic bacteria was analyzed for metals Zn, Cu, Cd, and Ni. The major observations discussed here may be summarized as below.

- There was a clear difference in the metal tolerance of PAB isolated from upstream and downstream, with more number of isolates from upstream showing tolerance to higher concentrations of Zn and Cd while tolerance to Ni and Cu was prevalent among downstream isolates.
- The PAB showed tolerance to very high concentrations ie. 250 mM of Zn and Ni, while they could tolerate upto 100 and 180 mM respectively of Cu and Cd.
- The *Acinetobacter* sp MMRF1051 isolated from the upstream showed tolerance up to 250 mM Zn, 100 mM Cd, and 250 mM Ni.
- The results showed that more than 55% of the PAB were tolerant to ≥3 metals and 14.3% of them were tolerant to all the metals tested.
- The maximum tolerance for Cd was observed in the genus *Acinetobacter sp, Brevundimonas* sp in upstream of the estuary. This was supported by the previous studies on the metal resistance capacity of these group of bacteria attributed to the production of exopolysaccharides.
- The study indicates that those bacteria which can adapt to higher concentrations of heavy metals are colonizing on the particulate matter in the upstream, while such segregation is not found among PAB from downstream.

The enzyme activities of particle associated bacteria (β-glucosidase, aminopeptidase, lipase and phosphatase) were analyzed using fluorescent assays and are described in Chapter 6. The major observations discussed in this chapter are summarized as below

- The PAB isolated from upstream and downstream differed in their enzyme activity. The enzyme expression profile of PAB from downstream was in the order of lipase > phosphatase > β-glucosidase > aminopeptidase, while it was in the order of β-glucosidase > lipase > aminopeptidase > phosphatase in the upstream of the estuary.
- Nearly 80 % of the isolates from downstream showed high or medium levels of alkaline phosphatase activity while it was restricted to less than 20 % of PAB from the polluted upstream. Alkaline phosphatase and lipase activity of PAB showed a decreasing trend towards the upstream i.e. the polluted region of the estuary. This may be due to the inhibition of enzyme expression by excess Zn and Cd in the upstream of the estuary.
- The lipase activity was high or medium among >90 % of PAB from downstream while it was restricted to among 60 % in the upstream of the estuary. The low lipase expression among bacteria isolated from upstream of the estuary may also be due to the high Zn pollution in this region or may be the substrate availability.

- The high aminopeptidase activity was observed among PAB from upstream (>30 %) compared those from downstream (< 20 %) of CE. The aminopeptidase activity of PAB was observed as higher in upstream compared to downstream may be due to the Zn in upstream may act as a cofactor for the enzyme.
- More than 60 % of the PAB from both upstream and downstream showed medium or higher levels of β-glucosidase activities.The β-glucosidase activity was more or less similar to the downstream one of the possible explanation is that microorganisms require carbon for the cell activity, repair, and maintenance under stressed conditions for their survival.

In short, the results of the present study clearly demonstrate that consequent to the increase in metal pollution in Cochin estuary, the metals accumulate in the suspended particulate matter, which in turn, affect the activity and diversity of the microorganisms associated with these suspended particles. This severely affects the ecosystem functioning, particularly the remineralization of organic matter in biogeochemical cycling and food web dynamics, eventually threatening human health and well being.

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Appendix

Appendix table No. 1

Formamide (%)	NaCl final concentration	µl of 5M NaCl in 50ml
0	0.900	9000
5	0.636	6300
10	0.456	4500
15	0.318	3180
20	0.225	2150
25	0.150	1490
30	0.112	1020
35	0.080	700
40	0.056	460
45	0.040	300
50	0.028	180
55	0.020	100
60	0.014	40

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List of Publications

[1] **Sheeba VA,** Abdulaziz Anas, Gireeshkumar TR, Anirudh Ram, Rakesh PS, Jasmin C, Parameswaran PS- Role of heavy metals in structuring the microbial community associated with particulate matter in a tropical estuary Environmental Pollution (2017) **231**: 589-600.NIO Contri. No. 6088.

[2] **Sheeba VA**, Abdulaziz Anas, Jasmin C, Manu Vincent, Parameswaran P S- Contrasting diversity, metal tolerance and enzyme expression profile of particulate associated bacteria in metal polluted and non-polluted regions of a tropical estuary-- under review.

[3] Anas, A., **Sheeba, V.A**, Jasmin C, Gireesh Kumar TR, Diana Mathew, Kiran, K., Muraleedharan, K.R., Jayalakshmy, K.V., Microbial abundance and community structure of a persistent mud bank in an Indian coastal region influenced by upwelling. Regional Studies in Marine Science **18** (2018):113–121 NIO Contri. No.6160.

[4] Anas A, Jasmin C, **Sheeba VA,** Gireesh TR, Shanta Nair. Heavy metal pollution influence the community structure of cyanobacteria in Cochin Estuary, a nutrient rich tropical estuary across the southwest coast of India., Journal Oceanography and Marine Research (2015) **3**:1.

[5] **Sheeba VA**, Saumya Nair, Diana Mathew, Gireesh TR, Anas A. Influence of upwelling on the distribution of bacteria and archaea in persistent mudbank regions of Alleppey, Southwest coast of India. OSICON 2015, Goa India (POSTER).

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Role of heavy metals in structuring the microbial community associated with particulate matter in a tropical estuary☆

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Estuary

ABSTRACT

Particulate matter (PM), which are chemically and biochemically complicated particles, accommodate a plethora of microorganisms. In the present study, we report the influence of heavy metal pollution on the abundance and community structure of archaea and bacteria associated with PM samples collected from polluted and non-polluted regions of Cochin Estuary (CE), Southwest coast of India. We observed an accumulation of heavy metals in PM collected from CE, and their concentrations were in the order Fe > Zn > Mn > Cr > Pb > Cu > Cd > Co > Ni. Zinc was a major pollutant in the water (4.36–130.50 μgL^{-1}) and in the particulate matter (765.5–8451.28 μgg^{-1}). Heavy metals, Cd, Co, and Pb were recorded in the particulate matter, although they were below detectable limits in the water column. Statistical analysis showed a positive influence of particulate organic carbon, nitrogen, PM-Pb, PM-Zn and PM-Fe on the abundance of PM-archaea and PM-bacteria. The abundance of archaea and bacteria were ten times less in PM compared with planktonic ones. The abundance of PM-archaea ranged between 4.27 and 9.50 × $10^7$and 2.73 to 3.85 × 10^7 cellsL^{-1} respectively for the wet and dry season, while that of PM-bacteria was between 1.14 and 6.72 × 10^8 cellsL^{-1} for both seasons. Community structure of PM-bacteria varied between polluted and non-polluted stations, while their abundance does not show a drastic difference. This could be attributed to the selective enrichment of bacteria by heavy metals in PM. Such enrichment may only promote the growth of metal resistant archaea and bacteria, which may not participate in the processing of PM. In such cases, the PM may remain without remineralization in the system arresting the food web dynamics and biogeochemical cycles.

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1. Introduction

Particulate matter (PM) are chemically and biochemically complex particles formed by various physical, chemical and biological processes such as weathering of bulk materials, resuspension of sediment particles, death and decay of large organisms, and biological repackaging of organic matter through feeding and excretion (Simon et al., 2002). The living part of PM is made up of phytoplankton, zooplankton, bacteria and archaea. PM plays key roles in the functioning of estuarine ecosystems, which includes serving as a micro-niche for the growth and activities of microorganisms, vertical transportation of organic matter and as food for organisms at higher trophic levels (GarneauÈ et al., 2009). The microorganisms mediate the remineralization of PM through the secretion of a variety of hydrolytic enzymes to digest the high-molecular-weight polymeric substances in PM into dissolved organic matter (Bong et al., 2010; Chróst and Rai, 1994). PM is also known for their ability to chelate heavy metals, which may alter the biochemical properties of the micro-niche and the diversity and functions of associated microorganisms. All microorganisms require certain heavy metals at optimum concentrations for the synthesis of structural proteins and pigments of metabolic importance in the redox processes, regulation of the osmotic pressure, maintaining the ionic balance and enzyme component of the cells (Bong et al., 2010; Kosolapov et al., 2004). On the other side, higher concentrations of heavy metals can interfere with the structural conformation of proteins and nucleic acids, leading to their malfunction (Bong et al., 2010). For example, microorganisms require

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10^{-5}–10^{-7} M concentrations of Zn ions in the environment for their optimal growth (Wilson and Reisenauer, 1970), whereas Zn binds with the cell membrane and inhibits cell division at higher concentrations (Nies, 1999; Silver and Phung, 2005). Although previous studies have reported on toxic effects of heavy metals on planktonic microorganisms (Anas et al., 2015; Jiya et al., 2011), their influence on the diversity of PM-associated microorganisms is less studied. It is possible that pollutants such as heavy metals could impinge on the diversity of PM-associated bacteria, which in turn could influence the digestion of particulate organic matter (POM) to dissolved organic matter (DOM).

Estuaries are the transition zones connecting fresh- and marine waters and are greatly influenced by terrestrial discharges and oceanographic processes. Estuaries receive organic matter, sewage, industrial effluents and pesticides from the terrestrial environment through runoff or river discharge. Cochin Estuary (CE) is the largest wetland ecosystem along the southwest coast of India, covering an area of ~25600 ha, extending from 9° 30′ to 10° 12′ N and 76° 10′ to 76° 29′ E. The nutrient composition of the estuary is greatly influenced by the anthropogenic and terrestrial inputs from six rivers, seawater influx from two bar mouths and the prolonged monsoon (Menon et al., 2000). It is estimated that significant fractions of ~260 million litres of industrial effluents and ~235 million litres of sewage being produced per day in the industrial belt and residential areas respectively of Cochin city reach the estuary through the river Periyar (Chakraborty et al., 2014; Menon et al., 2000), land runoff and other nonpoint source sources (Jeyaprasad, 2010). Under pristine conditions, the organic matter reaching the estuaries undergoes microbial processing before being released into the coastal waters. The major chemical pollutants reported from CE are heavy metals, polycyclic aromatic hydrocarbons and organic nutrients (Balachandran et al., 2005; Lallu et al., 2014; Ramzi et al., 2017). Heavy metal pollution has been reported from CE since the 1970s (Venugopal et al., 1982), and subsequent studies have reported their accumulation in the sediments which impinges on the trophic-level dynamics (Martin et al., 2012; Mohan et al., 2012).

Previously, we have reported on the influence of heavy metal pollution on the diversity and activities of heterotrophic bacteria and cyanobacteria in the water column of CE (Anas et al., 2015; Jiya et al., 2011). In the present study, we report on the accumulation of heavy metals in the PM of CE and its influence on the abundance and community structure of associated archaea and bacteria.

2. Materials and methods

2.1. Sample collection and preservation

Subsurface (~1 m below surface) water samples were collected across a pollution gradient in CE, southwest coast of India (Supplementary Fig. 1). The stations S1 (9°58″ 8′N& 76°13″ 42′E) and S2 (9°58″ 48′ N&76°15″ 46′E) are located near the bar mouth and are considered as the least polluted (Jiya et al., 2011). The station S3 (10° 2″ 24′N& 76°15″29′E) is at the midpoint where pollution levels are medium and S4 (10° 4″ 22′ N& 76°16″ 53′E), located near the discharge point of river Periyar, is considered to be in a highly polluted region (Aneesh and Sujatha, 2012). Water samples were collected during low tide on 20th September 2012 (wet season) and 26th February 2013 (dry season) using a Niskin water sampler (10 L capacity). Water samples (1 L) for chemical analyses were collected in plastic bottles, avoiding contamination from all possible sources. Sample (100 ml) for Fluorescent *in situ* hybridization (FISH) analysis were collected in sterile polypropylene bottles and preserved with 2% buffered formalin. One litre of the sample was collected for DNA extraction. PM was separated from the water sample, by passing through 10 μm nylon membrane followed by pre-combusted (450 °C 4 h) GF/F filters of 0.7 μm pore size for chemical analysis. The filtrates after separating PM were used for analyzing dissolved fractions. The filter papers were stored at −20 °C until further analysis.

2.2. Analysis of environmental variables

Environmental variables of water samples were measured in triplicate following the standard protocols. Salinity was determined using a Digi Auto Salinometer (Model TSK, accuracy ± 0.001) and pH was measured using an ELICO LI 610 pH meter. Samples for nutrients (Ammonium, nitrite, nitrate, phosphate and silicate) were filtered through Whatman No 1 filter paper and estimated spectrophotometrically (Grasshoff et al., 1983) within six hours of sampling. Ammonium was determined following indophenol blue method and the absorbance was measured at 630 nm. Nitrite was determined as the formation of highly colored azo dye (Abs 543 nm) in a reaction mixture containing N-(1-naphthyl)-ethylene diamine and a diazo compound formed through the reaction of nitrite in water samples with sulphanilamide in acidic condition. Nitrate in the water samples was measured after reducing it to nitrite by passing through the cadmium-copper column. Phosphate was measured spectrophotometrically (Abs 882 nm) following the reduction of phosphomolybdate complex with ascorbic acid. The phosphomolybdate complex was formed through the reaction of phosphate in the water sample with ammonium molybdate. Silicate was also measured in the same way, where the silicomolybdate complex was reduced with oxalic acid, and the optical density was measured at 810 nm. PM was collected on a pre-combusted 0.7 μm GF/F filter paper (Whatman, USA) and measured gravimetrically after achieving constant weight at 60 °C.

2.3. Biochemical characterization of PM

We analyzed Particulate organic carbon (POC), Particulate organic nitrogen (PON), total carbohydrate, protein and lipids in the PM sample following standard procedures. The filter paper maintained at −20 °C was thawed and used for the analysis. For the analysis of POC and PON, the filter paper was placed in scintillation vials and allowed to dry overnight at 65 °C. Subsequently, it was transferred into a desiccator saturated with HCl fumes to remove carbonates and air-dried in a clean fume hood. These filters were packed tightly in tin cups for the analysis using CHN analyzer (ElementarVario EL III) following standard protocols (Strickland and Parson, 1972).

The carbohydrate was extracted from PM using 5% trichloroacetic acid and analyzed spectrophotometrically using the phenol-sulphuric acid method. The reducing sugars were measured as the formation of yellow colored furfural at 490 nm using different concentrations of glucose as standard (Kochart, 1978). The proteins were extracted from samples using 1 N NaOH and analyzed spectrophotometrically using Lowry's method. The blue-to-purple-colored complex formed through the reaction of phenolic group in the amino acid residue of protein with Folin–Ciocalteau reagent at pH 9 to 10.5 was measured at 750 nm using Bovine serum albumin as the standard (Lowry et al., 1951).

Lipids were extracted in a solvent mixture of methanol: chloroform: water (5:10:4) following Bligh and Dyer's method (Bligh and Dyer, 1959). Further, the lipids were oxidized using acid dichromate solution (Strickland and Parson, 1972) and the reduction of yellow color was measured spectrophotometrically at 440 nm using stearic acid as standard. The concentrations of protein, carbohydrate and lipids were converted into carbon equivalents by multiplying with standard conversion factors 0.49, 0.40, 0.75 respectively (Fabiano and Danovaro, 1994). The sum total of the

V.A. Sheeba et al. / Environmental Pollution 231 (2017) 589–600 591

carbon equivalents of protein, carbohydrate, and lipids was referred as biopolymeric carbon (BPC) (Pusceddu et al., 2000).

2.4. Heavy metal analysis of water and PM samples

For metal analysis, PM collected in GF/F filters were extracted by repeated digestion with HF-$HClO_4$- HNO_3 suspended in 0.5 M HCl (20 ml). The metals, Fe, Mn, Cr, Pb, Cu, Cd, Zn, Co, and Ni, were analyzed using an inductively coupled plasma optical emission spectroscopy (ICP OES, Perkin Elmer) following the standard protocol (Loring and Rantala, 1992). Blank filters were also analyzed using the same method. Known volumes of water samples were filtered through pre-weighed GF/F filter paper (0.7 μm) and the filtrate was acidified using concentrated hydrochloric acid. The dissolved metals were concentrated from one-litre water samples using Ammonium Pyrrolidine Dithio Carbamate (APDC) and Methyl Isobutyl Ketone (MIBK) at pH 4.5 and brought back to aqueous layer by back-extraction with concentrated nitric acid and made up to 20 ml with sterile de-ionized water (Smith and Windom, 1972). The extracts were analyzed in the ICP OES (Perkin Elmer) for dissolved trace metals. The accuracy of the heavy metal analyses was checked using standard reference material PACS 2 (Supplementary Table 1). The results of Fe, Cr, and Cd were found to be accurate at 96% compared to the standard, while it was 87, 89, 82, 97, 81 and 98% respectively for Co, Cu, Mn, Ni, Pb, and Zn. Most of the results were within the uncertainty values certified by National Research Council of Canada.

