Influence of hydrological factors on bacterial community structure in a tropical monsoonal estuary in India

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Abstract

In the present study, we analysed variations in bacterial community structure along a salinity gradient in a tropical monsoonal estuary (Cochin estuary, CE), on the southwest coast of India, using Illumina next-generation sequencing (NGS). Water samples were collected from eight different locations thrice a year, to assess the variability in the bacterial community structure and to determine the physico-chemical factors influencing the bacterial diversity. Proteobacteria was the most dominant phyla in the estuary followed by Bacteroidetes, Cyanobacteria, Actinobacteria, and Firmicutes. Statistical analysis indicated significant variations in bacterial communities between freshwater, mesohaline and euryhaline regions, as well as between the monsoon (wet) and non-monsoon (dry) periods. Non-metric multidimensional scaling (NMDS) analysis demonstrated that the bacterial communities cluster according to different salinity regimes of the estuary. Canonical Correspondence analysis (CCA) showed a clear spatial and temporal variation in the distribution of bacterial communities in the CE. Abundance of Betaproteobacteria was high in the freshwater regions, while Gammaproteobacteria, Alphaproteobacteria and Epsilonproteobactera were more abundant in mesohaline and euryhaline regions of the estuary. Correlagram based on Pearson correlation analysis demonstrated the impact of different physico-chemical variables on the distribution of dominant phyla, class and genera. Spatial and temporal variations in bacterial community structure could be due to regional variations in environmental conditions imparted by allochthonous inputs, monsoonal rainfall, and tidal influence.

1. Introduction

Marine microbial communities are vital to global biogeochemical cycles of carbon, nitrogen, sulphur, and phosphorous. They are the engines of every ecosystem and constitute a massive biomass, diversity and activity in the global oceans (Graham et al. 2016). Hence, it is extremely important to understand the microbial community structure to appreciate the way in which ecosystems function and to recognize factors that control microbial communities. But even with modern tools (high-throughput amplicon sequencing, metagenomics and metatranscriptomics), it is cumbersome to determine microbial community structure and map its variations in space and time. Physicochemical factors directly affect microbial diversity and community composition and variations in microbial community structure affects ecosystem functioning. Extensive studies have been carried out to reveal the bacterial diversity and community structure in many marine and lacustrine ecosystems (Bowman et al. 2003; Acinas et al. 2004; Heijs et al. 2008; Bobrova et al. 2016; Jeffries et al. 2016). However, only few studies have addressed bacterial diversity from monsoonal estuaries (Crump et al. 2004, Bernhard et al. 2005, Khandeparker et al. 2017, Eswaran and Khandeparker 2019). Very little is known about the complex factors influencing bacterial community composition or the effects these communities have on estuarine ecosystems (Dolan 2005; Teira et al. 2008).

Estuaries make up some of the most complex and dynamic aquatic ecosystems due to freshwater influence from rivers and tidal influence from the seas. Mostly, the terrigenous riverine inputs together with the tidal mixing processes characterize the estuarine environments. Cochin estuary (CE) is a highly

dynamic tropical microtidal monsoonal estuary (Shivaprasad et al. 2013). The biodiversity in monsoonal estuaries is strongly influenced by monsoonal rains and riverine influx in addition to the estuarine variabilities in physical, chemical and biological factors due to tidal influx (Qasim 2003). Average monsoonal rainfall in the Cochin estuary is 2038 mm (CWC data, 2016). The river influx from six major rivers amount to 20000 mm^3/year and the annual precipitation varied between 630 mm to 916 mm (Revichandran et al. 2012). During monsoon (wet period), the riverine influx brings freshwater which accounts for 60–70% of the total annual river discharge to the system. The domestic sewage and industrial effluents dumped into the estuary results in nutrient enrichment in the CE (Madhu et al. 2007). The suspended matter brought in by the riverine influx turns the estuary turbid. During dry period (non-monsoonal months), the tidal influx is more pronounced due to reduced fresh water influx and precipitation (Madhu et al. 2007; Srinivas et al. 2003). Due to the variation in the monsoonal rains, tidal influx, riverine inputs, and the associated pollutants, water quality of the ecosystem and the associated bacterial community diversity is affected. The bacterial community diversity in the CE as well as the impact of physicochemical parameters on the distribution of these communities with respect to monsoonal rains has not been studied so far.

In the present study, the bacterial diversity in eight different locations along a salinity gradient in the CE was determined using Illumina Miseq sequencing. We also investigated the key environmental factors that influence the structure of bacterial communities. It is important to study the spatial and seasonal pattern in bacterial community structure as it reflects the selection mechanisms exerted by the dynamic environment on bacterial groups with specific functions and properties. Though microbial biogeography is addressed from many environments in recent years, principles that govern microbial distribution still remain poorly understood (Thompson et al. 2017, Nemergut et al. 2011). Furthermore, metagenomic analysis of bacterial communities from estuarine environments found that salinity is the most important factor influencing bacterial composition in estuarine environments compared to other physico-chemical factors (Crump et al. 2004, Dong et al. 2004, Wu et al. 2019, Herfort et al. 2017). We hypothesised that the distribution of different bacterial groups in the CE could be a function of spatial gradients and seasonal variabilities in salinity and monsoonal rains.

