

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

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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 to 9.50 $\times 10^7$ and 2.73 to 3.85 $\times 10^7$ cells L^{-1} respectively for the wet and dry season, while that of PM-bacteria was between 1.14 and 6.72 $\times 10^8$ cells L^{-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 remineralisation in the system arresting the food web dynamics and biogeochemical cycles.

Keywords: Particulate matter, Heavy metals, Particulate associated bacteria, Archaea, Estuary

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 (Garneau 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 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 Figure 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 (100ml) 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 hr) GF/F filters of 0.7µm 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)-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 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 (Elementar Vario 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 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-Ciocalteu 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 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-HClO₄- HNO₃ suspended 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 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 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 insitu hybridisation (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 (GTGCTCCCCGCCAATTCCT) (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 (50ng) and a mixture of 0.9 M NaCl, 20mMTrisHCl(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 20mMTrisHCl, 5mM EDTA, 0.01% SDS, 5M 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 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 $^{\circ}\text{C}$ for 1 hr in lysis buffer (NaCl 400 mM, Sucrose 750 mM, EDTA 20 mM and TrisHCl 50 mM), containing 1 mg ml^{-1} lysozyme. Subsequently, SDS (1 %) and proteinase K (100 $\mu\text{g ml}^{-1}$) were added to the solution and continued incubation for 5 hr at 55 $^{\circ}\text{C}$. Further 0.6 volume of isopropanol was added and DNA was precipitated by keeping at -20 $^{\circ}\text{C}$ for 60 min. DNA pellet was washed copiously with 70 % ethanol, dissolved in TE buffer and stored at -20 $^{\circ}\text{C}$ until used. The sample filters from station 4 (collected from the Eloor region) were pre-treated with 1 ml of 10mMTrisHCl and 40mM Na₂EDTA (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 hr at a constant voltage of 220 V in 1× TAE buffer (40 mM TrisHCl, 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 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 1-4 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 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 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 ($10622 \mu\text{g g}^{-1}$) and S4 ($10923.1 \mu\text{g g}^{-1}$) during the wet season. The level of PM-Fe collected from S3 and S4 during dry season were 1210.8 and $4116.7 \mu\text{g g}^{-1}$ respectively. The level of PM-Fe in S1 was $7827.1 \mu\text{g g}^{-1}$ during the wet season, while it decreased to $2130.6 \mu\text{g g}^{-1}$ 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 $22.69 \mu\text{g g}^{-1}$ respectively, with a maximum concentration of $22.69 \mu\text{g g}^{-1}$ 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 ($2439.49 \mu\text{g g}^{-1}$) and lowest ($21.27 \mu\text{g g}^{-1}$) 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 $109.67 \mu\text{g g}^{-1}$ respectively) compared with those of the dry season (i.e. 21.27 and $47.56 \mu\text{g g}^{-1}$ 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 $31.79 \mu\text{g g}^{-1}$ respectively) compared with those of the dry season (6.26 and $13.59 \mu\text{g g}^{-1}$ respectively). The lowest levels of PM-Pb was observed at S1 ($2.53 \mu\text{g g}^{-1}$) and S2 ($3.38 \mu\text{g g}^{-1}$) 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 $8451.28 \mu\text{g g}^{-1}$ respectively). The lowest level of PM-Zn was observed near the bar mouth stations (S1 and S2) during the dry season (765.45 and $788.20 \mu\text{g g}^{-1}$ 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 $4.83 \mu\text{g g}^{-1}$ respectively during the wet and dry seasons. Highest levels of

PM-Cu was observed in S3 and S4 (24.0 and 17.85 $\mu\text{g g}^{-1}$ 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 $\mu\text{g g}^{-1}$), followed by S1 (69.43 $\mu\text{g g}^{-1}$) and S4 (34.46 $\mu\text{g g}^{-1}$) 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 $\mu\text{g g}^{-1}$. PM-Ni was detected only at S3 (5.26 $\mu\text{g g}^{-1}$) 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. The abundance of planktonic archaea was ten times higher compared with the PM-associated ones (PM-archaea) as evident from FISH counts (Figure 1). There was no significant difference in the abundance of planktonic archaea between dry and wet seasons, and it ranged between $0.28 - 2.83 \times 10^8 \text{ cells L}^{-1}$, with the higher archaeal abundance being observed at station S4 during the wet season. The abundance of PM-archaea ranged between 4.27 to $9.5 \times 10^7 \text{ cells L}^{-1}$ and 2.73 to $3.85 \times 10^7 \text{ cells L}^{-1}$ 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 (Figure 2). The abundances of planktonic bacteria in both wet and dry seasons ranged from 0.55 to $3.4 \times 10^9 \text{ cells L}^{-1}$, while that of PM-bacteria ranged from 1.14 to $6.72 \times 10^8 \text{ cells L}^{-1}$.

The community structure of archaea and bacteria was studied using the PCR-DGGE technique (Supplementary Figure 2 & 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 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). 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 (Figure 3). The community structure of planktonic archaea of the near-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.

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 (Figure 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 (Figure 5). The abundance of PM-archaea and PM-bacteria showed a positive correlation with POC, PON, PM-Pb, PM-Zn, and PM-Fe (Figure 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 (Figure 6).