2.5. Abundance of archaea and bacteria using fluorescent in situ hybridization (FISH)

The abundance of total planktonic and PM associated archaea and bacteria were analyzed using FISH technique following the protocol of Glockner et al. (1999). The Cy3 labeled oligonucleotide probes ARCH 915 (GTGCTCCCCCGCCAATTCCT) (Stahl and Amann, 1991) and EUB 338 (GCTGCCTCCCGTAGGAGT) (Amann et al., 1990) were used for the enumeration of archaea and bacteria respectively. The buffered formalin-preserved water samples were prefiltered through 10 μm Nylon membranes and used for the enumeration of planktonic and PM associated archaea and bacteria. The PM-archaea and PM-bacteria collected on 0.8 μm (Millipore; ATTP02500) and planktonic ones collected on 0.2 μm polycarbonate filters (Millipore; GTTP02500) were analyzed separately. The archaea and bacteria collected on filters were hybridized with buffer containing respective probes (50 ng) and a mixture of 0.9 M NaCl, 20 mM Tris HCl (pH 8) and 0.01% of Sodium dodecyl sulphate (SDS), formamide (50% for eubacteria and 55% for archaea). The filters were washed with buffer containing 20 mM Tris HCl, 5 mM EDTA, 0.01% SDS, 5 M NaCl depending upon the formamide concentration to remove impurities and unlabelled probes. Fluorescent signals from the cells were counted using an epifluorescent microscope (Olympus BX41) with specific filter sets for Cy3. A blank filter was subjected to the same procedure without probe to remove error due to the background fluorescence.

2.6. Community structure of archaea and bacteria using DGGE

Nested PCR and Denaturing Gradient Gel Electrophoresis (DGGE) technique was used for analyzing the community structure of archaea and bacteria in water and PM samples. Genomic DNA from water samples was extracted following the protocol of Bostrom et al. (2004) with slight modification. Briefly, 1–2 L of water sample (pre-filtered through 10 μm Nylon membrane) was passed through 0.8 μm (Millipore; ATTP04700) and 0.2 μm polycarbonate membrane (Millipore; GTTP04700) filters sequentially to separate PM-attached and planktonic microbial fractions, followed by incubation at 37 ^{o}C for 1 h in lysis buffer (NaCl 400 mM, Sucrose 750 mM, EDTA 20 mM and Tris HCl 50 mM), containing 1 mg ml^{-1} lysozyme. Subsequently, SDS (1%) and proteinase K (100 μg ml^{-1}) were added to the solution and continued incubation for 5 h at 55 ^{o}C. Further 0.6 volume of isopropanol was added and DNA was precipitated by keeping at −20 ^{o}C for 60 min. DNA pellet was washed copiously with 70% ethanol, dissolved in TE buffer and stored at −20 ^{o}C until used. The sample filters from station 4 (collected from the Eloor region) were pre-treated with 1 ml of 10 mM Tris HCl and 40 mM Na_2EDTA (pH 7.0) to remove divalent metal inhibitors like Zn before cell lysis, because high concentrations of divalent cations, might contribute to premature precipitation of DNA (Kejnovsky and Kypr, 1997).

The DNA extracted from all samples were subjected to two-step PCR reaction with gene-specific primers for studying the community structure of archaea and bacteria. In the first step, archaeal 16S rRNA genes were amplified using two primer sets separately {A8F and A1492R (Casamayor et al., 2000), Arch 21F and Arch 958R (DeLong, 1992)} and the PCR products were pooled. Bacterial 16S rRNA genes were amplified using a primer set of 27F and 1492R (Lane, 1991) (Supplementary Table 2). The products of first step PCR reaction was used as template for the second step PCR using forward primers with GC clamp and reverse primers specific for bacteria (Muyzer F and Muyzer R) and archaea (Equimolar concentration of forward primers SAF (1) and SAF (2) and Parch 519r) (Supplementary Table 2) (Muyzer et al., 1993; Nicol et al., 2003; Overeas et al., 1997). Reactions were conducted in triplicate and PCR products were pooled during DGGE. The details of the gene specific primers and PCR conditions are illustrated in Supplementary Table 2. In the PCR, 5 μl DNA sample was used as template for 50 μl reaction mixture containing 2 μl each of primers (10 picomoles μl^{-1}), 5 μl of 10X Taq polymerase buffer (NEB, Canada), 1 U Taq DNA polymerase (NEB, Canada) and 200 μM dNTPs (NEB, Canada).

Denaturing Gradient Gel Electrophoresis (DGGE) of amplified PCR products of archaea (180 bp) and bacteria (200 bp) were performed using a D-code universal mutation detection system (Bio-Rad Laboratories Inc., USA). DNA concentration in the PCR products was quantified using Nanodrop (Thermo-Fisher, USA) and an equal concentration of DNA per sample (~400 ng) was loaded on the DGGE gel. The PCR products were run on an 8% polyacrylamide gel prepared with denaturing gradients of urea and formamide 30–70% and 40–65% respectively for archaea and bacteria, for 4.5 h at a constant voltage of 220 V in 1× TAE buffer (40 mM Tris HCl, 20 mM acetic acid, 1 mM EDTA). The bands separated were stained with ethidium bromide and observed in a gel documentation system (BioRAD, USA).

2.7. Statistical analysis

DGGE gel images were analyzed with Bionumerics software version 4.6 (Applied Maths USA). The software carries out a density profile analysis, detects the bands from each lane and calculates the relative intensity of each band. Numbers of operational taxonomic units (OTUs) in each sample were counted as a number of DGGE bands. Gels were cross-checked visually as well as for the number of bands per lane. An intensity matrix was constructed based on the relative contribution of the intensity of the band. The relative intensity of each band was used to calculate the Shannon-Wiener diversity index (H'). Cluster analysis of DGGE bands, based on square root transformed community data matrix through Bray-Curtis similarity was performed with PRIMER v.6 software package (Plymouth Marine Laboratory, UK). The multivariate relationship between community structure of archaea and bacteria with

592 V.A. Sheeba et al. / Environmental Pollution 231 (2017) 589–600

Table 1
Environmental variables and heavy metal of water column in study area during wet and dry season.

	Stations							
	Wet season				Dry season			
	S1	S2	S3	S4	S1	S2	S3	S4
PM (mgL^{-1})	36	18	6.8	2.8	32.4	26.8	23.2	17.6
Salinity (psu)	30	9	0	0	34.5	32	8	15
pH	7.8	7.8	7.6	7.1	7.83	7.82	7.92	7.84
Nutrients (μM)								
NH_4^+	11.3	7.9	16.1	4.8	1.68	2.23	3.20	5.24
NO_2^-	0.5	0.3	0.3	0.2	0.2	0.1	0.14	0.16
NO_3^-	4.4	6	13.6	19.3	0.996	0.45	1.40	0.54
SiO_4^-	31	56.7	35.4	36.1	10.25	8.5	36.25	22.63
PO_4^-	3.3	2.1	2.4	1.3	1.95	1.85	1.05	3.90
Dissolved Heavy Metals (μgL^{-1})								
Cd	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Co	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cr	0.47	0.07	0.09	0.09	0.22	0.08	0.34	0.20
Cu	0.68	0.27	0.44	n.d.	1.28	0.38	1.74	0.81
Fe	799	45.37	68.05	6.62	262.25	274.20	161.70	55.65
Mn	0.63	0.13	n.d.	n.d.	0.24	0.21	0.39	0.12
Ni	4.56	4.57	5.51	n.d.	3.55	2.97	4.21	2.02
Pb	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Zn	4.36	6.36	73.65	105.85	8.84	6.55	48.28	130.50

environmental variables and heavy metal concentrations of water and PM were analyzed separately using redundancy analysis (RDA) using Canoco (version 4.5, Centre for Biometry, Wageningen, Netherlands).

3. Results

3.1. Environmental variables and dissolved heavy metals

We observed salinity variation in stations during wet and dry seasons. CE was more limnetic during the wet season and the salinity was restricted only up to stations S1 (30 psu) and S2 (9 psu). Seawater influx was more evident during the dry season and it reached up to station S4 (15 psu). The pH of the water column remained neutral to slightly alkaline during both wet and dry season. The dissolved inorganic nitrogen (sum of ammonium, nitrite, and nitrate) load was higher in CE during the wet season, especially in S3 (30 μM) followed by S4 (24.3 μM), S1 (16.2 μM) and S2 (14.2 μM). It decreased to 2.9, 2.8, 4.7 and 5.9 μM respectively in S1, S2, S3 and S4 during the dry season. Silicate level also was higher during the wet season, with a maximum of 56.7 μM in S2 followed by S4 (36.1 μM), S3 (35.4 μM) and S1 (31 μM). During the dry season, higher levels of silicate were observed at S3 (36.25 μM), followed by S4 (22.63 μM), S1 (10.25 μM) and S2 (8.5 μM). Phosphate levels did not show much variations between the wet and dry seasons, ranging from 1 to 4 μM (Table 1).

We recorded Zn as the major dissolved heavy metal in the water column of CE. The concentration of Zn was higher in S4 during dry (130.5 μgL^{-1}) and wet (105.9 μgL^{-1}) seasons. Higher concentration of Zn was also found in the water column of S3 with 48.3 and 73.7 μgL^{-1} during dry and wet season respectively. The level of Zn was less than 10 μgL^{-1} in other stations during dry and wet seasons. High concentration of Fe was observed in S1 during the wet season (799 μgL^{-1}). Low concentration of Cr, Cu, and Ni was found during both wet and dry season while Cd, Co, and Pb were not detected in the water column of CE (Table 1).

3.2. Biochemical and chemical characterization of PM

The concentration of PM present in the water column of each station was calculated gravimetrically. The PM was higher in S1 during both wet (36 mgL^{-1}) and dry (32.4 mgL^{-1}) season, followed by S2, S3 and S4 (Table 1). Although the overall concentration of PM was higher in CE during the dry season, it contained low levels of POC, PON, proteins, carbohydrates, and lipids. The PM collected from S4 during wet season were rich in POC (17.86%), PON (10.71%), protein (7.55%) and lipids (2.39%). Similarly, the PM collected from S3 during the wet season also showed high levels of POC (10.29%), PON (4.41%), Proteins (7.81%), Carbohydrate (1.33%) and lipids (5.83%). The comparatively low level of POC and PON were observed in PM collected from stations S1 and S2 near the bar mouth. We also observed a seasonal variation in the biochemical characteristics of PM collected during wet and dry seasons. The POC levels in all the stations during the dry season was approximately half of that observed during the wet season. A similar trend was observed in PON levels in PM collected from all stations except S1. Such difference was more prominent in the levels of proteins in PM collected during the dry and wet season. The protein content in the PM collected during wet season ranged from 5.12 to 7.81%, while it reduced to 0.38–2.91% during the dry season. The carbohydrate and lipid content also showed the same trend in stations S1, S2 and S3 during dry and wet seasons. The carbohydrate concentration in PM collected from these stations ranged from 0.19 to 1.33% and 0.07–0.18% respectively during the wet and dry season. The carbohydrate concentration in PM collected from S4 during wet and

Table 2
Biochemical and chemical characterization of particulate matter.

	Stations							
	Wet season				Dry season			
	S1	S2	S3	S4	S1	S2	S3	S4
POC (%)	8.61	6.11	10.29	17.86	4.32	2.24	6.03	7.95
PON (%)	1.11	2.78	4.41	10.71	1.23	1.49	2.16	4.55
Protein (%)	5.12	5.20	7.81	7.55	0.38	0.41	1.12	2.91
Carbohydrate (%)	0.23	0.19	1.33	0.56	0.07	0.06	0.18	0.52
Lipid (%)	0.26	0.49	5.83	2.39	0.12	0.84	0.29	3.25
Cd (μgg^{-1})	1.44	4.01	4.64	7.57	1.22	5.25	4.28	22.69
Co (μgg^{-1})	0.43	n.d.	n.d.	n.d.	0.87	3.49	n.d.	5.90
Cr (μgg^{-1})	69.43	14.14	114.34	34.46	5.80	n.d.	35.14	n.d.
Cu (μgg^{-1})	8.57	2.26	24.00	17.85	3.05	4.83	2.12	1.15
Fe (μgg^{-1})	7827.14	3990.48	10662.00	10923.08	2130.55	3561.93	1210.79	4116.67
Mn (μgg^{-1})	65.43	109.67	384.55	188.72	21.27	47.56	22.09	2439.49
Ni (μgg^{-1})	n.d.	n.d.	5.26	n.d.	n.d.	n.d.	n.d.	n.d.
Pb (μgg^{-1})	27.29	5.06	15.69	31.79	2.53	3.38	6.26	13.59
Zn (μgg^{-1})	1600.00	2561.40	3998.07	8451.28	765.45	788.20	2086.33	2294.87

dry seasons were 0.56 and 0.52% respectively. The lipid concentration in PM collected from S4 during wet (2.39%) and dry (3.25%) seasons remained similar, while it ranged from 0.26 to 5.83% and 0.12–0.84% respectively in other stations.

The level of heavy metals was also higher in PM collected from all the stations compared with the dissolved levels of respective metals (Table 2). The levels of heavy metals were higher during the wet compared with the dry season, and maximum levels were observed in PM collected from stations S3 and S4. The level of Fe remained the same in PM collected from S2 during the dry and wet seasons, while it showed a 2.5 to 10 times increase in other stations during the wet season. The maximum level of Fe was observed in PM (PM-Fe) collected from S3 (10622 μgg^{-1}) and S4 (10923.1 μgg^{-1}) during the wet season. The level of PM-Fe collected from S3 and S4 during dry season were 1210.8 and 4116.7 μgg^{-1} respectively. The level of PM-Fe in S1 was 7827.1 μgg^{-1} during the wet season, while it decreased to 2130.6 μgg^{-1} during the dry season. The level of Cd, Mn, Pb and Zn showed higher accumulation in PM collected from the northern part of the estuary compared with those from the bar mouth (station S1) during both wet and dry seasons. The level of PM-Cd during wet and dry seasons ranged from 1.44 to 7.57 and from 1.22 to 22.69 μgg^{-1} respectively, with a maximum concentration of 22.69 μgg^{-1} recorded at S4 during the dry season. The lowest concentration of PM-Cd was observed near the bar mouth (station S1) during both wet and dry seasons. The highest (2439.49 μgg^{-1}) and lowest (21.27 μgg^{-1}) concentrations of PM-Mn were observed at S4 and S1 stations respectively during the dry season. The level of PM-Mn towards bar mouth stations (S1 and S2) was higher during the wet season (i.e. 65.43 and 109.67 μgg^{-1} respectively) compared with those of the dry season (i.e. 21.27 and 47.56 μgg^{-1} respectively). The concentrations of PM-Pb in the riverine end of the estuary, S3 and S4, were higher during the wet season (15.69 and 31.79 μgg^{-1} respectively) compared with those of the dry season (6.26 and 13.59 μgg^{-1} respectively). The lowest levels of PM-Pb was observed at S1 (2.53 μgg^{-1}) and S2 (3.38 μgg^{-1}) during the dry season. The level of PM-Zn was higher during wet season compared with the dry season, with particularly higher levels in the riverine end of the estuary (stations S3 and S4) during the wet season (3998.07 and 8451.28 μgg^{-1} respectively). The lowest level of PM-Zn was observed near the bar mouth stations (S1 and S2) during the dry season (765.45 and 788.20 μgg^{-1} respectively). Comparatively lower levels of PM-Cu were observed at all the stations. The level of PM-Cu ranged from 8.57 to 24.00 and 2.12 to 4.83 μgg^{-1} respectively during the wet and dry seasons. Highest levels of PM-Cu was observed in S3 and S4 (24.0 and 17.85 μgg^{-1} respectively) during the wet season. The PM-Cr was observed at all the stations during the wet season, but was not detectable at S2 and S4 during the dry season. The highest level of PM-Cr was observed at S3 (114.34 μgg^{-1}), followed by S1 (69.43 μgg^{-1}) and S4 (34.46 μgg^{-1}) during the wet season. Low levels of PM-Co were detected at S1, S2, and S4 during the dry season and at S1 during the wet season. The level of PM-Co ranged from 0.43 to 5.9 μgg^{-1}. PM-Ni was detected only at S3 (5.26 μgg^{-1}) during the wet season.