2. Materials And Methods

2.1. Station description and sampling details

The Cochin estuary is a complex shallow estuary with an average depth of 4 m (Fig. 1). Six major rivers, the Pamba, Achancovil, Manimala, Meenachil, Periyar, and Muvattupuzha along with their tributaries and several canals bring large volumes of fresh water in to the CE. The saline water from the neighbouring Arabian Sea enters the CE through the two inlets—one at Cochin and the other at Azhikode (Fig. 1). During the peak southwest monsoon (June–September), the rivers transport an enormous amount of freshwater in to the CE, which transforms it almost entirely into a freshwater lake except near the two inlet regions.

While in the dry period (non-monsoonal months, October-May), the riverine influxes gradually decrease, allowing salinity to build up in the estuary (Qasim 2003; Jyothibabu et al. 2006).

The water samples were collected from 8 distinct stations along the estuary during three months (August, November, and February in 2015–2016) (Fig. 1). The 8 stations were distinct with respect to inputs and outputs from the river and tides. Station 1 was located far upstream in a relatively unpolluted stretch of the Periyar river, while Station 2 runs through the Industrial Belt of the city. Station 3 is the region where the Periyar river empties into the estuary, and Station 4 or Kochi inlet is where the estuary meets the Arabian Sea. Stations 5, 6, and 7 are located further downstream of Kochi, which receives lot of sewage wastes from the urban population, similar to S3. Station 8 is situated beyond the Thanneermukkam tidal saltwater barrage near rice paddy plantations.

Water (5 L) samples were collected from the surface from each station in sterilized 1 L glass bottles, immediately placed on ice and shielded from sunlight. At each station, the salinity, water temperature, and pH were recorded. The subsamples (triplicate) were collected to reduce the sampling variability at each station during the study period. Upon returning to the lab, the samples were filtered through 0.2 µm filters (0.47 mm diameter, Millipore USA) using a sterilized vacuum filtration apparatus. Once filtration was complete, the filters were stored at -80^{o}C until further processing.

2.2. Environmental parameters

Temperature and salinity were measured using a Conductivity Temperature Density profiler (CTD, SBE, Seabird 19). The inorganic nutrients (nitrate, nitrite, phosphate, ammonia, and silicate) were estimated spectrophotometrically (Shimadzu UV 1800) using standard protocols (Grasshoff 1983). Dissolved oxygen (DO) content was determined following Winkler's titration method (Grasshoff 1983).

2.3 *Enumeration of Total Plate count (TPC)*

Total plate count was employed to enumerate the viable bacteria in water sample. Briefly the water samples were serially diluted using 0.85% physiological saline and plated on to a nutrient agar (NA). The plates were then incubated under room temperature for 24 hours. The colonies that developed on NA plates were enumerated and TPC was expressed as number of colony forming units (cfu) /ml for water sample.

2.4. Extraction of DNA from water samples

DNA was extracted from bacteria concentrated on 0.2 µm filters (Millipore, USA). Under aseptic conditions, the frozen filters were thawed, cut into small pieces using sterilized scissors. The total DNA was extracted using the Power-Soil DNA isolation kit following manufacturer's instructions (Qiagen, USA) (Cao et al. 2013). The DNA (triplicate) from each sampling stations was pooled and sent to GenePath Dx (Pune, India) for library construction and next-generation sequencing.

2.5. Amplicon library construction

The DNA samples were first quantified using a Broad Range Qubit System (Life Technologies, CA, USA), and the average concentration was 10 ng/µl. Library construction involved two PCR reactions. The first reaction ligated two 20 base pair (bp) proprietary tags on either end of the targeted V3/V4 region using 16S Illumina primers F: 5'- CCTACGGGNGGCWGCAG-3' and R: 5'- GACTACHVGGGTATCTAATCC-3' (Jasna et al. 2020, Parvathi et al. 2019) with the initial amount of 20 ng of DNA. Cycle conditions included an initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 20 sec, 72°C for 20 sec, followed by a final extension at 72°C for 10 min (Klindworth et al. 2013). The amplified products of 400–600 bp were visualized by gel electrophoresis, diluted 1:10 using 10 mM Tris-HCl (pH 8.0), and used as templates for the second PCR. This indexing PCR was completed using QuantiTect MultiPlex PCR kit (Qiagen, Germany). Both forward and reverse indexing primers contained a 100 bp tag, including the adapter and unique barcode sequences, and were used at 200 nM. Cycling conditions included, initial denaturation of 95°C for 15 min followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 63°C for 45 sec, and extension at 72°C for 90 sec. The amplified products were quantified using a Qubit Broad Range system (Life Technologies, CA, USA) (Parvathi et al. 2019; Ramanan et al. 2016).