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 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 $\mu\text{g g}^{-1}$ 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 $\text{Fe} > \text{Zn} > \text{Mn} > \text{Cr} > \text{Pb} > \text{Cu} > \text{Cd} > \text{Co} > \text{Ni}$. 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 $\text{Fe} > \text{Zn} = \text{Cu} > \text{Co} > \text{Mn} > \text{Ni}$. 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 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; 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 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 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 joins 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.

4. 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 remineralisation 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 area tends 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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Fig.1 Sheeba et al. 2017

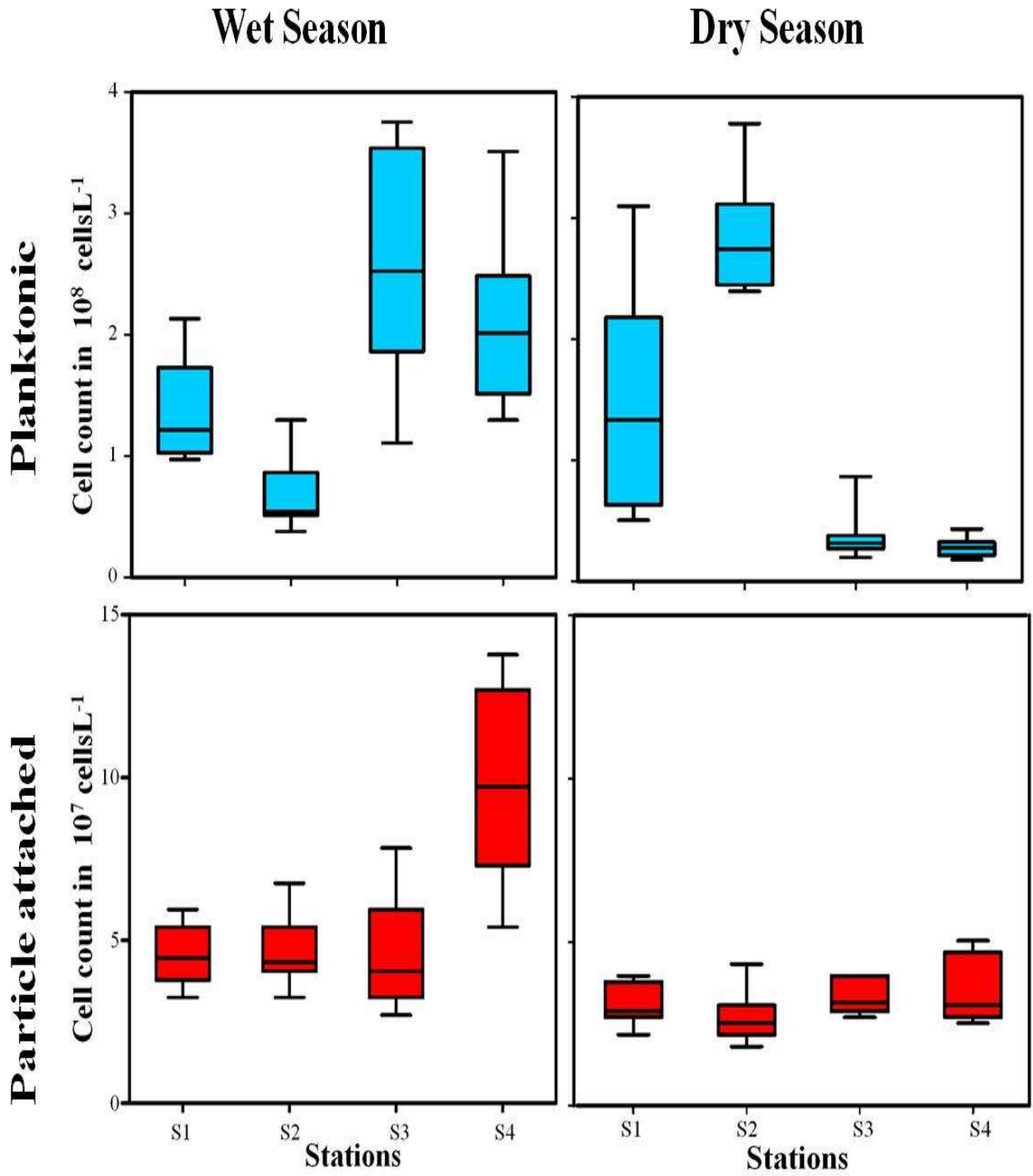


Fig.2 Sheeba et al.2017