3.3. Microbial abundance and community structure

The abundance of archaea and bacteria as plankton in the water and as components of PM were measured using the FISH technique.

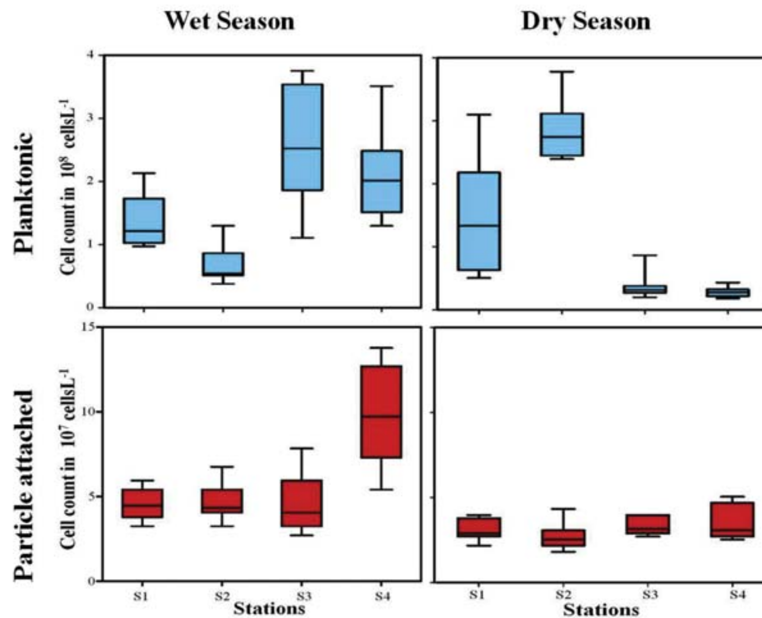

Fig. 1. Whisker box plot showing the abundance of Archaea.

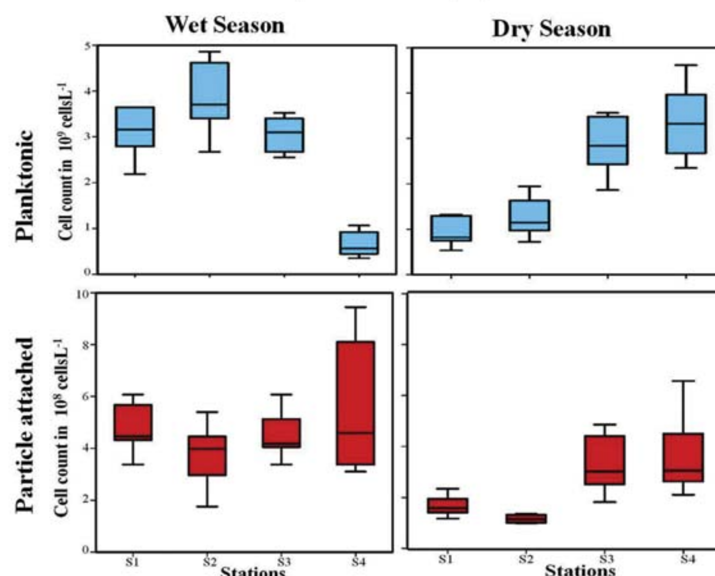

Fig. 2. Whisker box plot showing the abundance of Bacteria.

Table 3
Number of operational taxonomic units (OTUs) obtained from DGGE profile.

Number of Operational Taxonomic Units (OTU)	Wet season					Dry season				
	S1	S2	S3	S4	Total	S1	S2	S3	S4	Total
Planktonic archaea	11	15	13	11	23	6	7	10	15	19
PM- archaea	11	9	13	17	27	14	12	11	9	21
Planktonic bacteria	12	10	7	5	16	15	18	16	12	24
PM- bacteria	18	16	13	13	26	10	11	9	8	18

The abundance of planktonic archaea was ten times higher compared with the PM-associated ones (PM-archaea) as evident from FISH counts (Fig. 1). There was no significant difference in the abundance of planktonic archaea between dry and wet seasons, and it ranged between 0.28–2.83 × 10^8 cellsL^{-1}, with the higher archaeal abundance being observed at station S4 during the wet season. The abundance of PM-archaea ranged between 4.27 and 9.5 × 10^7 cellsL^{-1} and 2.73 to 3.85 × 10^7 cellsL^{-1} respectively in samples collected during the wet and dry seasons. The abundances of planktonic and PM-bacteria were tenfold higher compared with the archaeal abundance of their respective counterparts (Fig. 2). The abundances of planktonic bacteria in both wet and dry seasons ranged from 0.55 to 3.4 × 10^9 cellsL^{-1}, while that of PM-bacteria ranged from 1.14 to 6.72 × 10^8 cellsL^{-1}.

Table 4
Shannon-Weiner diversity index of planktonic and PM attached bacteria and archaea.

Diversity index	Wet season				Dry season			
	S1	S2	S3	S4	S1	S2	S3	S4
Planktonic bacteria	1.54	2.04	2.02	1.67	2.28	2.19	2.3	2.16
Planktonic archaea	1.74	2.49	1.88	1.87	1.56	1.55	1.97	2.37
PM- archaea	2.08	1.95	1.78	2.23	2.08	2.03	2.01	1.78
PM- bacteria	2.44	2.26	2.32	2.07	2.12	1.97	1.92	1.65

The community structure of archaea and bacteria was studied using the PCR-DGGE technique (Supplementary Figs. 2 and 3). Here each band in the DGGE was considered to have originated from one group of archaea/bacteria, and accordingly, each of them was considered as an independent operational taxonomic unit (OTU). The community structure of PM-archaea was more diverse (27 and 21OTUs respectively during the wet and dry seasons) compared with planktonic ones (23 and 19 OTUs respectively during the wet and dry seasons) (Table 3). Shannon-Wiener diversity (H') index of planktonic archaea was higher during the wet season, while that of PM-archaea was higher during the dry season (Table 4). The cluster analysis of DGGE band pattern of planktonic and PM-archaea showed spatial difference in their community structure (Fig. 3). The community structure of planktonic archaea of the near-riverine-end stations S3 and S4 showed nearly 80% similarity

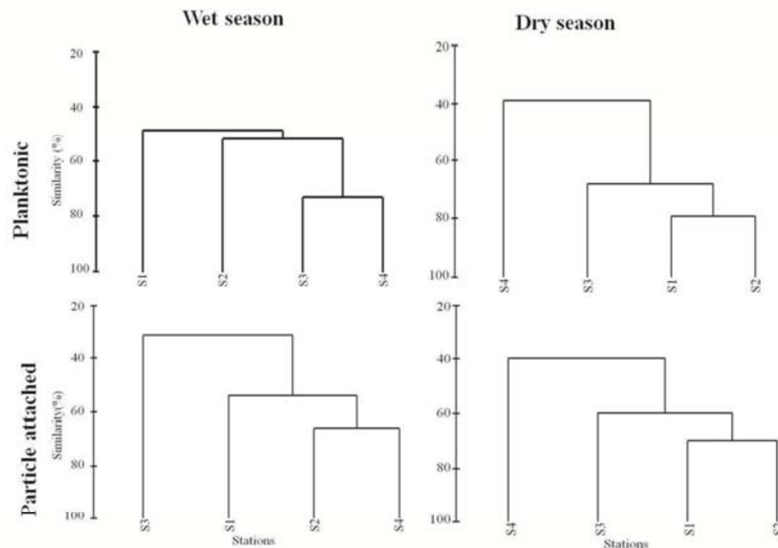

Fig. 3. Cluster analysis of planktonic and PM-attached archaea during wet and dry season.

during the wet season, while such a clustering was observed between the bar-mouth stations S1 and S2 during the dry season. In the case of PM-archaea, similarities were observed between S4 and S2 (~60%) during the wet season and between S1 and S2 (~60%) during the dry season.

We observed a seasonal difference in community structure between planktonic and PM-bacteria. The community structure of planktonic bacteria was less diverse (16 OTUs) compared with that of PM-bacteria (26 OTUs) during the wet season. On the contrary, the community structure of planktonic bacteria was more diverse (24 OTUs) compared with that of PM-bacteria (18 OTUs) during the dry season. During the wet season, the highest number of OTUs corresponding with planktonic (12 OTUs) and PM-bacteria (18 OTUs) were found in station S1. During the dry season, highest numbers of OTUs corresponding to planktonic (18 OTUs) and PM-bacteria (11 OTUs) were found in station S2 (Table 3). Cluster analysis of DGGE profile of planktonic and PM-bacteria showed clear demarcation between the bar mouth and riverine end stations in the estuary during wet and dry seasons (Fig. 4). The community structure of planktonic bacteria in bar mouth stations (S1 and S2) formed a separate cluster of ~80% similarity during wet and dry seasons. In the case of PM-bacteria, more than 80% similarity was observed between S2 and S3 during the wet season and more than 60% similarity between S3 and S4 during the dry season. Differences were also evident in the Shannon-Wiener diversity index between planktonic and PM-bacteria. Higher diversity index of planktonic bacteria was found during the dry season, while that of PM-associated bacteria were higher during the wet season.

3.4. Environmental influence on microbial distribution

Two-tailed correlation analysis was done to understand the influence of environmental characteristics on the abundance and community structure of planktonic and PM-associated archaea and bacteria. The levels of PM-Pb and PM-Zn were correlated with protein concentrations of PM (r value for Pb = 0.73 and Zn = 0.75) while the levels of PM-Ni (r = 0.90) and PM-Cr (r = 0.76) were well correlated with carbohydrates. The levels of PM-Cu and PM-Fe were correlated with both protein and carbohydrates. We observed no significant influence of soluble metals on the abundance and community structure of planktonic archaea and bacteria. But the abundance and community structure of PM-archaea and PM-bacteria were strongly influenced ($p < 0.05$) by biochemical properties and heavy metal concentrations of PM (Fig. 5). The abundance of PM-archaea and PM-bacteria showed a positive correlation with POC, PON, PM-Pb, PM-Zn, and PM-Fe (Fig. 5). The biochemical characteristics and heavy metal concentration do not show any significant correlation with the diversity of PM-archaea while that of PM-bacteria were negatively correlated with PM-Co and PM-Cd (r value for Co = −0.75 and Cd = −0.77). The RDA indicates that heavy metal variables have a pronounced impact on the abundance and diversity of archaea and bacteria associated with PM (Fig. 6).

4. Discussion

In the present study, we report the influence of heavy metal pollution on the abundance and community structure of PM-archaea and PM-bacteria in CE, a tropical estuary along the southwest coast of India. Samples were collected along a pollution gradient from the riverine end to the bar mouth of the estuary. CE is a monsoon-driven estuary in which the hydrographic variables and the level of pollution may vary depending upon the seasonal flushing (Vinita et al., 2015). The estuary flushes multiple times during the wet season, because of high river-water influx as evident from salinity measurements. The fresh-water influx was reduced during the dry season and hence we could record the intrusion of

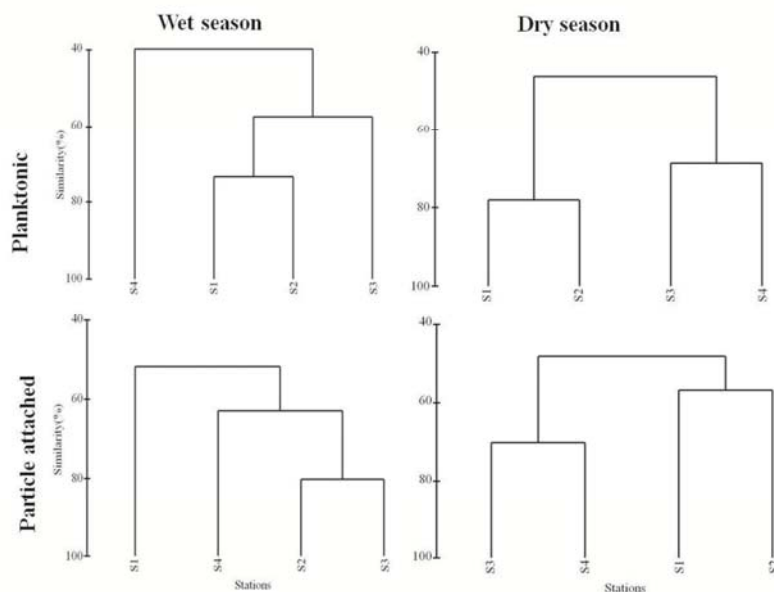

Fig. 4. Cluster analysis of planktonic and PM-attached bacteria during the wet and dry season.

saline water up to station S4. The concentrations of nutrients, viz. ammonia, nitrite, and nitrate, were also high in the CE during the wet season. The high nutrient load and associated phytoplankton productivity in CE during wet seasons were attributed to high terrigenous input and effluent discharge (Madhu et al., 2010). The present and previous studies confirm that the level of Zn in the CE has been increasing over the past decade (Jiya et al., 2011; Martin et al., 2012).

We observed high levels of all heavy metals tested, except Ni, in PM. The major heavy metal pollutant in the CE, PM-Zn,varied from 1600 to 8451 and 766 to 2295 $\mu g g^{-1}$ during wet and dry seasons, respectively. The observed concentration of PM-Zn was 2.5 times higher compared with that reported from the CE in 1992 (Ouseph, 1992). Accumulation of heavy metals in PM have been reported from the world estuaries such as the Seine estuary in France (Ouddane et al., 1992), Texas estuary in USA (Benoit et al., 1994), Humber estuary in UK (Comber et al., 1995), Scheldt Estuary in Belgium (Baeyens et al., 1998), Tagus estuary in Portugal (Duarte and Caçador, 2012) and Jade Bay in Germany (Beck et al., 2013). Based on the toxicity classification of heavy metals by Long et al. (1995), the levels of PM-Zn in CE are highly toxic, and PM-Cd is toxic at the intermediate level at certain locations. We observed the presence of Cd, Co, and Pb in PM, although they were below detectable limit in the water column. Generally, heavy metals showed a tendency to accumulate in PM, but the level of accumulation varied between different metals. This may be attributed to the differential affinity of heavy metals towards different biochemical components of PM. Statistical analysis showed the affinity of Zn and Pb to protein, Ni and Cr to carbohydrate, and Cu and Fe towards both protein and carbohydrate. The concentrations of heavy metals accumulated in PM were in the order Fe > Zn > Mn > Cr > Pb > Cu > Cd > Co > Ni. Previously, Fang and Lin (2002) have also reported the differential binding of heavy metals with PM, and they observed a decrease in affinity of heavy metals to PM in the order Fe > Zn=Cu > Co > Mn > Ni. Heavy metals have a tendency to form metal complexes with organic matter, which is mediated through the interaction of metal cations with anionic ligands, such as hydroxyl groups (OH^-), carboxyl groups ($COOH^-$), phosphates (PO_4^{2-}), sulphates (SO_4^{2-}), sulfonates, ketal-linked pyruvates, acetyl groups, amine groups etc. of proteins, lipids and carbohydrates of PM (Decho, 2000; Diop et al., 2014; Hernandez and Jimenez, 2012; Lenoble et al., 2013). The high plankton biomass and nutrient levels (Gireeshkumar et al., 2013; Madhu et al., 2010) could contribute to the production of organic-rich PM in CE and subsequent attachment of heavy metals.