2.6. Illumina sequencing

PCR products were molar-normalized, pooled into a single tube and purified to a minimum of 300 bp using PureLink PCR Purification kit (Invitrogen, CA, USA). The purified, pooled sample was then diluted to 4 nM final concentration using Resuspension Buffer (RSB – Illumina, CA, USA). The sample was denatured for 5 minutes and neutralized using 0.2 N NaOH and HT1 Buffer, respectively (Illumina, CA, USA). It was then pooled with other libraries prepared for NGS in a ratio dependent on amplicon size/total panel size, desired sequencing depth, and number of samples pooled in each sub-library. Pooled libraries were further diluted down to a final 15 pM and spiked with 5% phiX (Illumina, CA, USA) as a control and diversity enhancer. Samples were then loaded into an Illumina MiSeq v3 cartridge (Illumina, CA, USA) and run in 2*300 mode on an Illumina MiSeq next generation sequencer (Illumina, CA, USA) (Parvathi et al. 2019; Ramanan et al. 2016).

2.7 Initial processing of sequence reads

The bcl2fastq Conversion Software embedded in the MiSeq was used for demultiplexing. The quality reads (> Q30) were filtered out using the automated FASTQ Tool Kit application on Illumina BaseSpace Labs website for downstream analysis. Within the application, TagCleaner software was used to remove the adapter sequences.

2.8 Data analysis

The QIIME pre-processing application (Version 1.0.0, Illumina BaseSpace) was used for the analysis of raw sequence data (Caporaso et al. 2010). Pre-processing included demultiplexing, quality filtering, OTU picking, and taxonomic assignment using Greengenes. QIIME outputs were used to create a BIOM file. Before succeeding analysis, OTUs abundance was normalized. QIIME software (version 1.7.0) was used to analyse alpha diversity and richness). The data were normalized and transformed by the Bray-Curtis method. PERMANOVA was performed in order to understand the significant variations in species

abundance. The potential relationship between bacteria and environmental variables were tested using Pearsons correlation analysis. A correlation matrix or correlogram was generated for analysing the relationship between environmental factors and dominant phyla, class and genus using software Origin pro (Developed by Origin Lab Corporation). Pearson correlation was adopted for the analysing of data and represented in correlogram, wherein, colour indicates the strength of the correlation in which red indicates positive and blue indicates negative correlation. Canonical correspondence analysis (CCA), a multivariate method was used to elucidate the relationships between biological and environmental variables (CANOCO 4.5) (Lepš and Šmilauer 2003). A Monte Carlo test was used to determinate the significance of each axis and to evaluate the influence of the environmental variables upon the overall distribution of bacterial species and their distribution at each sites and sampling seasons. (Salles et al. 2004; Sapp et al. 2007). Similarity percentage breakdown (SIMPER) was used to calculate the partition of the average Bray-Curtis dissimilarity between different clusters into components from different genera (Clarke 1993). This allowed identification of genera that are the most important in creating observed patterns in similarity.

2.9. Nucleotide sequence accession number

Paired end Illumina sequence data from this study was submitted to the NCBI Sequence Read Archive (SRA) under bio project number PRJNA595444

3. Results

3.1 Environmental parameters

Observations on environmental parameters were made on wet and dry seasons in the Cochin estuary. Seasons were classified based on the rainfall in this region (**Fig. S1**). The duration from June to September was considered as wet period (i.e. monsoon season), which received an average rainfall of 528.75 mm, whereas the dry period, sub classified into the Dry period I and II, received an average rainfall of 151.75 mm and 133 mm, respectively (**Fig. S1**). The entire estuary was freshwater dominated (salinity = 0-3.6) during the wet period, except at the inlet station, S4, which sustained a salinity of 16. With the retreat of southwest monsoon, the riverine influx reduced by 45% resulting in salinity stratification in the estuary with a salinity of 28 at the inlet (S4) by May (Table 1). During dry seasons, freshwater conditions prevailed in stations S1, S2, S7 and S8 (salinity = 0 to 5), while mesohaline salinities (salinity = 6–20) were observed in stations S3, S5, and S6. Euryhaline salinity of > 20 was observed only in the inlet (S4) during this season. Spatial variations in salinity and temperature were highest during the dry period II and least during wet period (Table 1). Salinity was the most fluctuating variable in the CE ranging from 0–28. The major inorganic nutrients were high during the sampling period in the CE (Table 1).

Table 1

Table showing physicochemical parameters in different stations during three periods such as Wet period (Monsoon), Dry period I and (c) Dry period II in the Cochin estuary (CE).