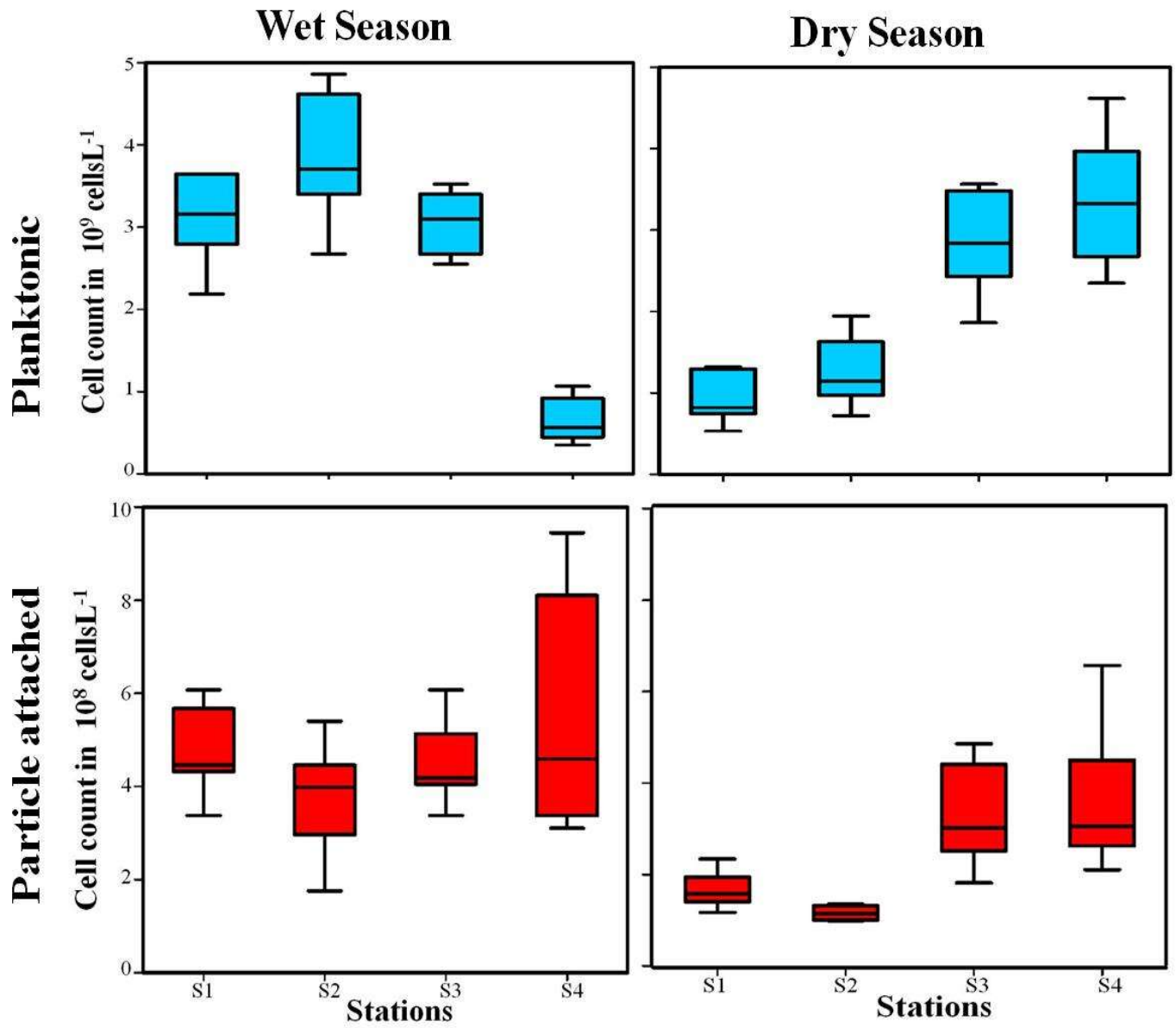


Fig.3 Sheeba et al.2017

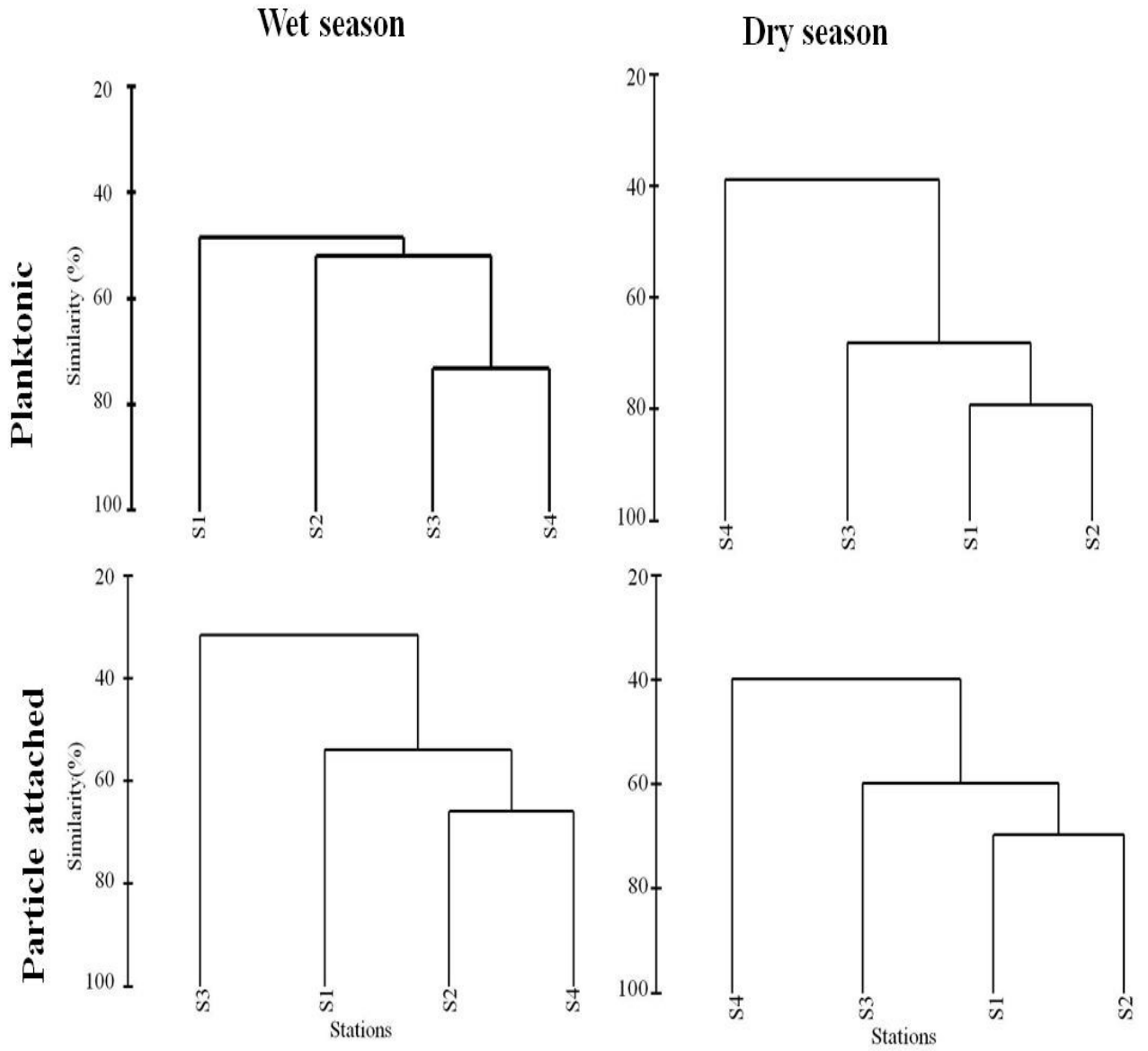


Fig.4Sheeba et al.2017

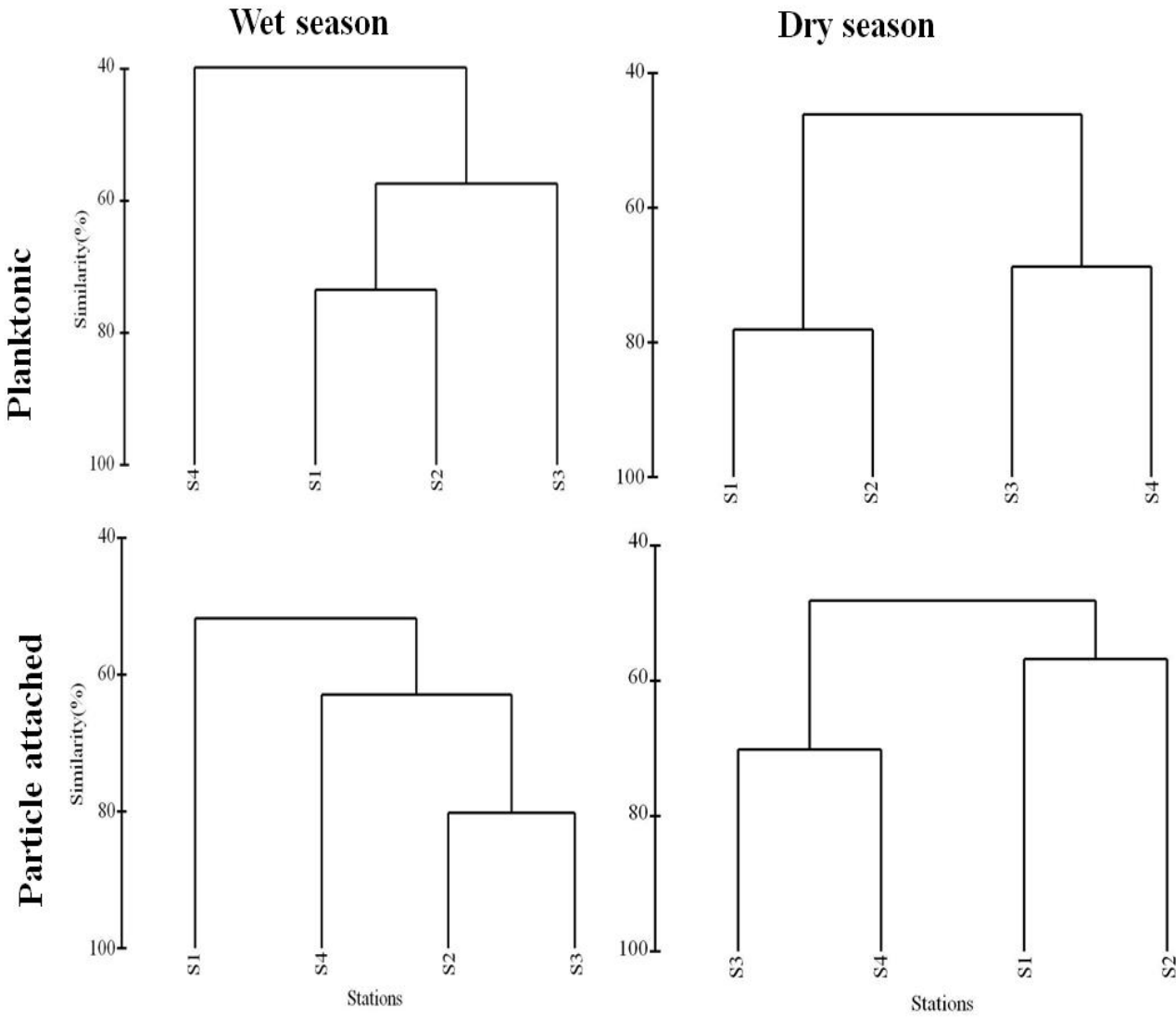


Fig.5 Sheeba et al. 2017

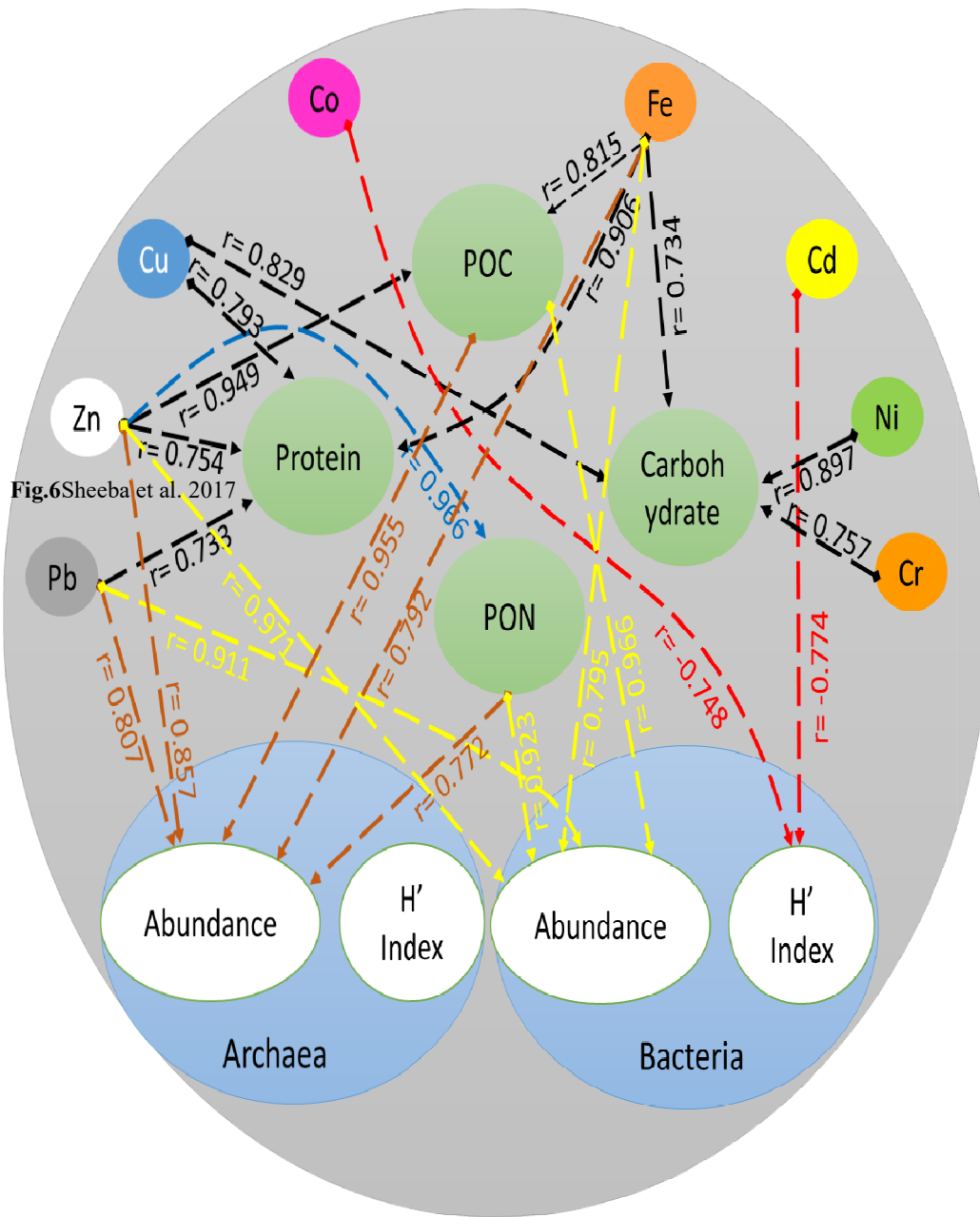
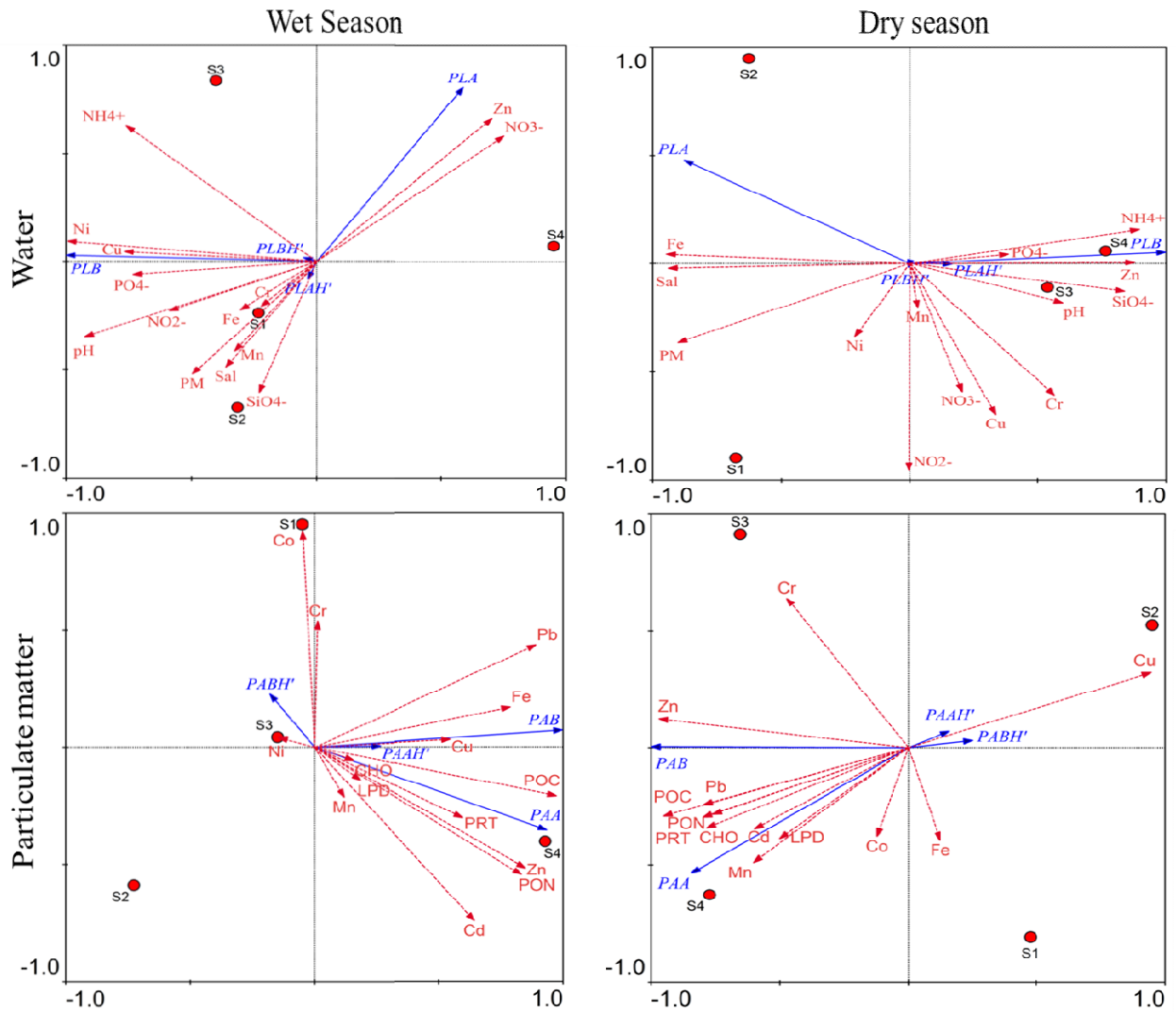