We observed a high protein-to-carbohydrate ratio in PM collected during the wet season, which indicates the presence of newly generated organic matter. Since the proteins are more amenable to bacterial digestion compared with carbohydrates, the protein-to-carbohydrate ratio decreases with the age of PM. This is considered as an indication of seasonal transition of the ecosystem from autotrophic to heterotrophic nature (Fabiano and Danovaro, 1994). In a previous study, Shoji et al. (2008) showed the influence of freshwater influx during wet season on bacteria-mediated carbon flux and autotrophic-to-heterotrophic shift in CE. The protein contributes more to the biopolymeric carbon composition (BPC) during the wet season, while the lipid levels dominated the BPC of PM during the dry season. A possible reason for higher BPC in stations near the riverine end (S3 and S4) during both wet and dry seasons is the low microbial remineralization due to heavy

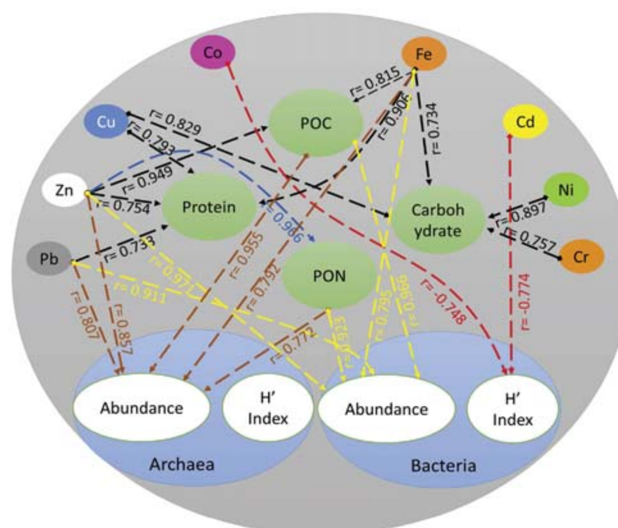

Fig. 5. Schematic diagram showing the two-tailed correlation of the abundance and diversity index of archaea and bacteria with heavy metal concentration and biochemical characteristics of PM. The parameters with significant correlation are connected through dotted lines and coefficient of correlation (r value) are given along the line.

metal pollution. A reduction in hydrolytic enzymes, involved in remineralization of organic matter, has been reported in planktonic bacteria isolated earlier from these locations (Jiya et al., 2011). The heavy metal pollution may impinge microbial remineralization of PM (Zampieri et al., 2016) and also become toxic to humans through bioaccumulation in higher trophic levels of the food web. Many organisms cannot tolerate heavy metals beyond certain concentrations, which may force them to reside in a viable but non-cultivable (VBNC) status or to die (Hobman and Crossman, 2015; Nies, 1999). Those organisms which can tolerate the pollutants at higher concentrations may flourish under such conditions and a reduction or adaptation in the diversity and activities of microorganisms have been reported (Jiya et al., 2011).

In the present study, we monitored the impact of heavy metal pollution on the distribution and community structure of planktonic and PM-associated archaea and bacteria. Among the different metals observed in the particulate matter, Fe, Zn and Mn are reported to function as electron acceptors during anaerobic respiration and as cofactor for metalloenzymes in bacteria and archaea (Lovley, 2013; Voica et al., 2016). On the contrary, the metals such as Pb and Cd do not have any significant biological role and can become toxic to the organism by entering the cytoplasm by replacing the essential metals from their natural binding sites or by interacting with other specific ligands (Voica et al., 2016). There are different mechanisms through which archaea and bacteria acclimatize with the heavy metal pollution, which includes extracellular sequestration by biopolymers, regulating intracellular concentration by efflux pump mechanisms and enzymatic detoxification (Nies, 1999; Voica et al., 2016). The results showed that the planktonic and PM-archaea were less abundant than bacteria in CE. This is in agreement with previous reports from various aquatic ecosystems across the world (Bouvier and Giorgio, 2002; GarneauÈ et al., 2009). A possible explanation is that archaeal metabolism slows down at eutrophic conditions (Vipindas et al., 2015). Among archaea, the abundance of planktonic ones was higher than that associated with PM. We observed a positive correlation of PM-archaeal abundance with biochemical (PON and POC) and heavy metal (Pb, Zn, and Fe) concentrations of PM. However, we did not find any significant correlation between archaeal diversity and biochemical or chemical characteristics of water column and PM. The difference in the abundance of archaea observed between dry and wet seasons in S4 may be due to the difference in the level of heavy metals. The influence of heavy metal pollution on the community structure of archaea was earlier reported from sewage-sludge contaminated soil (Sandaa et al., 1999), marine sediments (Besaury et al., 2014), wetland soil (Wu et al., 2016) and mine tailings (Tan et al., 2008). More detailed studies are required to understand the influence of heavy metal accumulated in PM on the ecophysiology of archaea in marine and estuarine environments.

The abundance and community structure of planktonic bacteria were not influenced by heavy metal concentration, while that of PM-bacteria were significantly influenced by the chemical and biochemical characteristics of PM. The abundance of PM-bacteria varied between estuaries depending on the particle size and chemical composition of PM. The abundance of PM-bacteria observed in CE was comparable with Ria de Aveiro estuary in Portugal (Santos et al., 2014) and less than that reported from Columbia River estuary in the USA (Crump et al., 1998) and Zuari estuary in India (Gonsalves et al., 2017). The metabolic activities of PM-bacteria was reported to be higher in many studies, although their abundance was less compared to the planktonic counterparts (Ghiglione et al., 2009; Grossart and Simon, 1998). The level of POC, PON, PM-Zn, PM-Pb and PM-Fe had a positive influence on the abundance of PM-bacteria and the PM-Co and PM-Cd had a

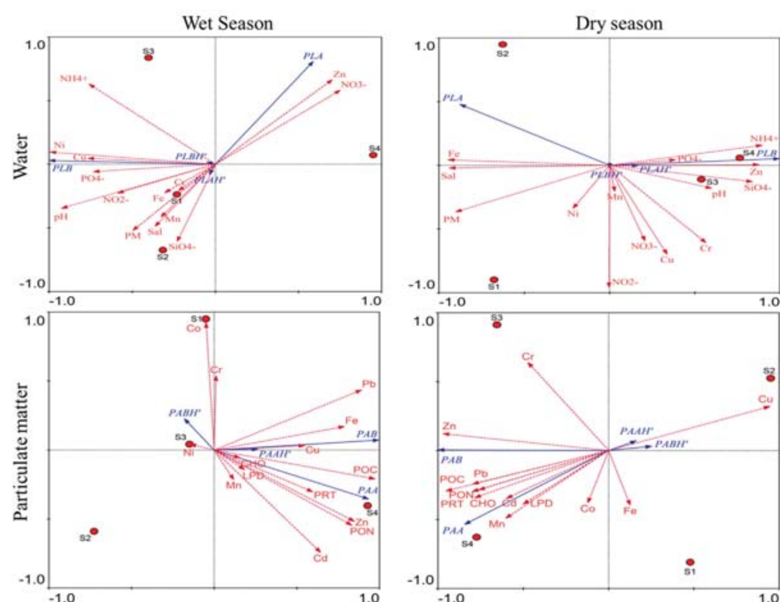

Fig. 6. Redundancy analysis showing the influence of hydrographic variables and heavy metal concentrations on the abundance (PLA, PLB, PAA, PAB) and diversity (PLAH', PLBH', PAAH', PABH') of planktonic (PLA, PLB, PLAH', PLBH') and PM (PAA, PAB, PAAH', PABH') associated archaea (PLA,PLAH',PAA,PAAH') and bacteria (PLB,PAB,PLBH',PABH'). (Protein PRT; Carbohydrate CHO; Lipid LPD; Particulate organic carbon POC; particulate organic nitrogen PON; particulate matter PM; salinity sal).

negative influence on the diversity index of PM-bacteria in CE. Interestingly the differences in the abundance of PM-bacteria between different stations were not drastic. At the same time, the DGGE band pattern showed differences in the community structure of PM-bacteria between stations. There were smaller number of OTUs in stations near the industrial zone (S3 and S4), during the wet and dry season compared with other stations. Station S4 is the point where the river Periyar carrying the industrial effluents joints with the estuary. The redundancy analyses showed that the heavy metal pollution impinges the community structure of PM-bacteria in CE. The reduction in the community structure of bacteria in response to heavy metal pollution has been reported earlier from different marine and estuarine environments (Bååth et al., 1998; Bezverbnaya et al., 2005; Gillan et al., 2005; Naik et al., 2012; Shi et al., 2002). These studies also reported an increase of abundance and a decrease of community structure in sediments contaminated/amended with heavy metals such as Cd, Cu, Pb, Cr, and Zn. It is possible that heavy metals may function as a selective factor on bacterial community structure in polluted environments and may lead to the proliferation of metal-resistant bacteria. Under such conditions, the diversity index remains low at polluted regions while keeping the abundance comparable between stations.

5. Conclusion

In the present study, we have reported on the chemical, biochemical and microbiological characteristics of particulate matter collected from different stations along a pollution gradient in a nutrient-rich tropical estuary. The study showed the accumulation of heavy metals in particulate matter in the Cochin Estuary, which may invoke serious threats to the ecosystem functioning, as PM is considered as hotspots of microbial processes. We observed low diversity index of PM-bacteria in polluted regions of CE, which indicates the role of heavy metals in structuring the bacterial community associated with PM. The bacteria under polluted environment may utilize most of its energy for maintaining its essential cellular processes, and may not be participating in the processing of PM through secretion of extra-cellular enzymes. In such cases, the PM may remain without remineralization in the system, arresting the food web dynamics and biogeochemical cycles. The toxic effects of heavy metals may be aggravated further with nanoparticles, as their surface areas tend to be higher compared with bulk materials, which create opportunity for increased uptake and interaction with biological tissues (Nel et al., 2006). Recent studies have characterized the toxicities of metal ions such as cadmium released from quantum dots in animal cells, silver nanoparticles on microorganisms and engineered nanoparticles on food web dynamics (Anas et al., 2012; Maurer Jones et al., 2013; Wiecinski et al., 2013). Studies focusing on the implications of heavy metals and nanoparticles on food web and biogeochemical cycles in estuarine and marine environment may need further attention.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.08.053.

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Regional Studies in Marine Science 18 (2018) 113–121

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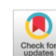
Upwelling induced changes in the abundance and community structure of archaea and bacteria in a recurring mud bank along the southwest coast of India



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HIGHLIGHTS

- Upwelling and mud bank occur simultaneously in coastal waters.
- Dissolved nutrients in the water column increase during upwelling.
- Upwelling induces changes in abundance and community structure of bacteria.
- Microbial distribution is controlled by interactive effect of hydrographic variables.

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ABSTRACT

The present study reports the influence of upwelling on the abundance and community structure of archaea and bacteria in a mud bank formed along the coastal waters of Alappuzha, the southwest coast of India. The signatures of upwelling were observed with the onset of southwest monsoon, characterized by a decrease in sea surface temperature and dissolved oxygen in bottom waters and increase in salinity. Nitrite, nitrate, and phosphate showed a significant increase during upwelling compared to pre-upwelling conditions. There were no significant differences in the abundance of archaea across seasons (pre- and during upwelling conditions) and regions (Station S1, inside and Station S2, outside the mud bank), while the bacterial abundance showed a six-fold increase for S1 and four-fold increase for S2 during upwelling. The PCR–DGGE results show the existence of a transient population of archaea and bacteria in the mud bank region during upwelling. The step up multiple regression models could explain 66.5% and 56.3% of the temporal variations in archaeal and 96.5% and 53.63% in bacterial distribution inside and outside of mud bank respectively, at pre-upwelling conditions. About 67% of the temporal variations in the distribution of archaea and 41.4% in bacteria inside and 49.9% in bacteria outside of mud bank during upwelling conditions could be explained by the same model. The temporal variation in the distribution of archaea outside mud bank during upwelling conditions could not be explained by the present regression model. Our studies indicate that the upwelling induces changes in the abundance and community structure of bacteria in the mudbank, which are modulated by the interactive effects of hydrographic variables. Further studies to unravel the interactive effect of hydrographic variables on the biogeochemical activities of microorganisms would be appreciated.

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1. Introduction

The ecosystem at interfaces linking terrestrial and aquatic or aquatic and marine systems undergo drastic seasonal changes in environmental conditions, which may induce changes in community structure and activities of microorganisms (Naiman and Decamps, 1997). Such types of drastic environmental changes are observed in coastal environments, where multiple oceanographic processes happen simultaneously. Upwelling and mud bank formation are two processes that occur simultaneously in coastal areas of southeast Arabian Sea during the southwest monsoon (Rao et al., 1992). An interesting feature of this region is that the upwelling induces dramatic changes in the nutritional characteristics, which in turn influence the functioning of the ecosystem. The coastal upwelling is controlled by the strengthening of the alongshore wind stress towards southward direction and continental

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morphology. Such systems are characterized by intense primary biomass production in the surface waters after the outcropping of the nutrient-rich bottom water. Studies from other locations of coastal upwellings such as the southwest coast of Africa, have demonstrated the ecological importance of upwelling which is known to cause high nitrification (Codispoti et al., 2001; Kuypers et al., 2005; Woebken et al., 2007), increased biological productivity (Gonzalez-Rodriguez, 1994; Gonzalez-Rodriguez et al., 1992; Pereira et al., 2009), stimulate fishing activities (Cury et al., 2000) and response of microorganisms (Cury et al., 2011). Previous studies have confirmed the existence of a typical upwelling system in the southwest coast of India, with Kerala coast as the location where strongest upwelling occur (Gupta et al., 2016; Rao et al., 1992). During upwelling, the oxygen minimum zones of Arabian Sea extend to the coastal waters, creating hypoxic conditions and causing rapid changes in biogeochemical properties (Helly and Levin, 2004; Naqvi and Jayakumar, 2000). The physical forces inducing the formation of upwelling off the southwest coast of India and its implications for biological processes at different trophic levels are reported earlier, but there have been few reports on bacterial response during the process (Habeebrehman et al., 2008; Malik et al., 2015).

The biological implications of upwelling may become more complex in a mud bank, a unique feature appearing in the coastal waters of Kerala, India, during southwest monsoon (Dinesh Kumar, 2016). The waters above mud banks are characterized by high suspended matter which attenuates the waves to retain the water calm even during roughest monsoon, and copious fish catch. Historically, the mud banks have been reported across about 21 locations along the coastal areas of Kerala, of which Alappuzha is the only location where a mud bank appears every year during the southwest monsoon (Dinesh Kumar, 2016). There are several hypotheses regarding the formation of mud banks, which includes subterranean passage, water-bearing stratum, river deposition, upwelling, rip current wave convergence and hydrocarbon explosion. Although there is a lack of clarity over the formation of this phenomenon, mud banks have been accepted as a region of high organic matter content and a potential platform for fishing activities. Previous studies have focused on the physical factors influencing the formation of mud banks, their chemical characteristics and fishery potential (Dinesh Kumar, 2016; Jacob and Qasim, 1974; Noujas et al., 2016; Parvathy et al., 2015). The upwelling-induced high nutrient conditions in a mud bank region may create an oxygen minimum condition which provokes the conversion of microbial processes from aerobic to anaerobic. The influence of upwelling on abundance and community structure of microorganisms in a mud bank region would be important to understand, as microorganisms have central roles in food webs and biogeochemical cycles. Microorganisms with their small size and high growth rate are considered to respond quickly to environmental changes (Tuyet et al., 2015). This study forms the first detailed observation made with the goal of understanding the influence of upwelling on abundance and community structure of archaea and bacteria in a recurring mud bank region along the southwest coast of India.

2. Materials and methods

2.1. Study sites, sample collection, and analysis of hydrographic variables

We choose two stations, one inside (S1, depth 6 m) and another outside (S2, depth 12 m), a recurring mud bank region in Alappuzha, Kerala on the southwest coast of India (Fig. 1).