Parameters	Wet Period							
	S1	S2	S3	S4	S5	S6	S7	S8
Nitrite ((μM)	0.32	0.16	0.42	0.42	0.26	0.26	0.12	0.06
Nitrate (μM)	28.06	23.22	14.41	13.06	6.79	5.76	4.37	4.19
Ammonia (μM)	26.55	12.05	35.05	2.25	2.25	81.45	1.15	16.45
Phosphate (μM)	0.7	0.3	0.45	0.7	0.55	0.85	0.2	0.4
Silicate (μM)	115.2	119.6	61.5	15.9	86.1	138	106	101
Temp (°C)	28.5	31.5	32.5	29	26	25	27	29
Salinity	0	0	4	16	3.6	0	0.1	0.1
DO (mL/L)	3.25	4	4.19	3.58	3.83	3.96	3.94	4.05
	Dry period I							
Nitrite ((μM)	0.48	0.25	1.17	1.15	1.97	0.66	0.3	0.2
Nitrate (μM)	27.14	17.03	6.47	5.25	3.85	4.25	13.37	16.11
Ammonia (μM)	10.04	19.99	41.35	28.74	42.06	29.89	17.08	13.49
Phosphate (μM)	1.09	1.46	2.38	3.27	3.75	1.35	0.35	0.45
Silicate (μM)	98.71	126.5	119.7	76.39	89.74	78.9	60.95	94.9
Temp (°C)	28.5	31.5	32.5	29	26	25	27	29
Salinity	0	4	10	26	16	13	7	5
DO (mL/L)	4.86	4.11	4.96	4.2	4.14	4.4	5.33	5.3
	Dry period II							
Nitrite ((μM)	0.16	0.24	0.53	0.44	0.8	0.9	0.18	0.02
Nitrate (μM)	26.84	11.87	2.55	2.55	3.42	3.86	15.04	18.22
Ammonia (μM)	3.57	7.46	18.77	11.93	23.35	58.5	6.75	13.3
Phosphate (μM)	0.61	1.27	1.95	2.99	1.55	1.61	0.05	0.1
Silicate (μM)	114.83	91.19	43.01	43.01	33.9	40.27	81.55	106.3
Temp (°C)	30.9	22.88	28.88	29.15	23.28	30.43	23.58	30.5
Salinity	0	5	21	28	19	16	6.5	2

Parameters	Wet Period							
DO (mL/L)	4.28	5.73	3.62	3.59	4.12	4.3	5.07	5.1

3.2 Total Plate count (TPC)

Total plate count (TPC) of bacteria ranged from 0.22to 5.98× 10^5 cfu/ml **(Fig S2).** The bacterial abundance was higher in the Dry period II and especially in the euryhaline region of the estuary.

3.3 Taxonomic richness and α-diversity of the prokaryotic community

The bacterial community diversity was highest in the dry period I compared to other periods **(**Table 2**)**. The overall species coverage of the samples was highest (≥ 68.60%) for dry period I, followed by wet period (≥ 66.68%) and the dry period II (≥ 63.19%). The Good's average was high (0.99) indicating that most of the bacterial diversity was attained by sequencing. However, Shannon diversity, Chao richness and Simpson diversity indices, which represent abundance and evenness of the species distribution, varied slightly with sampling period and with stations (Table 2). There was significant difference in α-diversity between the dry and wet seasons at all stations ($p > 0.05$) and bacterial diversity significantly differed both spatially and temporally ($p < 0.05$).

Table 2

This table shows the summary of the Illumina sequencing: the number of reads, % reads classified, and OTU identified, Chao-1, Shannon and Simpson species diversity are listed. Sequencing was completed for 8 samples in 3 sampling periods: Wet period (Monsoon), Dry period I and Dry period II.

Wet period	No of reads	% Reads, classified to genus	Shannon Species Diversity	Cho-1 species Diversity	Simpson Species Diversity	Number of OTUs identified
S1	304436	75.65%	2.898	1782	0.9768	1421
S2	322227	64.49%	2.446	1798	0.9523	1324
S3	281047	65.69%	2.34	1527	0.9729	1215
S4	179025	66.01%	2.718	1388	0.9767	1121
S5	252310	64.20%	2.599	1746	0.9745	1225
S6	275375	65.91%	2.663	1720	0.977	1329
S7	236879	66.95%	2.336	1355	0.8978	948
S8	167417	64.57%	2.408	1088	0.9453	795
Dry period I						
S1	274745	70.93%	2.556	1930	0.9723	1543
S2	367764	67.31%	2.792	2016	0.9805	1651
S3	173542	64.60%	2.69	1600	0.9764	1203
S4	237272	71.13%	3.065	1819	0.9802	1462
S5	260109	71.13%	2.992	1879	0.9643	1575
S6	323762	66.62%	2.971	1815	0.9746	1483
S7	337895	66.74%	2.779	1887	0.9791	1558
S8	130689	70.79%	2.716	1541	0.9645	1188
Dry period II						
S1	171453	66.93%	2.825	1588	0.976	1078
S2	165909	52.63%	2.481	1285	0.9512	954
S3	127522	57.53%	2.239	1422	0.9706	1036
S4	189601	59.06%	2.417	1514	0.9779	1025