Fig.6 Sheeba et al. 2017

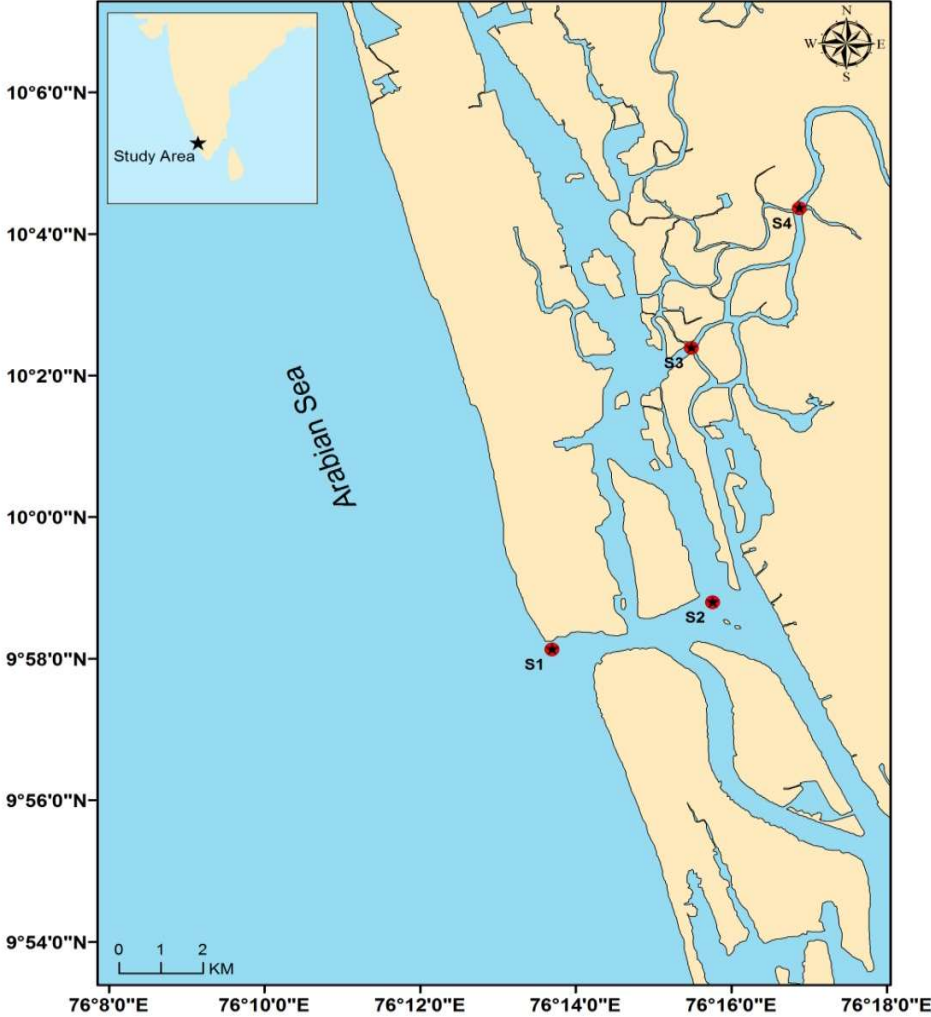


Supplementary Fig.1 Study Area of Cochin Estuary

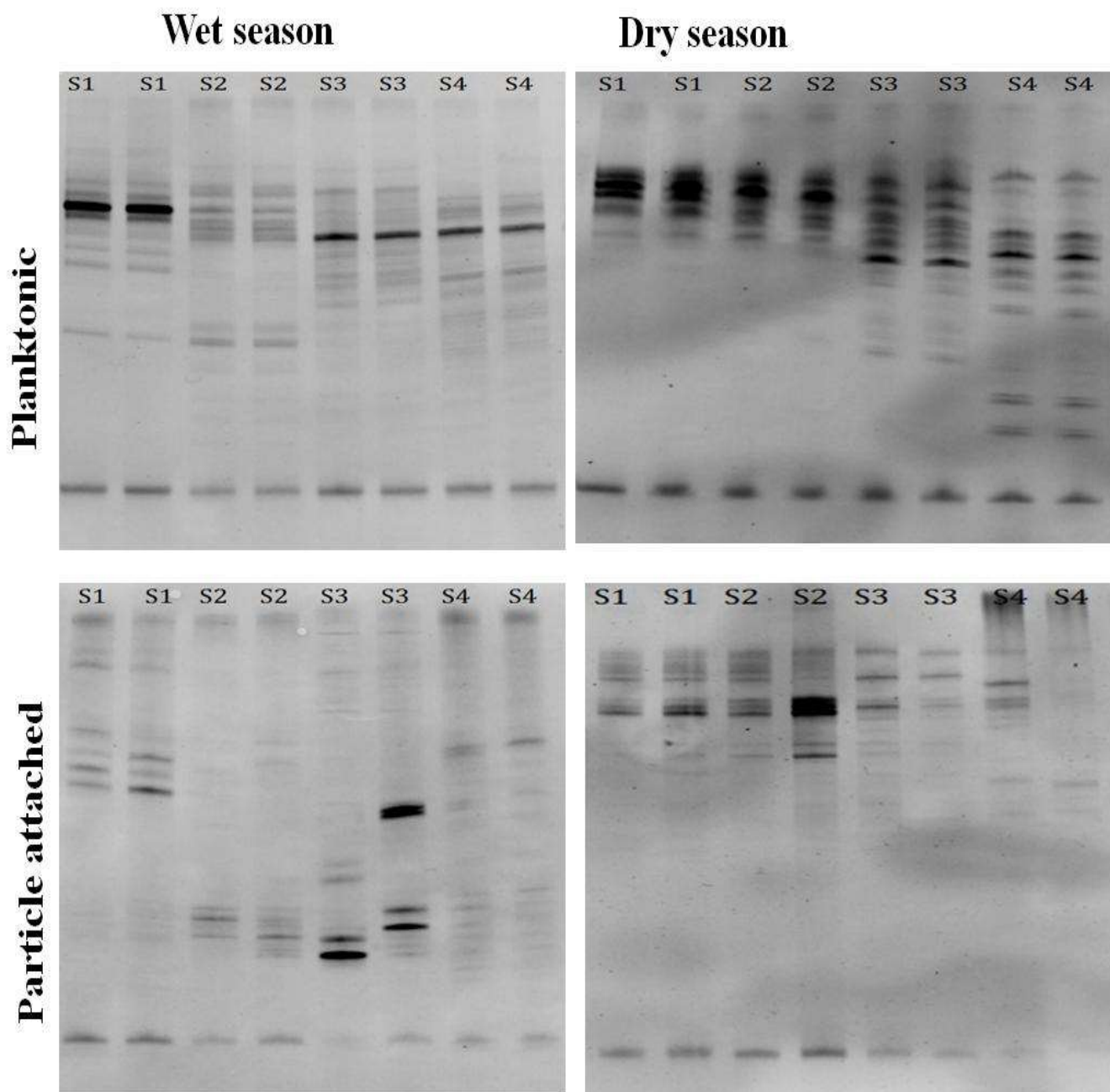
Supplementary Fig.2 DGGE profile of Particle attached archaea and planktonic archaea during wet and dry season

Supplementary Fig.3 DGGE profile of Particle attached bacteria and planktonic bacteria during wet and dry season .