Sampling was carried out 16 times at 10–15 days interval from April to September 2014 onboard a fishing vessel (FV Silver pompano, CMFRI Kochi). Water samples were collected at 3 m intervals from the surface to bottom using a 5 L capacity Niskin water sampler. For microbiological analysis, multiple water samples were removed aseptically into sterile polypropylene bottles and maintained at 4 °C for further analysis. Water samples for chemical analysis were transferred into polypropylene bottles avoiding contamination from all possible sources. A portable Sea-Bird CTD (Conductivity–Temperature–Depth) (Model Seacat-26) was used to profile the physical properties of the water column. During each cast, salinity and temperature profiles were measured and binned into 0.2 m depth intervals. Salinity was determined using a Digi Auto Salinometer (Model TSK, accuracy ± 0.001). Dissolved oxygen (DO) content was determined following Winkler's titration method (Grasshoff et al., 1983). Samples for nutrients (ammonium, nitrite, nitrate, and phosphate) were filtered through Whatman No 1 filter paper and concentrations estimated spectrophotometrically (Grasshoff et al., 1983) within six hours of sampling. Ammonium was measured following the indophenols blue method and the absorbance was measured at 630 nm. Nitrite was measured as the formation of highly colored azo dye (Abs 543 nm) in a reaction mixture containing N-(1-naphthyl)-ethylenediamine and a diazo compound formed through the reaction of nitrite in water samples with sulphanilamide in acidic condition. Nitrate in the water samples was measured after reducing it to nitrite by passing through a cadmium-copper column. Phosphate was measured spectrophotometrically (Abs 882 nm), following the reduction of phosphomolybdate complex, formed through the reaction of phosphate in a water sample and ammonium molybdate, with ascorbic acid.

2.2. Abundance of archaea and bacteria

Abundance of archaea and bacteria were quantified using fluorescent *in situ* hybridization (FISH), following the protocol of Glöckner et al. (1999) with Cy_3labeled oligonucleotide probes such as ARCH 915 (GTGCTCCCCCGCCAATTCCT) (Stahl and Amann, 1991) and EUB 338 (GCTGCCTCCCGTAGGAGT) (Amann et al., 1990) respectively. Briefly, water samples preserved in paraformaldehyde (final concentration 4% v/v) were passed through 0.2 μm white polycarbonate membrane filter (Millipore GTTP2500) for bacteria and through 0.1 μm white polycarbonate membrane filter (Millipore VCTP02500) for archaea. The membrane filters were hybridized for 90–120 min in a hybridization solution (0.9 M NaCl, 20 mM Tris-HCl (pH 7.4) and 0.01% SDS), containing 50 ng nucleotide probes and formamide (55% and 50% respectively for archaea and bacteria). The cells were counterstained with DAPI to localize the nuclei and distinguish nonspecific bindings. Fluorescent signals from labeled cells were counted after exciting under an epifluorescence microscope equipped with a 100 W Hg lamp and filter sets specific for DAPI and Cy_3. The cell counts from 20 fields each from duplicate filter sets of each sample were counted and the average cell counts were expressed in cells ml^{-1}. Hybridization conditions were optimized with positive and negative samples of *E. coli* cells with and without recombinant plasmids of the respective genes. A filter section of the sample without hybridization was used for subtracting auto-fluorescence from FISH counts.

2.3. Community structure of archaea and bacteria

2.3.1. Extraction of DNA from water samples

Genomic DNA from water samples was extracted following Boström et al. (2004) with slight modification. Briefly, 1 to 2 L of water sample was passed through 0.2 μm polycarbonate membrane filter (Millipore, GTTP2500). The membrane filter was incubated at 37 °C for 1 h in 600 μl lysis buffer (NaCl 400 mM, Sucrose 750 mM, EDTA 20 mM and Tris-HCl 50 mM), containing 1 mg ml^{-1} lysozyme. Subsequently, SDS (1%) and proteinase K (100 μg ml^{-1}) were added to the solution and incubation continued

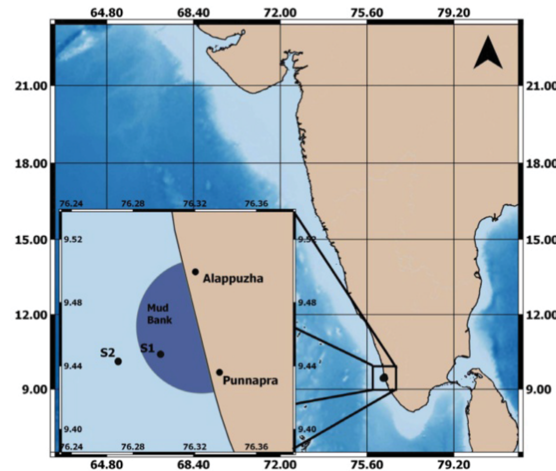

Fig. 1. Map showing study area and sampling locations.

for 5 h at 55 °C. A 360 μl of isopropanol was added and DNA was precipitated by keeping at −20 °C for 60 min. The DNA pellet was washed with 70% ethanol, dissolved in TE buffer and stored at −20 °C until used.

2.3.2. PCR and denaturing gradient gel electrophoresis (DGGE)

Community structure of archaea and bacteria was studied using nested PCR–DGGE techniques. Briefly, a 2 μl DNA sample was used as a template for a 25 μl PCR reaction mixture containing 1 μl each of primers (10 pmol μl^{-1}), 2.5 μl Taq polymerase (10X concentration, NEB, Canada), and 100 μM each dNTPs (NEB, Canada). Approximately 1400 bp 16S rRNA gene of archaea and bacteria were amplified with primer set of A8F (5´CGGTTGATCCTGCCGGA) & A1492R (5´GGCTACCTTGTTACGACTT) and 27 F (5´AGAGTTTGATCCTGGCTCAG) 1492 R- (5´TACGGCTACCTTGTTACGACTT) respectively. The reaction mixture was subjected to thermal conditions as follows: initial denaturation at 95 °C for 2 min, followed by cycle denaturation at 95 °C for 40 s, annealing at 51.5 °C for 60 s for archaea/ 55 °C for 40 s for bacteria, and 72 °C for 15 s for a total 30 cycles. The PCR products were further used as template for nested PCR using forward primers with the GC clamp at the 5′ end and reverse primer sets for archaea [equimolar concentration of two forward primers (SAF 1 (5´ACGGGGGGCCTAYGGGGCGCAGCAGG) and SAF 2 (5´ACGGGGGGCCTACGGGGCGCAGAGGG and reverse primer Parch 519R (5´TTACCGCGGCKGCTG)] and bacteria [Muyzer F GC (5´ACGGGGGGCCTACGGGAGGCAGCAG and Muyzer R (5´ATTACCGCGGCTGCTGG)]. The PCR reaction mixture (50 μl) was subjected to thermal conditions mentioned above with different annealing conditions for archaea and bacteria.

DGGE of amplified PCR products of archaea (∼200 bp) and bacteria (∼200 bp) were performed with the D-Code universal mutation detection system (Bio-Rad, USA) as per the manufacturer's instructions. DNA concentration in the PCR products was quantified using Nanodrop (Thermo-Fisher, USA) and an equal concentration of DNA per sample (1000 ng) was loaded on the DGGE gel. The PCR products were run on an 8% polyacrylamide denaturing gradient gel prepared with 30% to 70% (for archaea) and 45% to 65% (for bacteria) denaturing gradient of urea and formamide for 5 h at a constant voltage of 220 V in 1× TAE (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA). The bands separated were stained with ethidium bromide and observed in a gel documentation system (BioRAD, USA).

2.4. Statistical analysis

Since the sample size was not equal at different stations and under different conditions, we applied two-way nested ANOVA using Gaylor and Hopper conditions with Satterthwaite's approximation (Gaylor and Hopper, 1969) to compare the significance of difference in hydrographic variables and abundance of archaea and bacteria between different depths and between conditions (pre- and during-upwelling) at each station (Rohlf and James Sokal, 1981). Linear step up multiple regression models was applied to determine the influence (contribution, %) of hydrographic variables on the distribution of archaea and bacteria in the study area. The DGGE band patterns were compared using bionumerics software version 4.6 (Applied Maths USA). The software carried out a density profile analysis, detected the bands from each lane and calculated the relative contribution of each band to the total lane intensity. Numbers of operational taxonomic units (OTUs) in each sample were counted as a number of DGGE bands. Gels were cross-checked visually as well for a number of bands per lane. The relative intensity of each band was used to calculate the Shannon–Wiener diversity index (H' index). Cluster analysis of DGGE bands based on square root transformed community data matrix through Bray–Curtis similarity was performed.

3. Results

3.1. Environmental characteristics

We observed the signatures of upwelling in mud bank regions with the onset of southwest monsoon in early June 2014. This was characterized by a decrease in sea-surface temperature to below 28 °C in June, which extended to September (Fig. 2). Salinity

116 A. Anas et al. / Regional Studies in Marine Science 18 (2018) 113–121

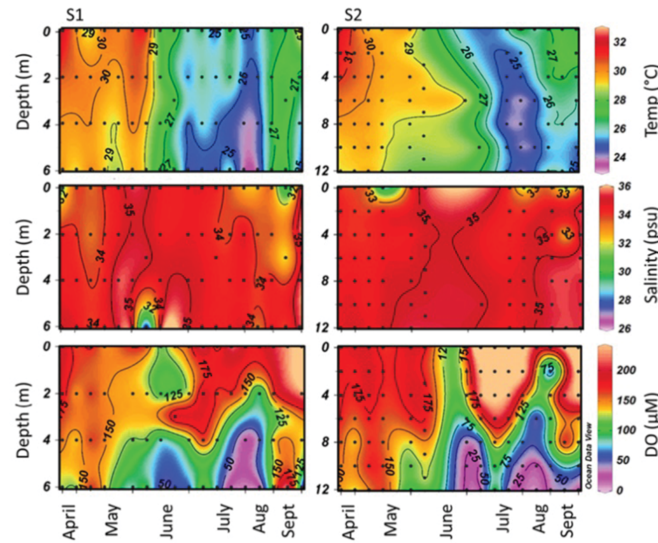

Fig. 2. Profile of temperature, salinity and dissolved oxygen in S1 and S2 during the study period.

increased with the onset of upwelling, while reductions in sea-surface salinity were observed in the latter stage when the wind became weak (Fig. 2). The low salinity pool of less than 30 psu observed in the bottom water (6 m) in early June at station S1 is erroneous and is attributed to the blinding effect of conductivity sensor of CTD when lowered into the fluid mud layer (Gireeshkumar et al., 2017). Winds were weak and directed towards the coast during April and May, while they acquired strength and were moving parallel to coast during June to July resulting in strong upwelling (Supplementary Fig. 1). During the late upwelling period (August and September), the wind weakened while remaining parallel to the coast. During April and May, the surface and bottom waters recorded more than 100 µM of dissolved oxygen, whereas the DO levels in bottom waters decreased with the upwelling in June (Fig. 2). Based on the pattern of temperature, salinity and DO, the study period was broadly classified into pre-upwelling and upwelling periods, and nutrient characteristics and microbial abundance were plotted accordingly. The Box–Whisker plot showing the distribution of ammonium, nitrite, nitrate, and phosphate during pre-upwelling and upwelling conditions in stations S1 and S2 are given in Fig. 3.

We observed a slight increase in the level of ammonium during upwelling at S2 ($P > 0.05$), while the increase was higher in the levels of nitrite, nitrate, and phosphate ($P < 0.05$, Table 1). Statistical analysis of nutrient levels in S1 did not depict any significant difference between surface and bottom ($P > 0.05$, Table 1) while temporal variations observed between pre- upwelling and during-upwelling periods were significant ($P < 0.05$, Table 1). All nutrient levels except ammonium in S2 showed significant spatial and temporal variations.

3.2. Abundance and community structure of archaea and bacteria

Box–Whisker plot showing the variation in the abundance of archaea and bacteria are given in Fig. 4. Archaeal abundance in S1 ranged from 1–16 × 10^4 cells ml^{-1}, at both pre- and during upwelling conditions, with an average cell count of 5.3 × 10^4 and

Table 1
Nested ANOVA analysis of the significance of variation in hydrographic variables and abundance of archaea and bacteria in stations S1 and S2. The variables were tested for different depths and between pre- and during upwelling conditions. The values in bracket indicate degrees of freedom of Snedecor's *F* statistic. *R* indicates constant determining Gaylor and Hopper condition. F_S indicates *F* statistic among depths and F_S' is *F* statistic among depths calculated when Gaylor and Hopper's condition is satisfied. F_{SA} is the *F* statistic calculated among pre- and during upwelling conditions.

Variables	F_S	F_{SA}	R	F_S'
Station S1				
Temperature	1.89(2,44)^{NS}	49.41(3,44)**	977.7	0.04(2,2)^{NS}
Salinity	1.14(2,44)^{NS}	1.06(3,44)^{NS}	20.9	1.07(2,2)^{NS}
DO	2.81(2,42)^{NS}	1.42(3,42)^{NS}	16.97	1.97(2,2)^{NS}
Ammonium	0.18(2,44)^{NS}	2.88(3,44)*	56.95	0.06(2,2)^{NS}
Nitrite	0.89(2,44)^{NS}	4.24(3,42)*	83.98	0.20(2,2)^{NS}
Nitrate	3.03(2,44)^{NS}	6.04(3,44)**	119.61	0.48(2,2)^{NS}
Phosphate	0.18(2,44)^{NS}	12.18(3,44)**	241.014	0.01(2,2)^{NS}
Archaea	1.83(2,44)^{NS}	3.17(3,44)*	75.52	0.56(2,2)^{NS}
Bacteria	1.10(2,49)^{NS}	3.01(3,44)*	62.51	0.35(2,2)^{NS}
Station S2				
Temperature	1.79(4,47)^{NS}	19.67(5,47)**	9975.2	0.09(4,4)^{NS}
Salinity	7.37(4,47)**	0.24(5,47)^{NS}	121.71	30.70(4,4)^{NS}
DO	28.54(4,61)**	8.89(5,61)**	126.18	3.01(4,4)^{NS}
Ammonium	1.03(4,60)^{NS}	1.20(5,60)^{NS}	14.65	0.84(4,4)^{NS}
Nitrite	6.17 9(4,60)**	2.73(5,60)*	41.76	2.16(4,4)^{NS}
Nitrate	3.39(4,61)*	7.33(5,61)**	104.10	0.43(4,4)^{NS}
Phosphate	3.729(4,58)**	3.54(5,61)**	50.19	0.99(4,4)^{NS}
Archaea	1.33(4,58)^{NS}	1.07(5,58)^{NS}	14.39	1.24(4,4)^{NS}
Bacteria	0.71(4,62)^{NS}	4.81(5,62)**	45.75	0.14(4,4)^{NS}

NS means not significant.
* The level of significance is indicated as significant ($P < 0.05$).
** The level of significance is indicated as highly significant ($P < 0.01$).

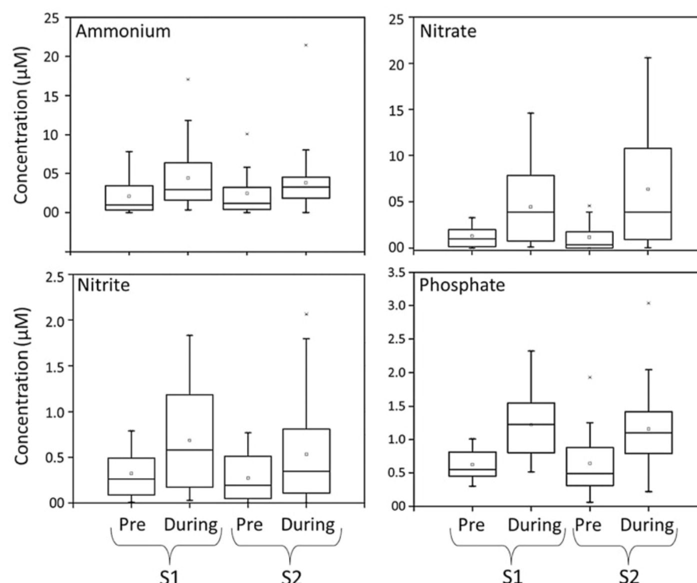

Fig. 3. Box–Whisker plots showing the quartile deviation in the level of ammonium, nitrite, nitrate, and phosphate in the stations S1 and S2 at pre- and during upwelling conditions.