Wet period	No of reads	% Reads, classified to genus	Shannon Species Diversity	Cho-1 species Diversity	Simpson Species Diversity	Number of OTUs identified
S5	210901	57.34%	2.41	1370	0.9769	1038
S6	176353	62.86%	2.192	1077	0.9595	829
S7	93251	63.19%	2.36	1073	0.9468	827
S8	179775	85.98%	2.376	915	0.9524	645

3.4 Seasonal and spatial variations in bacterial diversity at phylum and class level

The relative abundances of different phyla in the eight locations from three different sampling periods (24 samples) are shown in Fig. 2. Total bacterial diversity was distributed among 32 different phyla, with Proteobacteria (5.53–57.18%), Bacteriodetes (7.62–27.11%), Firmicutes (2.09–26.76%), Actinobacteria (2.99–14.34%), Cyanobacteria (3.37–36.91%) and Verrucomicrobiae (0.53–7.85%) accounting for more than > 95% of the total OTUs in all the samples (Fig. 2). It was found that the phylum Proteobacteria dominated in all the stations irrespective of sampling period, followed by Actinobacteria, Bacteroidetes and Firmicutes in the wet period and dry period I. Proteobacteria was followed by Cyanobacteria Actinobacteria, Bacteroidetes and Firmicutes in order, in dry period II (Fig. 2). Phylum Proteobacteria was dominated by Alphaproteobacteria, Gammaproteobacteria, and Betaproteobacteria, with Betaproteobacteria being more abundant in the freshwater regions and Alphaproteobacteria in the meso- and euryhaline regions of the estuary. Betaproteobacteria was the most dominant class throughout the freshwater-dominated estuary during wet period, in all stations except S4 (Fig. 3). Alphaproteobacteria was the most dominant class in the inlet station, S4, during wet period and in S4 to S6, during dry periods. Similarly, Gammaproteobacteria was found in high proportions in S4 and S6 during dry periods, while during wet period, it was most abundant in the upstream station, S1.

3.5 Bacterial diversity at generic level

A total of 975 genus contributing to 60 to 75 % of total generic diversity was considered and represented in Fig. 4. The most dominant genus were *Sanguibacter, Saccharopolyspora, Prochlorococcus, Arcobacter*, and *Ruegeria*, with significant spatial variations in abundance. Most dominant genus in the freshwater regions were *Sanguibacter, Saccharopolyspora, Demequina, Chthoniobacter, Bifidobacterium, Paucibacter, Flavobacterium, Limnohabitans, Chitinophaga, Acinetobacter, Oxalobacter, Prochlorococcus*, etc. In Mesohaline regions, dominant genera included *Microcystis, Prochlorococcus, Agromyces, Saccharopolyspora, Actinocatenispora, Peptoniphilus, Acidiphilium, Polaribacter, Aquimarina, Arcobacter, Gramella, Leucobacter, Flavobacterium, Ruegeria, Calothrix* etc. *Actinocatenispora, Agromyces, Prochlorococcus, Microcystis, Acidiphilium, Ruegeria, Saccharopolyspora, Vibrio, Peptoniphilus, Gramella, Aquimarina, Leucobacter, Acidocella, Tenacibaculum, Marivita, Nisaea, Arcobacter*, were most dominant

in the euryhaline regions of the estuary. Pollution indicators such as *Bacteroides, Microcystis, Agromyces, Vibrio, Clostridium, Prevotella, Enterobacter, Ruminococcus, Lachnospira, Pseudomonas* were detected.

3.6 Statistical Analysis

NMDS analysis revealed two clusters of bacterial communities during wet period (Fig. 5). During wet period, the entire estuary behaved like a freshwater lake and hence formed two clusters, S4 in one cluster and all other stations in another cluster. During dry months, the bacterial communities were clustered in to three, euryhaline stations (S4, salinity = > 25), mesohaline stations (S3, S5, and S6, salinity = 5–20) and freshwater stations comprising of stations S1, S2 and S8 (salinity = < 5). Based on SIMPER analysis, the average similarity within the cluster was 73 % and the degree of dissimilarity between freshwater and mesohaline cluster was 40 %. DP1 yielded three clusters with an average similarity of 75% in the freshwater cluster. The average dissimilarity between FW and MH stations was 32% and that between FW and EH was 38%. Average dissimilarity between MH and EH was 26%. NMDS revealed two clusters in DP II. The average dissimilarity between FW and MH was 42% and that between MH and EH was 21% in DP II.