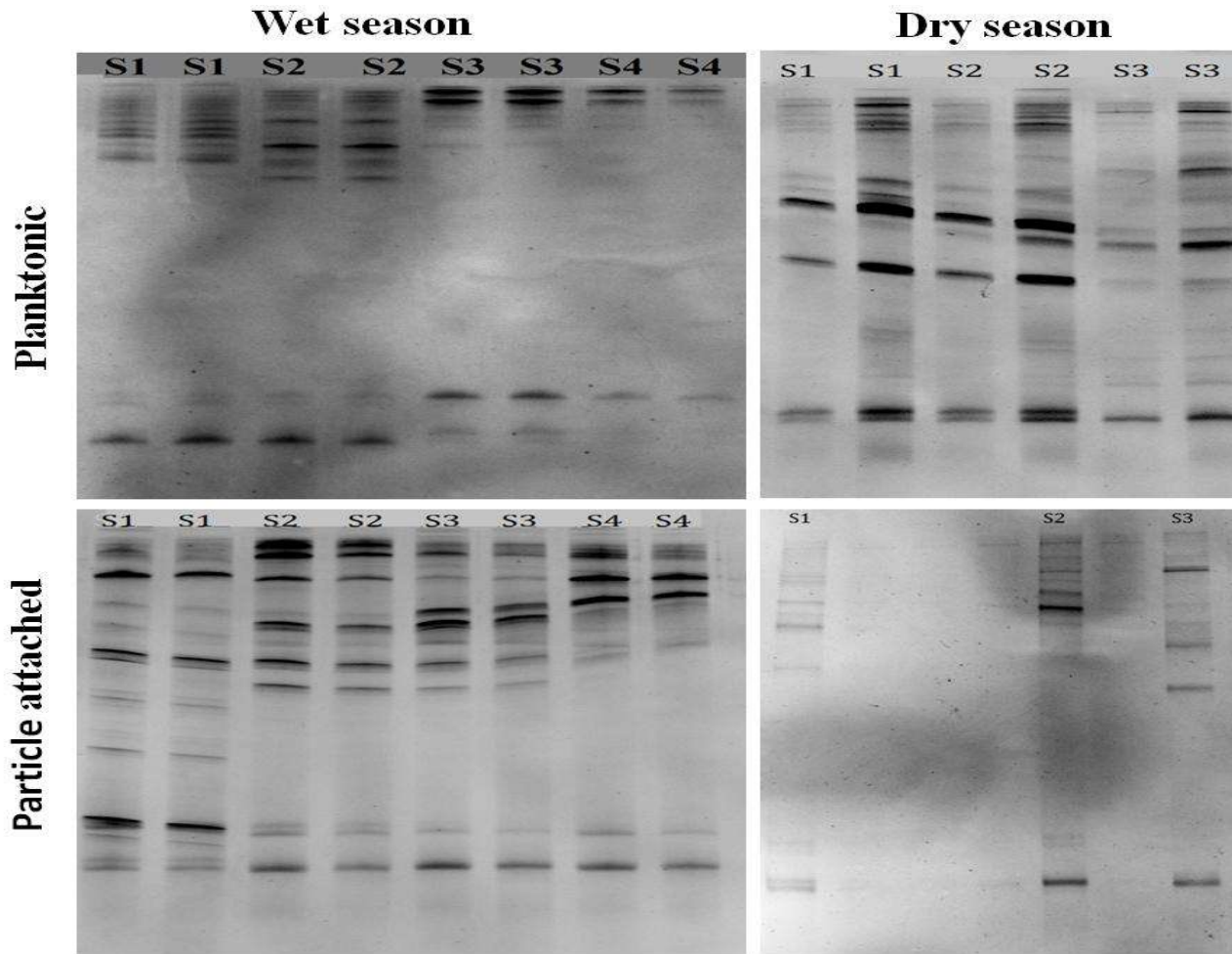
Supplementary fig .1 Sheeba et al 2017



Supplementary fig .2 Sheeba et al 2017



Supplementary fig .3 Sheeba et al 2017



Tables

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)								
NH₄⁺	11.3	7.9	16.1	4.8	1.68	2.23	3.20	5.24
NO₂⁻	0.5	0.3	0.3	0.2	0.2	0.1	0.14	0.16
NO₃⁻	4.4	6	13.6	19.3	0.996	0.45	1.40	0.54
SiO₄⁻	31	56.7	35.4	36.1	10.25	8.5	36.25	22.63
PO₄⁻	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

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

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

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

	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 ($\pm\%$))	4.0	13.0	4.0	11.0	18.0	3.0	19.0	2.0	4.0

Supplementary Table 1: Trace metal extracted from the standard reference material PACS-2 (n=4)

Supplementary Table 2: Details of PCR primers used in the study

Domain	Primer combinations	T_m °C
Archaea	A8F (cggttgatcctgccgga) A1492R (ggctacctgttacgactt)	51.5 °C
	Arch 21 F (ttccggtgatccygccgga) Arch 958 R(yccggcgtgamtccaatt)	55.0 °C
	Parch 519R (ttaccgcgckgctg) SAF(1)(ctayggggcgagcagg) SAF (2)(ctacggggcgagaggg)	53.5 °C
	27 F (agagttgatcctggctcag) 1492 R(tacggctacctgttacgactt)	55.0 °C
	MuyzerF (cctacgggaggcagcag) Muyzer R (attaccgggctgctgg)	60.0 °C