6.6×10^4 cells ml^{-1} respectively. In S2 also, the archaeal abundance ranged from 1–16 $\times$ 10^4 cells ml^{-1}, during both pre-upwelling and during upwelling conditions, with an average cell count of 5.4×10^4 and 6.2×10^4 cells ml^{-1} respectively ($P > 0.05$, Table 1). The bacterial abundance showed a six- and four-fold increase respectively for S1 and S2 during upwelling compared with that of pre-upwelling condition ($F_{(5,62)} = 4.81$, $P < 0.05$, Table 1). In S1, the bacterial abundance during pre-upwelling condition ranged from 0.5–4.5 $\times$ 10^5 cells ml^{-1} with an average of 2.97 $\times$ 10^5 cells ml^{-1}. These values showed significant increase at during upwelling ($F_{(3,44)} = 3.17$, $P < 0.05$, Table 1), with bacterial abundance ranging from 0.7 to 72×10^5 cells ml^{-1} with an average of 16.5×10^5 cells ml^{-1} ($F_{(3,44)} = 3.01$, $P < 0.05$, Table 1). At S2, the range in abundance of bacteria increased from 0.7 to 6.0 $\times$ 10^5 cells ml^{-1} during pre-upwelling to 0.5 to 72 $\times$ 10^5 cells ml^{-1} at during upwelling condition, and the average increased from 2.3 $\times$ 10^5 to 9.4 $\times$ 10^5 cells ml^{-1}.

In all the cases the bacterial abundance was approximately ten times higher than that of archaea. Statistical analysis showed significant variation in the abundance of archaea and bacteria at S1 ($P < 0.05$, Table 1) between pre-upwelling and during upwelling periods, while such a difference was observed only for bacteria at S2 ($P < 0.05$, Table 1).

The community structure of archaea and bacteria, at pre- and during upwelling conditions was analyzed using the PCR–DGGE technique. Since there was no statistically significant difference in the abundance of bacteria between samples from the surface to bottom ($P > 0.05$, Table 1), we pooled the samples. The community structure of archaea and bacteria were analyzed in samples collected during May, June, July, August, and September. About four to six bands were present in DGGE gel of archaea (Supplementary Fig. 2), which did not show significant variations between pre-upwelling and during upwelling conditions (Fig. 5). Shannon–Wiener diversity index for archaea ranged from 1.1 to 1.5 in both S1 and S2. Nine to fourteen bands were observed in the bacterial DGGE of S1 and S2.

The dendrogram of cluster analysis prepared based on DGGE band pattern showed that the community structure of bacteria varied during different months (Fig. 5). In both S1 and S2, the bacterial DGGE band pattern of samples collected during May (Pre-upwelling) and September (end of upwelling) formed one cluster (Bray–Curtis similarity index up to 60%), while other samples formed a separate cluster (Bray–Curtis similarity index up to 70%). Shannon–Wiener diversity index for bacteria in S1 and S2 at pre-upwelling was 2.39 and 2.15 respectively, while it ranged from 1.89 to 2.35 and 2.18 to 2.58 respectively during upwelling indicating a more stable and less dynamic environment during the pre-upwelling period at both mud bank and non-mud bank stations.

3.3. Environmental influence on abundance of archaea and bacteria

Linear multiple regression models were employed to explain the role of hydrographic variables and their first-order interaction effects on the distribution of archaea and bacteria at S1 and S2. During pre-upwelling condition, the distribution of archaea in mudbank station S1 was influenced by temperature ($r = 0.253$), salinity ($r = -0.566$), DO ($r = -0.458$), nitrate ($r = 0.0026$) and ammonium ($r = -0.278$). The individual effect of these hydrographic variables along with nitrite ($r = -0.366$) and phosphate ($r = -0.468$) could explain the variation in archaeal distribution at 32. 91% confidence. Further, the variation in archaeal distribution could be explained at 66.5% confidence by considering the interaction effect of the above five hydrographic variables ($F_{(15,5)} = 3.6478$, $P < 0.05$, $N = 21$) among which interacting effect

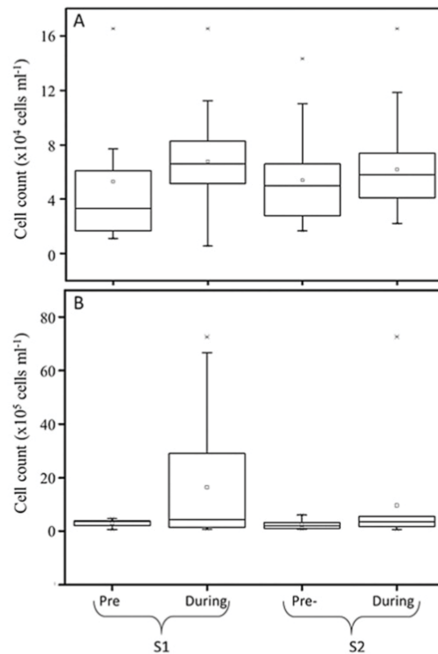

Fig. 4. Box–Whisker plot showing the quartile deviation in the abundance of archaea (A) and bacteria (B) in the stations S1 and S2 at pre- and during upwelling conditions.

of salinity & nitrate (26.73%), temperature & nitrate (25.76%) and nitrate & ammonium (3.56%) are the significant ones. Archaeal abundance in S1 during upwelling conditions were also influenced by individual and interaction effects of temperature, salinity, DO, nitrite, nitrate, ammonium, and phosphate. On an individual scale, the archaeal abundance was negatively influenced by temperature ($r = -0.2530$), DO ($r = -0.3273$) and nitrate ($r = -0.0904$), while salinity ($r = 0.2610$), nitrite ($r = 0.2169$), ammonium ($r = 0.115$) and phosphate ($r = 0.0745$) showed positive influence. About 67.01% of the temporal variability of archaea in S1 at during upwelling condition could be explained by modeling using the individual and interaction effect of the seven hydrographic variables listed above ($F_{(28,2)} = 3.1764, P < 0.05, n = 31$). The major interactions which contributed to the distribution of archaea in S1 were: DO & phosphate (8.77%), nitrite & phosphate (8.13%), temperature & nitrite (7.98%), salinity & phosphate (5.83%), salinity & nitrite (5.3%) and temperature & ammonium (4.1%).

The temporal variation in the distribution of archaea in S2 at pre-upwelling conditions also could be explained to 56.3% ($F_{(15,13)} = 3.4$), $P < 0.02, n = 29$), contributed largely by the interaction effects of temperature & nitrite (12.99%), Salinity & nitrite (10.78%), DO & nitrite (10.21%), salinity & ammonium (6.92%), temperature & DO (4.21%), nitrite & ammonium (3.74%), and temperature & ammonium (2.38%). On the other hand, only 8% of the variability could be explained when the effect of hydrographic variables is considered individually. Interestingly, step up multiple regression models could not explain the distribution of archaea in S2 at during upwelling conditions at a significant level.

When the individual contributions were considered, only 22.9% of the temporal variability of bacterial distribution ($F_{(7,13)} = 1.8484, P < 0.16, n = 21$) in S1 station at pre-upwelling conditions were controlled by temperature ($r = 0.34$), salinity ($r = -0.28$), DO ($r = -0.053$), nitrite ($r = 0.086$), nitrate ($r = -0.046$), ammonium ($r = -0.35$) and phosphate ($r = -0.29$). On the other hand, 96.46% of the temporal variability in bacterial distribution ($F_{(15,5)} = 37.6634, p < 0.01, n = 21$) at S1 could be explained with the step up multiple regression model. The major contributing interaction effects include nitrite & ammonium (31.62%), DO & nitrite (3.34%) and salinity & ammonium (3.23%). The hydrographic variables such as salinity, DO, nitrite, nitrate, ammonium, phosphate and their first order interaction effects could explain only 41.4% of the temporal variability of bacteria ($F_{(21,9)} = 2.01, P < 0.05, n = 31$) in S1 during-upwelling condition. The hydrographic variables phosphate ($r = -0.547$), nitrite ($r = -0.467$) and salinity ($r = -0.121$) influenced the bacterial distribution negatively while temperature ($r = 0.369$) and DO ($r = 0.3354$) imparted positive influence.

At pre-upwelling conditions, the temporal variability of bacterial abundance ($F_{(21,7)} = 2.5417$, p, 0.10, $n = 29$) at S2 could be explained with 53.63% confidence by step up regression model. The interaction effects of salinity & nitrite (23.42%), salinity & phosphate (9.14%), DO & nitrite (7.4%) and salinity (4.12%) were identified as the significant hydrographic variables to be considered. The hydrographic variables such as salinity ($r = -0.1190$), nitrite ($r = -0.0327$), nitrate ($r = -0.1412$) and phosphate ($r = -0.2073$) imparted negative influence, while temperature ($r = 0.254$), DO ($r = 0.217$) and ammonium ($r = 0.31$) imparted positive influences on bacterial abundance. It was complicated to explain the distribution of bacteria in S2, at during upwelling conditions, and only 28.19% ($F_{(7,35)} = 3.36, P < 0.008, n = 43$) of the temporal variability in the bacterial distribution could be explained by the individual variables. This was further improved to 49.92% with the contributions of temperature, salinity, DO, nitrite, ammonium and their first order interactions ($F_{(15,27)} = 3.79, P < 0.001, n = 43$).

4. Discussion

Results of the present study provide information, for the first time, on the influence of upwelling on the abundance and community structure of archaea and bacteria in a recurring mud bank in the coastal waters of Alappuzha, southwest coast of India. The southwest coast of India is strongly influenced by monsoons (Gupta et al., 2015). Wind-driven upwelling occurs along a broad region parallel to the coast during the summer monsoon, which brings cold and nutrient-rich waters to the surface. The first signals of upwelling in the surface waters of the southwest coast of India have been recorded in May, which intensifies during June–July and withdraws completely and abruptly by October (Gupta et al., 2015). Mud banks are another unique phenomenon occurring on the southwest coast of India during the same period, characterized by clearly demarcated areas of calm water even during the roughest monsoon (Dinesh Kumar, 2016). The sampling stations S1 and S2, selected in the present study, are placed in the upwelling path. The station, S1 is within a recurring mudbank and hence is influenced by multiple oceanographic processes such as mudbank and upwelling. The station S2 is influenced only by upwelling. The observations in the present study started in April, before the occurrence of intense upwelling (pre-upwelling), and extended till the withdrawal of upwelling in September. The signals of upwelling were observed since June, characterized by the reduced surface temperature. Dissolved oxygen showed an interesting pattern during the study period, with decreasing values at surface and bottom

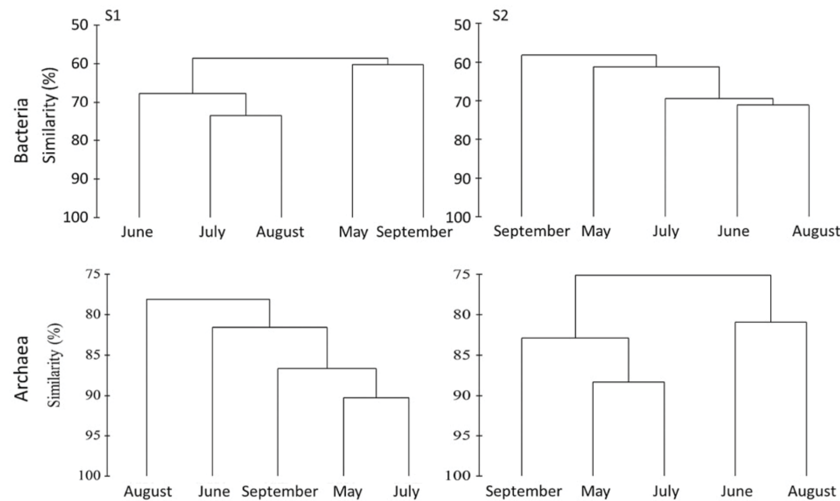

Fig. 5. Brey–Curtis similarity cluster based on DGGE pattern of archaea and bacteria in S1 and S2 during pre- (May) and during-upwelling (June–September) conditions.

waters during upwelling due to the incursion of hypoxic waters. The transportation of hypoxic waters to Alappuzha mud banks during southwest monsoon at varying levels depending on local winds have been discussed recently by Gireeshkumar et al. (2017). The nutrient levels also increased concurrently. Mudbank formation may not have a significant role in nutrient enrichment as the same trend was observed in both the stations. This was supported by a previous report by Jacob and Qasim (1974), who reported that mud banks in Alappuzha are formed by re-suspension of sediment particles with poor nutrient content. The seasonal fertilization due to upwelling and subsequent increase in productivity have been reported extensively from the coastal upwelling regimes located along the eastern boundaries of Pacific and Atlantic ocean basins (Anabalon et al., 2014; Antonio Cuevas et al., 2004; Capone and Hutchins, 2013; Vergas et al., 2007). There are only limited reports from coastal regions along the southeast Arabian sea on upwelling and associated changes in nutrient characteristics and food web (Habeebrehman et al., 2008; Malik et al., 2015). In both these studies, upwelling is associated with high nitrate concentrations. We also observed an increase in the level of oxidized nitrogen species (nitrite and nitrate) and phosphate during upwelling.

During upwelling, nutrient-rich waters are physically transported to the surface, which initiates a cascade of energy transfer process from microorganisms to other organisms in a higher level of food web through phytoplankton and zooplankton (Postel and Packard, 2014). However, our understanding of the influence of upwelling on abundance and community structure of microorganisms are limited. We observed a significant increase in the abundance of bacteria during upwelling. The bacterial abundance found in the study area at pre-upwelling and during upwelling conditions were in agreement with previous reports from elsewhere on the Kerala coast (Malik et al., 2015) and other upwelling waters (Barbosa et al., 2001). In a two-year data sets, Tsai et al. (2010) observed the higher abundance of bacteria during upwelling and suggested the more pronounced influence of available nutrients than the temperature in subtropical Ilan Bay in Taiwan. A coupling between available nutrients and temperature in determining the bacterial distribution in Cabo Frio upwelling region in Brazillian coast was also suggested by Coelho-Souza et al. (2015). An increase in the abundance and growth rate of prokaryotes in response to temperature has been reported earlier from coastal waters of the temperate and subtropical zone (Tuyet et al., 2015). Chen et al. (2012) reported a unimodal relationship between bacterial abundance and temperature in the South China Sea using generalized additive models. Although many studies reported the individual effect of nutrients on the distribution of microorganisms in the marine environment, the interaction effects are rarely discussed (Sneha et al., 2016). The linear step up multiple regression models used in the present study indicates that the interaction effects of nutrients have a more pronounced influence on the distribution of archaea and bacteria in the system than the individual nutrients. For example, the confidence level of explaining bacterial distribution in S1 station at pre-upwelling condition increased from 22.9% to 96.5%, when the individual influence of hydrographic variables was replaced with their interaction effect. However, the step up regression modeling using interaction effects of hydrographic variables monitored in the study were also not successful in explaining the distribution of archaea or bacteria at during upwelling condition to more than 70% confidence. The percentage of the temporal variation in bacteria distribution at station S1 that can be explained by the regression model decreased from 96.5% in pre-upwelling conditions to 41.4% during upwelling conditions. This clearly indicates the existence of complex characteristics of the system during upwelling. The major limitation of the present model is that we did not include the bacterivory and viral lysis, which can also play a significant role in regulating bacterial distribution (Chen et al., 2012). A coupled physical–biogeochemical model would give a more realistic view of the complex interactions taking place during upwelling. Interestingly, the abundance of archaea remained unchanged before (pre-upwelling) and during upwelling condition in S2, while it shifted slightly during upwelling in S1. Also, the diversity of archaea was found to be less compared to bacteria, which is in agreement with the observations from tropical upwelling regions of the Brazilian coast (Cury et al., 2011).

The mud banks, being calm and hypoxic water bodies, may function as the bioreactor for the mineralization of organic matter brought up by upwelling. The pileup of organic matter was indicated by high levels of Chlorophyll-a during upwelling (7–8 mg m^{-3}) compared to pre-upwelling (3–4 mg m^{-3}) condition (Madhu et al., 2015). The increase in substrate availability enhances the abundance and activity of microorganism (Antonio Cuevas et al., 2004). It was perceived earlier that the conditions such as high organic load and upwelling induced suboxic/anoxic conditions across the coastal waters of India are conducive for the biogenic production of methane (Jayakumar et al., 2001). However, an abrupt reduction in methane during upwelling was reported recently in the mud banks of Alappuzha (Gireeshkumar et al., 2017). The recurrence of hypoxic water with the accumulation of nitrite and ammonium in highly productive systems like mud banks sets a favorable condition for enriching anaerobic microorganisms. This may be discussed in line with the observed shift in the community structure of archaea and bacteria during upwelling. The two clusters of the bacterial community, i.e cluster 1 (May and September) and cluster 2 (June, July, and August), strongly suggest the existence of a transient population of bacteria during upwelling which participates actively in degradation of detrital organic matter. The community structure returns to the native stage after the process, and accordingly the bacterial community structure in May and September clustered with nearly 60% similarity. A community shift of bacteria due to upwelling was also reported from the Brazilian coast (Cury et al., 2011). It is evident in the literature that different groups of bacteria thrives in different depths and nutrient conditions in the sea (Cury et al., 2011; DeLong et al., 2006; Massana et al., 1997; Murray et al., 1998). Under the hypoxic conditions prevailing over a mud bank, it is possible that the oxygen-dependent conversion of ammonium and methane could be shifted to a nitrite dependent anaerobic methane (n-damo) and ammonium (anammox) oxidation. It is proved in closed system experiments that the anaerobic reactions are more efficient compared to oxygen-dependent reactions (Ni et al., 2013) and releases fewer amounts of greenhouse gases. This could be a possible reason for reduced methane over a mud bank during upwelling observed by Gireeshkumar et al. (2017), and if so mud banks could function as a sink of greenhouse gases like methane.