CCA analysis of the phylum, class and generic level diversity showed a distinct spatial and seasonal pattern in the distribution of bacterial communities (Fig. 6). Distribution of bacterial communities at each station was influenced by different environmental parameters, such as DO, silicate, and nitrate in the freshwater regions, high inorganic nutrients such as ammonia, phosphate, and nitrite in the mesohaline regions and salinity in the euryhaline station, S4. Correlogram based on Pearson correlation analysis revealed influence of physico-chemical parameters on distribution of dominant phyla, class and genus (Fig. 7). Salinity was an important factor determining the distribution of Cyanobacteria, Gammaproteobacteria, Alphaproteobacteria and Epsilonproteobacteria. At the phylum level, Proteobacteria and Bacteriodetes showed positive correlation with nitrite, nitrate, ammonia and phosphate. Bacteriodetes showed negative correlation with dissolved oxygen, silicate and temperature during dry period. Alphaproteobacteria showed positive correlation with salinity, nitrite and ammonia, and negative correlation with DO and silicate during dry months. Betaproteobacteria showed positive correlation with nitrite, nitrate and temperature during wet period and with nitrate, silicate and DO during dry period. Betaproteobacteria showed negative correlation with salinity, nitrite, ammonia and phosphate during dry period (Fig. 7**).** Actinobacteria showed positive correlation with DO and silicate. Cyanobacteria showed positive correlation with DO and salinity in dry months and negative correlation with nitrite and phosphate during wet period and with silicate and nitrate during dry period. Salinity played an important role in the distribution of certain major genera such as *Alteromonas, Ruegeria, Anaeropsora, Marvita, Polaribacter, Vibrio* and *Acrobacter* (Fig. 8). Various other factors such as concentrations of silicate, temperature and nitrite, nitrate and ammonia also played significant roles in the distribution of important bacterial genera in different salinity regimes of the estuary (Fig. 8)

The number of shared and unique OTUs at the genus level in dry and wet periods in indicated in the Venn diagram (**Fig. S3**). Comparative analysis showed that ~ 1621 OTUs were shared during all the seasons, 172 OTUs were unique to WP, and 375 OTUs to dry period I and 87 OTUs to dry period II. PERMANOVA

analysis demonstrated that bacterial community structure significantly varied temporally during wet and dry seasons (Pseudo F = 3.342, $p = 0.002$) and spatially (Psuedo F = 1.615, $p = 0.016$).

4. Discussion

Estuaries, being dynamic mixing zones of ocean and freshwater masses, are characterized by steep spatial and temporal gradients of physical, chemical and biological parameters. Hence, it is essential to fathom the impact of these gradients on local bacterial community, their metabolism and on the water quality in an estuarine system. Salinity has been demonstrated as an important environmental factor structuring bacterial communities in coastal ecosystems (Ortega-Retuerta et al. 2013; Liu et al. 2015; Herlemann et al. 2016). The present study examines seasonal and spatial variations in bacterial diversity with respect to physico-chemical gradients in the Cochin estuary. Though CE comprised of similar aquatic microbial phyla found within other aquatic environments (Eswaran and Khandeparker 2019; Meziti et al. 2016; Savio et al. 2015), it hosted variations in the relative abundance of bacterial communities with changes in estuarine hydrography and pollutants. The estuarine bacterial genera clearly fell into three distinct categories, Euryhaline/marine (Salinity > 20), mesohaline/brackish (Salinity = 5–20) and freshwater (Salinity = < 5) (See Station details and description for seasonal hydrography in the CE) (Fig. 5). Previous studies in the Cochin estuary has demonstrated existence of three distinct zones based on salinity variations during dry months and two zones during wet monsoonal months and has indicated unique biological responses to these gradients (Parvathi et al. 2015, Jasna et al. 2017). In addition to the variations imparted by salinity influx, there are regional variations in the input of industrial wastes, agriculture wastes and sewage inputs, especially from many non-point sources. Large inputs of fresh organic matter in to the estuary from riverine inputs and other non-point sources impact heterotrophic production (Jasna et al. 2017) and bacterial diversity. We detected fluctuations in bacterial richness within the three different salinity regimes of the estuary (Table 2). This indicates that factors other than salinity influenced bacterial richness. Freshwater regions of the estuary detected the highest and lowest richness during dry period. We assume that variations in the lability of organic matter in these regions might be responsible for the proliferation of several adapted bacterial taxa (Bunse et al. 2016). The results of this study corroborated with recent studies on distinct bacterial communities in estuarine environments of Delaware Bay, Chesapeake Bay, Columbia River estuary and Baltic Sea (Herfort et al., 2017; Herlemann et al., 2011; Hugerth et al., 2015).

Proteobacteria was the most dominant phyla in all the stations irrespective of the sampling period, with only marginal variations in the percentage of occurrence, with Betaproteobacteria, Alphaproteobacteria, and Gammaproteobacteria being the dominant classes. The dominance of Proteobacteria was in concurrence with reports from other tropical estuaries (Bouvier and del Giorgio 2002; Ghosh and Bhadury 2019; Ortmann and Santos 2016; Eswaran and Khandeparker 2019) and also from coastal waters of India (Sachithanandam et al. 2020, Parvathi et al. 2019). High abundance of Betaproteobacteria and Alphaproteobacteria in our study corroborated with previous studies from other estuarine regions (Sekiguchi et al. 2002). While, Betaproteobacteria was found in high proportions in the freshwater regions of the estuary, Alphaproteobacteria, Gammaproteobacteria and Epsilonbacteria were more dominant in

the mesohaline and euryhaline regions of the estuary during dry seasons. Betaproteobacteria was present in high proportions at salinities below 4 and Alphaproteobacteria at salinities above 13. This shows that salinity transitions play a significant role in the abundance and distribution of Beta- and Alphaproteobacteria. However, the mechanisms causing changes in bacterial community composition at different salinities are currently unclear. Few studies in the Cochin estuary have linked salinity to differences in the key metabolic capabilities of bacteria (Shoji et al. 2007). Similar studies from Baltic sea have reported differences in the relative abundance of bacterial genes associated with respiration, glycolysis quinone biosynthesis, and osmolyte transport to variations in salinity (Dupont et al., 2014). Due to short generation times of bacteria together with their remarkable trophic versatility, environmental variabilities directly reflect upon the distribution and abundance of bacterial communities.

Dominance of Proteobacteria, Bacteriodetes, Gammaproteobacteria, Alphaproteobacteria, and Epsilonproteobacteria was high in regions with high concentrations of ammonia and nitrite. Mesohaline regions were characterized high concentrations of ammonia, nitrite, and phosphate. The mesohaline regions of the estuary, especially, S3, S5, and S6 receive high amount of agriculture wastes, industrial effluents and sewage from the urban population through many nonpoint sources. This region has the highest amount of ammonia, and nitrite compared to upstream fresh water stations, like S1, S2 and S8, indicating increased sewage input and agricultural runoff. These regions lie between the inlet and the freshwater region/Vembanad Lake, which makes these regions less dynamic with low flushing rates during the dry seasons with no rainfall and less riverine influx (Jasna et al. 2017). Dissolved organic carbon is also more (340 ± 108 to 193 ± 102 μmol kg^{-1}) in the mesohaline regions compared to freshwater regions (< 200 μmol kg^{-1}) of the estuary (Gupta et al. 2009). Moreover, due to the closure of Thannermukkam bund during dry period II, the riverine influx from the Vembanad Lake is blocked to prevent salinity intrusion to the paddy fields in the southern regions (Fig. 1). This has reduced flushing out of organic pollutants from the estuary, especially from these less dynamic mesohaline regions. The mesohaline regions support high bacterial respiration (Jasna et al. 2017), indicating that these regions have heavy nutrient load which in turn supports the growth and dominance of Gammaproteobacteria, Alphaproteobacteria and Epsilonproteobacteria. Saline water allows dissolution of dissolved inorganic phosphorus (DIP) from iron bound fractions in sediment, leading to subsequent increase in phosphate observed in surface waters (Slomp, 2011). On the contrary during wet season, river discharge brings in DIP into estuaries from anthropogenic as well as natural sources (Slomp, 2011). Phosphate is a major constituent of nucleic acid and lipids, hence is an important nutrient required for the growth of microorganisms. Gammaproteobacteria were also abundant in the river mouth station, S1 during wet season, indicating that this group also takes advantage of allochthonous material loadings and nutrient enriched conditions. In addition to these, variations in other biotic factors, such as high phytoplankton (Madhu et al. 2007), grazing (Sooria et al. 2016) and viral lysis (Jasna et al. 2017) can also shape bacterial community composition in the CE. Since these factors also change along the salinity gradient of the CE, the observed bacterial community composition patterns may be a result of factors that co-correlate with salinity.

We report on various dominant bacterial genera in different salinity regimes, which are either indicative of water quality and water pollution or as strains with bioactive potential. Salinity had a significant influence on the bacterial genera such as *Vibrio, Faecalibacterium, Paracoccus Rugeria, Arcobacter, Microbacterium Gremalla*, and *Polaribacter*. Apart from salinity, spatial variations in temperature and other inorganic nutrients also influenced the bacterial community structure and distribution. Temperature, nitrate, nitrite and phosphate positively influenced the abundance of *Vibrio* species, whereas factors like DO and temperature had a negative correlation with *Vibrio.* DO declined in the mesohaline regions due to enhanced bacterial heterotrophic activity to process the high organic matter in this region (Jasna et al. 2017). The growth of autochthonous bacteria, for instance Vibrios are supported by dissolved organic carbon released from diatom-dominated phytoplankton as well as zooplankton communities in the CE (Madhu et al. 2007). Diatom have been primary determinant for *Vibrio* population in coastal water of Arabian sea along southwest coast of India (Asplund et al. 2011). However, it would be misleading to extrapolate *Vibrio* genus to specific *Vibrio* strains, since the growth response of different *Vibrio* spp. to organic matter, temperature and nutrients is variable (Eiler et al., 2007). However, results of our study point to the potential that *Vibrio* strains are sustained as free-living populations in the CE. Dissolved oxygen had positive correlation with the abundance of dominant genera such as *Demequina, Actinocarenispora, Chthoniobacter, Sanguibacter*, and *Bifidobacterium*. DO was high in the CE, with higher concentration in the freshwater regions of the estuary. Solubility of oxygen in water decreases with increased temperature and salinity. High concentrations of DO in the freshwater regions could be due to high solubility of oxygen in colder and less saline waters