5. Conclusions

In short, the observation of the present study indicates the influence of upwelling on the abundance and community structure of archaea and bacteria in a persistent mud bank region in the southwest coast of India. Step up multiple regression models shows that the microbial distribution during upwelling is controlled by the interaction effects of different hydrographic factors. Further studies may focus on understanding the influence of upwelling on microbial biogeochemistry in mud bank ecosystems, and their impact and climate change.

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Conflict of interest

All authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found online at https://doi.org/10.1016/j.rsma.2018.01.006.

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Heavy Metals Pollution Influence the Community Structure of Cyanobacteria in Nutrient Rich Tropical Estuary

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Abstract

Anthropogenic inputs influence the community structure and activities of microorganisms, which may impinge the functioning of estuarine and coastal ecosystem. The aim of the present study was to understand the influence of dissolved heavy metals (Cr, Mn, Fe, Ni, Cu and Zn) on community structure of cyanobacteria in a nutrient rich tropical estuary, Cochin Estuary (CE), across the southwest coast of India. Dissolved heavy metals were higher in CE during dry season, with Zn as major pollutant. The level of dissolved Zn was higher in stations near river head during dry (130 µg L^{-1}) and wet seasons (105 µg L^{-1}). The community structure of cyanobacteria was higher in CE during wet season (H' index: 1.4 to 3.1) compared to that in dry season (H' index: 1.1 to 2.2). The 16S rRNA gene sequence analysis showed the prevalence of *Synechococcus* sp in CE. PCA analysis indicated the positive influence of Zn and nutrients on community structure of cyanobacteria. We postulate that the Zn pollution may be a critical factor to be monitored and controlled in nutrient rich estuaries, otherwise it may accelerate the eutrophication and associated ecological constraints.

Keywords: Heavy metal; Cochin estuary; Eutrophication; Cyanobacteria; Phytoplankton

Introduction

Influence of heavy metals on the community structure and activities of microorganisms in aquatic systems, varies depending on their concentration and available chemical forms [1,2]. At optimum concentration, heavy metals like Cu, Zn and Mn support major life processes such as maintenance of osmotic balance, stabilization of cellular organelles, they function as electron acceptors in many processes and cofactors for metallo-enzymes [3-5]. The heavy metal pollution can induce long lasting effects on health of estuaries as they are not easily or rapidly eliminated from these ecosystems by natural degradative processes. Estuaries, being the transition zone between fresh- and marine-water systems, function as receiving points of heavy metals and other pollutants of anthropogenic origin and regulate their supply to the coastal environment. Heavy metal pollution in estuaries has increased significantly in the recent past through enhanced discharge of industrial and domestic wastes [6-9]. Previous studies showed that heavy metal pollution causes unintended alterations in the functioning of estuarine ecosystems [10-13]. The direct impact of heavy metal pollution on microbial ecosystem includes the alterations in the physiology, diversity and abundance of microorganisms, which indirectly affect the biogeochemical cycles and ocean productivity [10-13].

Cyanobacteria are the major picoplankton distributed in estuarine environment, where they occupy a key position at the base of the food web and play a central role in carbon, nutrient and oxygen cycling [14]. The cyanobacteria *Prochlorococcus* and *Synechococcus*, alone are responsible for an estimated 20-40% of global carbon fixation, although they account for only one percent of photosynthetic biomass [15]. In spite of this, the effect of metal pollution on diversity of cyanobacteria is least studied from estuarine environments and most of the reported studies on heavy metal–cyanobacteria interactions have been conducted in small volume culture flasks in laboratories [2]. Field level observations that include the whole microbial food web are important to understand the impact of heavy metal pollution on cyanobacterial diversity. In a pristine environment heavy metals are transported across the cell membrane through energy independent or dependent pathways to participate in cellular processes. If this open gate condition pertains under polluted environment, heavy metals may accumulate in cytoplasm leading to toxic effects at cellular and subcellular level. Interestingly, microorganisms acquired several mechanisms to maintain the homeostasis between the available metal concentration and microbial physiology [12,16,17]. In oligotrophic marine environments, cyanobacteria secrete ligands that bind with biologically important metals like copper, iron, cobalt and zinc [18]. The laboratory scale experiments showed that trace metals above optimum levels can influence the general growth, pigment composition, photosynthesis and enzyme expression of cyanobacteria [19]. Several strains of cyanobacteria have shown the ability to accumulate, detoxify or metabolize heavy metals up to certain concentration range [20]. Cyanobacteria are effective biological metal sorbents, which represent an important sink for metals in aquatic environments and have the ability to control the speciation of many biologically active metals [20-22]. Although metal accumulation is a promising strategy for the survival of cyanobacteria in metal polluted environments, their impact on the food web will be disturbing.

Cochin Estuary (CE) is the largest wetland ecosystem along the southwest coast of India, covering an area of ~25600 ha, extending from 9° 30'-10° 12' N to 76° 10'-76° 29' E. CE is reported to be nutrient rich, and its composition is greatly influenced by anthropogenic and terrestrial inputs from six rivers, seawater influx from two bar mouths and prolonged monsoon [23]. CE also receives high concentrations of industrial effluents (104 × 10^3 m^3 per day) and untreated domestic

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waste (260 m^3). Like most estuaries, the anthropogenic contribution of heavy metals has increased significantly in CE over the past three decades [9]. However, the influence of heavy metal pollution on diversity of photosynthetic microorganisms are not studied till date. In a previous study we reported the influence of heavy metal pollution on the diversity and enzyme expression profile of heterotrophic bacteria in CE [9]. In the present study, we report the influence of dissolved heavy metals on the community structure of cyanobacteria in CE.

Materials and Methods

Sample collection

Water samples were collected from four locations across a pollution gradient in CE using 5 L capacity Niskin samplers during dry (February) and wet (September) season. The positions of the sampling stations, S1, S2, S3 and S4 are given in Figure 1. S1 is situated near the Cochin bar mouth and is designated as least polluted station. S4 is designated as a grossly polluted station in this study and located near the point where the river Periyar, carrying effluents, joins the estuary. S2 and S3 are considered as intermediately polluted stations.

Analysis of environmental parameters

Environmental variables were measured following standard protocol. Salinity was determined using a Digi Auto Salinometer (Model TSK, accuracy ± 0.001) and the pH using an ELICO LI 610 pH meter. Dissolved oxygen content in the ambient water was determined following Winkler's titration method [24]. Samples for nutrients (ammonia, nitrite, nitrate, phosphate and silicate) were filtered and estimated spectrophotometrically within six hours of sampling [24].

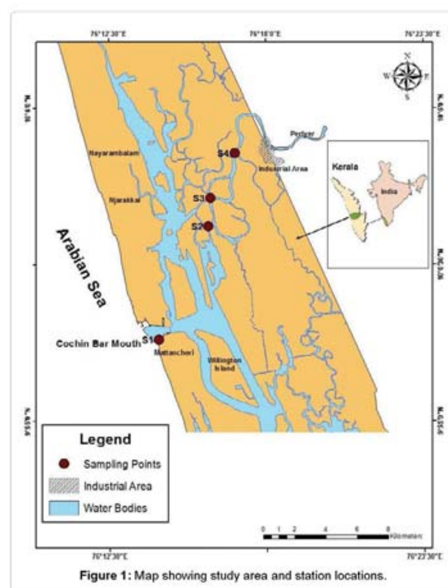

Figure 1: Map showing study area and station locations.

Suspended particulate matter (SPM) was collected on a pre-combusted 0.45 µ GF/F filter paper (Whatman, USA) and measured gravimetrically after achieving constant weight at 70°C.

Analysis of heavy metals

Water samples were analyzed for dissolved heavy metals, Cr, Mn, Fe, Ni, Cu and Zn, using inductively coupled plasma optical emission spectroscopy (ICP-OES) following standard protocol [24]. Heavy metals present in water samples (400 ml) were acidified using concentrated nitric acid. The dissolved metals were extracted using ammonium pyrrolidine dithiocarbamate (APDC, 2%) and chloroform at acidic pH, brought back to aqueous layer by back-extraction with concentrated nitric acid and made up to 20 ml with sterile di-ionized water. The precision and accuracy of the analytical procedure was monitored by the repeated analysis of standard reference material BCSS-1.

Community structure of cyanobacteria

The community structure of cyanobacteria in CE was studied using nested PCR-DGGE technique. Genomic DNA was extracted from water samples following standard protocol with slight modification [25]. Here the water samples (2 L) were passed through 0.2 µ polycarobonate membrane filter (Milliipore; GTTP2500), followed by incubation at 37°C for 1 hr in lysis buffer (NaCl 400 mM, Sucrose 750 mM, EDTA 20 mM and Tris HCl 50 mM) containing 1 $mgml^{-1}$ lysozyme. Subsequently, SDS (1%) and proteinase K (100 μgml^{-1}) were added to the solution and incubation was continued for 5 hr at 55°C. Further DNA was precipitated with 0.6 volume of isopropanol at -20°C for 60 min. DNA pellet was washed copiously with 70% ethanol, dissolved in TE buffer and stored at -20°C until used.

Approximately 2000 bp of 16S rRNA gene of cyanobacteria were amplified using primers 359F (GGGGAATTTTCCGCAATGGG) and 23S30R (CTTCGCCTCTGTGTGCCTAGGT). The genomic DNA was amplified with 10 picomol each of forward and reverse primers in a 25 µl reaction volume containing 1 µl of DNA, 2.5 µl 10 × Taq polymerase buffer (NEB, Canada), 0.5 U Taq DNA polymerase (NEB, Canada), and 200 µM each dNTP (Sigma Aldrich, USA). The reaction mixture was subjected to thermal cycling conditions as follows: initial denaturation at 95°C for 2 min, followed by cycle denaturation at 95°C for 40 sec, annealing at 58°C for 40 sec, and extension at 72°C for 15 sec for a total of 30 cycles. Nested PCR was done using cyanobacterial specific DGGE primers CYA359F and an equimolar combination of reverse primers, CYA781R (a) (GACTACTGGGGTATCTAATCCCATT) and CYA781R (b) (GACTACAGGGGTATCTAATCCCTTT), following PCR conditions of initial denaturation at 95°C for 2 min, followed by cycle denaturation at 95°C for 40 sec, annealing at 58°C for 40 sec, and extension at 72°C for 2 min for a total of 30 cycles. The resulting 400bp PCR products were run on a denaturing gradient gel (30-50% formamide) prepared in 8% polyacrylamide for 17.5 hr at constant voltage of 75 V in 1X TAE buffer (40 mM tris-HCl, 20 mM acetic acid, 1 mM EDTA). The bands formed in the gel were stained with SYBR green and observed in a gel documentation system (BioRAD, USA). The bands were picked, incubated overnight in a 50 µl TE and re-amplified. The PCR products were treated with EXOSAP-IT (Affymetrix, Ohio USA) and used as template for sequencing on an ABI sequencer. Sequence data obtained were analyzed and edited using Sequencher V4.10.1 (GeneCodes, USA). Subsequently, the sequences were compared with those in the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm to determine approximate phylogenetic affiliations. The nucleic acid sequences

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showing the closest similarities were used as Reference sequences while constructing a neighbor -joining tree using the software MEGA (version 5.0). DGGE approach will give an insight into the overall distribution of cyanobacteria in the CE. We acknowledge the limitation of DGGE which originated from efficiency of DNA extraction methodology, selective amplification of genes from mixed DNA samples and low resolution in the band pattern of phylogenetically related species. Maximum care was taken to keep these errors to the minimum possible levels. Despite these shortcomings, the PCR-DGGE approach has been proved to be a powerful method allowing a comprehensive picture of the community structure and constraints associated with it.

Statistical analysis

Difference in band pattern between stations was compared using Bionumerics software. The software carried out a density profile analysis, detected the bands from each lane and calculated the relative contribution of each band to the total lane intensity. Numbers of Operational Taxonomic Units (OTUs) in each sample were counted as number of DGGE bands. Gels were cross-checked visually as well as for number of bands per lane. The relative intensity of each band was used to calculate the Shannon Wiener diversity index (H' index). Cluster analysis of DGGE bands based on square root transformed community data matrix through Bray-curtis similarity were performed. Multivariate statistics, Principal Components Analysis (PCA), was carried out to understand the influence of environmental variables and heavy metals on DGGE band diversity of cyanobacteria. All statistical analysis were performed with the software Statistica (Version 6.0).

Results and Discussion

Environmental variables in CE

We observed higher levels of nutrients in CE during wet season (Table 1). Ammonia was the major nutrient in CE and its level ranged from 1.9 to 5.2 and 4.8 to 11.3 µmol L^{-1} respectively in dry and wet season. Nitrate (4.4. to 19.3 µmol L^{-1}), silicate (31 to 36.1 µmol L^{-1}) and phosphate (1.3 to 3.3 µmol L^{-1}) were also higher in the CE during wet season compared to dry season (nitrate 0.5 to 1.4, silicate 8.5 to 36.3 and phosphate 1.05 to 3.9 µmol L^{-1}). The level of dissolved nitrogen (sum of ammonia, nitrite and nitrate) are increased four to six times in the estuary during wet season. Nutrient levels are reported to be higher in tropical estuaries during wet season, and is attributed to increased river water discharge and rainfall associated with southwest monsoon [26-28]. CE receives 60–70% of its annual freshwater discharge i.e., 22.41×10^3 Mm^3 during southwest monsoon [29], which carries the nitrogen rich effluents discharged from fertilizer and fish processing industries [30]. It is estimated that 260 million litre per day of effluents are produced in the industrial belt of Cochin and a significant fraction of it reaches CE through river Periyar [31]. Also, 235 million litre per day of untreated sewage are generated in Cochin city and a significant fraction of this may reach the estuary through land runoff and other nonpoint sources [30]. CE has been recognized as a nutrient rich estuary and the nutrient levels are always maintained at sufficient levels to support the nourishment of phytoplankton [32]. The nutrient levels in the estuary are comparable with Godavari estuary, India [33], Carolina's Neuse river estuary (NRE) USA [34], Seine estuary in France [35] and Schelde estuary in Belgium [36]. Higher SPM levels in CE during dry seasons was attributed earlier to high seawater influx and mixing of water column [37]. The distribution of nutrients in the estuary are directed by seasonal changes in river water discharge and seawater influx. CE is dominated by seawater during dry season and its influence was recorded up to station S4 (salinity 15) (Table 1), while it remains limnetic during wet season due to heavy river water inflow and the influence of seawater was restricted at station S2 (salinity 9). The average river water discharge was reported to be minimum in CE from January to May in a year, it increases in June with the onset of southwest monsoon and reaches its plateau during August [29]. Accordingly the CE shifts between seawater and fresh water dominance in dry and wet season respectively.

Heavy metal pollution in CE

The concentrations of dissolved heavy metals in the water column of CE are given in Table 2. The dissolved heavy metals were higher in CE during dry season, which may be attributed to changes in flushing rate. Flushing activity would be inactive in Indian estuaries during dry season, while it experiences multiple flushing in addition to heavy rain fall during wet season [38]. In concurrence with this, the level of dissolved heavy metals were found higher during dry season and lower during wet season. Although the fresh water influx and industrial discharge are higher during wet season, the high flushing rate, i.e., one flushing in 1-2.5 day [39], will result in the dilution of dissolved heavy metals in CE during wet season. We recorded higher levels of Zn in stations near to the river head i.e., S3 and S4 during dry (48. 28 and 130.5 µgL^{-1} respectively) and wet season (73. 65 and 105.85 µgL^{-1} respectively). The S4 is located geographically close to the industrial belt of Cochin and functions as discharge point of effluents carried by river Periyar. The level of heavy metal pollution gradually reduces from S4 at river mouth to S1 at bar mouth, owing to its accumulation in sediment [9]. Earlier studies recorded accumulation of heavy metals in the sediment samples and benthic organisms collected from CE [9,23,40-42]. In a previous study, we recorded an 18 times increase in the level of Zn in sediments of CE during the past three decades [9]. Although the heavy metals accumulated in sediment samples can cause serious problems to both estuarine and human health, phytoplanktons are most affected by dissolved heavy metals. Since the purpose of this work was to understand the impact of heavy metal pollution on

	Environmental factors							
	Dry season				Wet season			
	S1	S2	S3	S4	S1	S2	S3	S4
pH	7.8	7.8	7.9	7.8	7.8	7.8	7.6	7.1
Salinity	34.5	32	8	15	30	9	0	0
SPM(mg L^{-1})	32.4	26.8	23.2	17.6	36	18	6.8	2.8
NH_3 (µmolL^{-1})	1.9	2.5	3.2	5.2	11.3	7.9	16.1	4.8
NO_2 (µmolL^{-1})	0.2	0.1	0.1	0.2	0.5	0.3	0.3	0.2
NO_3 (µmolL^{-1})	1	0.5	1.4	0.5	4.4	6	13.6	19.3
SiO_4 (µmolL^{-1})	10.3	8.5	36.3	22.6	31	56.7	35.4	36.1
PO_4 (µmolL^{-1})	1.9	1.6	1.05	3.9	3.3	2.1	2.4	1.3

Table 1: Environmental variables observed in Cochin estuary during the study period.

Heavy metal	Concentration (µg L^{-1}) at different stations							
	Dry season				Wet season			
	S1	S2	S3	S4	S1	S2	S3	S4
Cr	0.22	0.08	0.34	0.2	0.47	0.07	0.09	0.09
Cu	1.28	0.38	1.74	0.81	0.68	0.27	0.44	0
Fe	262.3	274.2	161.7	55.7	799	45.37	68.1	6.62
Mn	0.24	0.21	0.39	0.12	0.63	0.13	BDL	BDL
Ni	3.55	2.97	4.21	2.02	4.56	4.57	5.51	BDL
Zn	8.84	6.55	48.28	130.5	4.36	6.36	73.65	105.85

Table 2: Distribution of heavy metals in sampling stations during dry and wet season.

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community structure of cyanobacteria and the level accumulated in sediments are recorded in previous studies, only the dissolved heavy metals are monitored in the present study.

The level of Fe and Mn showed interesting fluctuations in CE. Although their levels were higher during dry season, maximum concentrations of Fe and Mn were recorded in S1 during wet season. The higher levels of Mn and Fe during dry season was reported earlier in CE [43], and was attributed to the higher salinity, which facilitates the formation of comparatively soluble Fe and Mn sulphides [44]. Under normal conditions, the heavy metals discharged to the estuary are immobilized in the sediment or accumulated in benthic organisms. Disturbing the system by either physical or chemical forces, i.e., resuspension of sediments by dredging operation or tidal mixing or adjusting the pH or redox conditions by discharging acidic wastes, may trigger the release of significant amount of heavy metals to the water column [45-47]. The resuspension of sediment due to continuous dredging operations could be the reasons for pulsed level of heavy metals in S1 during wet season. Previous studies placed CE among the most impacted estuaries in the world based on high levels of Zn, and recorded sediment as a sink of heavy metals [48].

Community structure of cyanobacteria in CE

We used standard PCR-DGGE technique to identify the community structure of cyanobacteria in CE. PCR DGGE have many advantages over morphological or culture dependent methods in analyzing the community structure of cyanobacteria. The morphological methods mainly depend on the size and pigmentation of cyanobacteria, which may vary with the nutrient compositions while culture dependent method is limited by the lack of a universal media which can accommodate all cyanobacteria. In PCR-DGGE, the PCR product of each species from the sample is segregated into distinct bands in a gradient gel [49]. Hence each band in a gradient gel can be considered as an operational taxonomic unit (OTU). Twenty representative DGGE bands were eluted, cloned in TOPO cloning vector and sequenced. The sequences were submitted to NCBI GenBank (accession numbers KP694048 – KP694067). The number of OTUs of cyanobacteria in the water column of CE was higher during wet season (4- 8) compared to dry season (2-4) (Figure 2). The Shannon Weiner diversity index calculated from the DGGE image also showed comparatively higher diversity of cyanobacteria in CE during wet season. During this season, maximum Shannon Weiner diversity index was observed in S4 (3.1), followed by S1 and S2 (1.8) and S3 (1.4). During dry season, maximum diversity of cyanobacteria was observed in S1 (H' 2.2) followed by S2 (H' 1.6). The lowest diversity of cyanobacteria was recorded in samples collected from S3 and S4 (H' 1.1) during dry season. Based on the similarity of PCR–DGGE band pattern, the stations were clustered differently during dry and wet season (Figure 2). During dry season, S3 remained as a different cluster while other stations showed nearly 40% similarity in DGGE band pattern. The dissimilarity in cyanobacterial diversity may be due to the difference in both environmental variables and heavy metals at different stations in the estuary. DGGE band pattern of S1 and S2 showed more than 60% similarity during wet season, while S2 and S3 remained different clusters. The phylogenetic tree derived from PCR-DGGE analysis showed the presence of *Synechococcus* sp in all the stations (Figure 3). This is in agreement with a previous culture dependent study from CE [50]. A recent study based on pigment fluorescence also confirmed *Synechococcus* sp as a dominant picoplankton in CE [51]. *Synechococcus* sp has been reported from many estuaries and bays [52,53] and its bloom has been reported

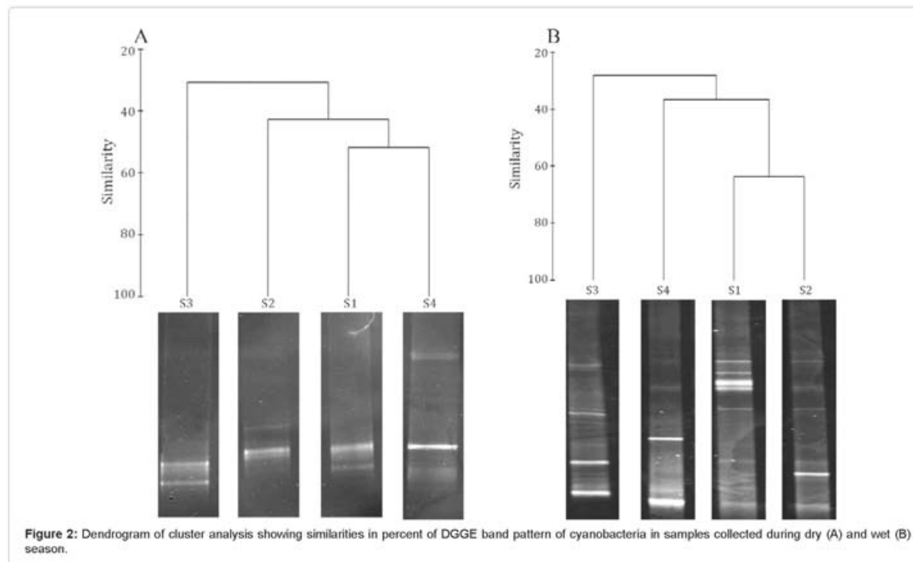

Figure 2: Dendrogram of cluster analysis showing similarities in percent of DGGE band pattern of cyanobacteria in samples collected during dry (A) and wet (B) season.

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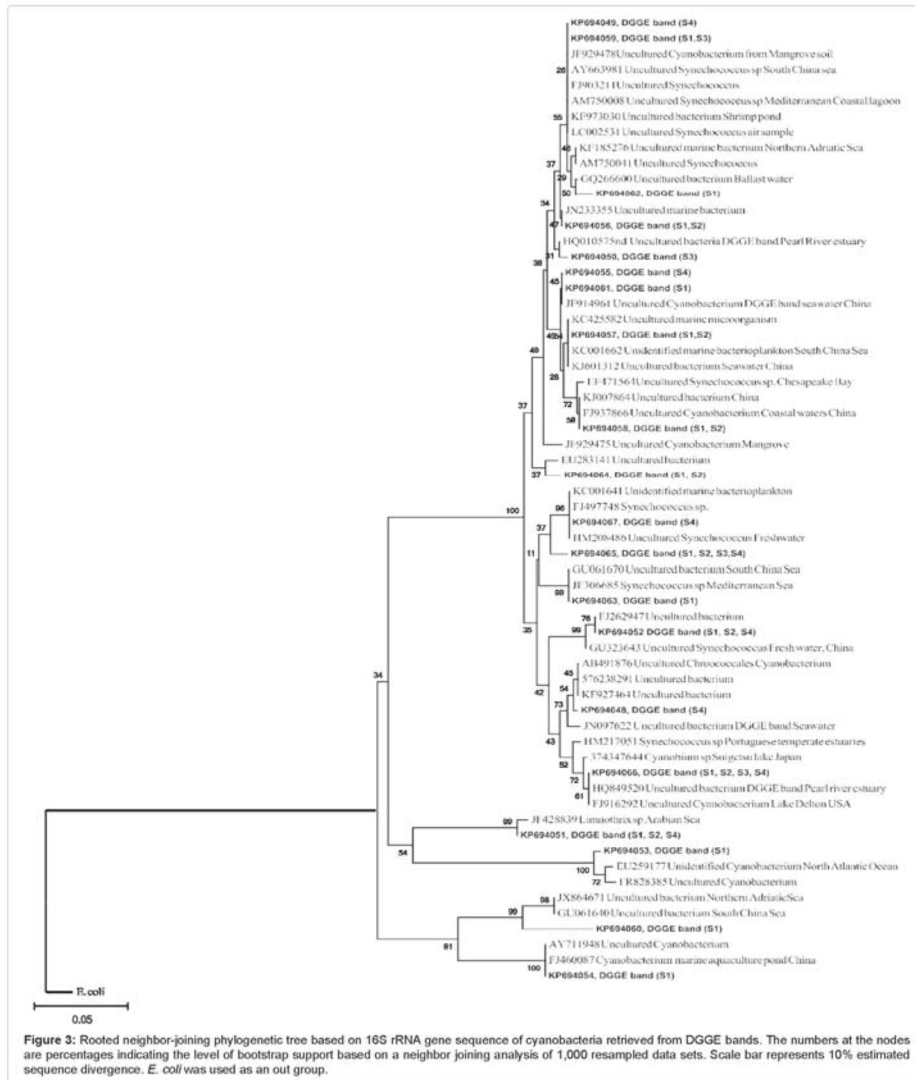

Figure 3: Rooted neighbor-joining phylogenetic tree based on 16S rRNA gene sequence of cyanobacteria retrieved from DGGE bands. The numbers at the nodes are percentages indicating the level of bootstrap support based on a neighbor joining analysis of 1,000 resampled data sets. Scale bar represents 10% estimated sequence divergence. *E. coli* was used as an out group.

in Chesapeake Bay during summer when the salinity was high [52]. The *Synechococcus* sp has been recorded as a dominant contributor of biomass and phytoplankton productivity in nutrient rich North Carolina's Neuse River Estuary (NRE) [34]. Our study also confirm the prevalence of *Synechococcus* sp in nutrient rich CE.

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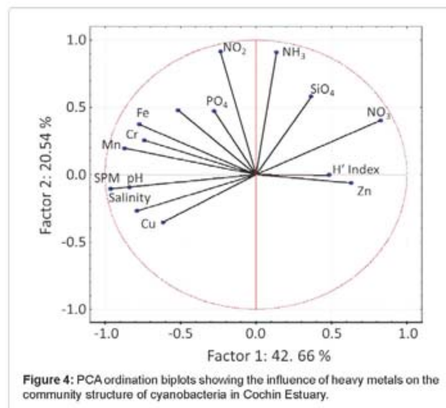

Figure 4: PCA ordination biplots showing the influence of heavy metals on the community structure of cyanobacteria in Cochin Estuary.

Here we used three 16S rRNA gene primers specific for cyanobacteria Cya359F, Cya781R (a) and Cya781R (b), for the assessment of community structure of cyanobacteria in CE. The reverse primer Cya781R(a) preferentially targets filamentous cyanobacteria whereas Cya781R(b) targets unicellular cyanobacteria [49]. Interestingly, the 16S rRNA gene sequence derived from majority of bands (13 number) were matched with previously uncultivated cyanobacteria sequence data available in NCBI. This happens because a majority of the cyanobacteria are still uncultivated in laboratory condition and hence its taxonomical positions are not assigned completely. This indicates the need for further studies in isolation and taxonomical classification of cyanobacteria in marine environments.

Effect of heavy metal pollution on community structure of cyanobacteria

The PCA analysis showed that the community structure of cyanobacteria in CE was influenced by the levels of heavy metals and nutrients (Figure 4). Ammonia and nitrate are reported as the preferable source of nitrogen for natural phytoplankton communities in estuaries and their influence on phytoplankton productivity is well documented from different estuaries across the world [54-57]. CE is reported as a nutrient rich estuary and the nutrients are always maintained at sufficient levels to support the growth of phytoplankton [32,58]. Therefore the variations in level of dissolved Zn may function as the selective pressure on community structure of cyanobacteria in CE. Previous studies also supported our hypothesis that availability of Zn in the water column influences the community structure of cyanobacteria in marine and estuarine environments [59,60]. Zn is required at the active site of carbonic anhydrase, as it catalyses the conversion of HCO_3^- to CO_2, and hence plays a pivotal role in photosynthetic carbon synthesis [61]. The available concentration of Zn is very low in many parts of the oceanic environment compared to that of estuaries [59] and the Zn absorption mechanisms of oceanic cyanobacteria are well reviewed [15,60]. However, very little is known about the response of cyanobacteria to elevated Zn levels in the estuarine environment. It is possible that up to certain levels the Zn promotes the growth and activities of cyanobacteria, but different cyanobacteria will have varying tolerance limits. Further mesocosm studies are therefore required to understand the tolerance limit of different groups of cyanobacteria present in CE to elevated Zn. Mesocosm studies using *Synechococcus* PCC 7942 demonstrated the synthesis of a 56 aminoacid cysteine rich metal binding protein, SmtA, which binds and sequesters metal ions within the cells and thereby maintains the homeostasis between the metal concentration and cyanobacterial viability [61]. Beyond tolerable limits, the Zn may out compete less competitive metal ions such as Fe and Mn for their protein binding sites [18]. In a mesocosm study, it was reported that Zeaxanthin level in the water collected from Godavari River decreased with increasing Zn concentration from 1×10^{-7} to 2.5×10^{-7} M [62]. We observed maximum diversity of cyanobacteria in CE at S4 during wet season, when the levels of nitrate, silicate and Zn were high. Although the Zn level increased further at S4 during dry season, the diversity of cyanobacteria was low, which may be due to the comparatively low levels of nitrate and silicate at that time or due to the toxicity of Zn. High levels of primary productivity and chlorophyll contents were also reported from S4 during wet season (i.e., June to September), and was attributed to high enormous inputs of nutrients [63].

Conclusion

Although our results are specific with CE, the influence of Zn pollution on community structure of cyanobacteria and its implications regarding environmental impacts are of significant to tropical estuaries. In CE the Zn levels are high near the river end and is attributed to the anthropogenic input. *Synechococcus* sp was recorded as the dominant cyanobacteria in CE. The present study postulates that the anthropogenic inputs of Zn may impart critical control on the community structure of cyanobacteria in nutrient rich tropical estuaries like CE. In short, the present study points towards the importance of controlling Zn pollution in CE, as it may otherwise impinge the community structure of cyanobacteria, which may leads to unintended alterations in the functioning of estuarine ecosystem. Further studies to elucidate the tolerance limit of cyanobacteria to different heavy metals and the influence of metal pollution on photosynthetic efficiency of cyanobacteria will be appreciated.

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