The presence of some genera indicated the extend of pollution in this estuarine environment. Some strains of genus *Sanguibacter* has been reported from blood of healthy cows while others were found in soil, sand, and sediment from terrestrial and aquatic ecosystems (Schumann and Stackebrandt, 2014). Members of the genus *Bifidobacterium* are known to colonize the human gastrointestinal tract and they naturally occur in a range of ecological niches that are either directly or indirectly connected to the animal gastrointestinal tract, such as the human oral cavity, the insect gut and sewage. *Oxalobacter* is found in rumens of animals such as cattle and in feces of other animals and humans. They are also isolated from marine and fresh water environments (George et al., 2005). *Acinetobacter* species are a key source of infection in debilitated patients in the hospital, while *Peptoniphilus* are frequently encountered as a part of vaginal and gut microbiota (Brown et al. 2014). The most dominant genus in mesohaline regions was *Microcystis* which include members that can produce neurotoxins and hepatotoxins, such as microcystin and cyanopeptolin. Hence, presence of this genera pose a threat to drinking water quality. Anthropogenic nutrient over-enrichment (eutrophication), and other factors including expansion of intensive agriculture, rapid industrialization, and urbanization, enhance the occurrence of microcystin-producing HABs in most regions and thus poses deleterious effects on human health (Garrity et al., 2020).

Bacteria involved in faecal contamination such as *Bacteroides, Clostridium, Faecalibacterium, Enterococcus, Pseudomonas, Vibrio, Prevotella, Enterobacter, Klebsiella, Campylobacter* are also detected in CE (Boehm and Sassoubre 2014). *Bacteroides* are an important indicator group which are exclusive to warm-blooded animals and constitutes a larger portion of pollution indicator bacteria in CE, especially

during dry period. Human and animal associated *Bacteroides* marker are been extensively used for fecal identification studies (Harwood et al. 2014). In the present study, *Bacteroides* showed significant positive correlation with nitrate and ammonia and did not show any correlation with salinity. Another genus, *Clostridium* are anaerobic spore forming bacteria can often been found in the intestinal tract of mammals and were present high abundance in S6 during dry period. Other indicator genera like *Prevotella* and *Bifidobacteria* were also highly abundant in brackish water regions during dry period that are often present in the gastrointestinal tract microbiota in mammals. *Faecalibacterium* are anaerobic commensal bacteria found in the human gut microbiota. *Bergeyella* is also a bacterium isolated from the upper respiratory tract of dogs, cats and other mammals. Other fecal contamination indicators included *Enterococcus, Pseudomonas*, and *Vibrio*. The presence of these pathogens in the CE indicate the presence of sewage or fecal contamination from other animal sources.

Presence of many potential actinobacterial species indicates that there is a plethora of bacterial communities with potential for industrial applications. The genus *Saccharopolyspora* and *Actinocatenispora*, belonging to Actinomycetes has been chemically investigated for the production of novel natural products with pharmacological effects (Sayed et al. 2020). The genus *Demequina* is also an actinobacterial strain characterized by having discriminative menaquinone(s), namely demethylmenaquinone(s) (diagnostic isoprenoid quinone) (Hamada et al., 2013; Park et al., 2015). Genus *Leucobacter* has been studied extensively for its capabilities for reducing toxic chromium compounds into less toxic forms in the environment (Zhu et al., 2008). Metabolic potential of *Polaribacter* strains for degrading carbohydrates and complex algal polysaccharides has been demonstrated (Xing et al. 2015) and grow well in diatom dominated environments (Teeling et al. 2012). They were present in high proportions in the mesohaline regions of this estuary, which are largely dominated by diatoms (Madhu et al. 2007).

Several genera in the CE could be considered as indicators of eutrophication, such as those involved in ammonia oxidation, nitrite reduction, N_2O reduction. Genus such as *Paracoccus*, *Comamonas. Nitrosomonas*, and *Nitrobacter* are involved in the nitrogen cycle and were more abundant in the mesohaline regions of the estuary (Wang et al. 2014, Kim et al. 2015). Species involved in nitrogen cycle such as *Pseudoalteromonas denitrificans, Alicycliphilus denitrificans, Thiobacillus denitrificans, Sulfurimonas denitrificans, Sterolibacterium denitrificans, Paracoccus denitrificans, Microvirgula aerodenitrificans, Halomonas halodenitrificans, Flavobacterium denitrificans and Thauera mechernichensis* were identified in the CE in the mesohaline and euryhaline regions of the estuary. Dissolve inorganic nitrogen is high in the Cochin estuary with higher nitrogen fixation rate (Bhavya et al. 2016). In fact, these regions receive 7–11 times high organic matter as lateral input as compared to the inputs through rivers (Shoji et al, 2008, Gupta et al. 2009). The input of industrial and domestic waste discharges as a result of large human settlement and industrial growth and agriculture has resulted in excess nutrients in the system. The TRIX scores showed that CE is highly eutrophic (Martin et al., 2012). The nutrient enrichment triggers massive growth of phytoplankton and blooms in the CE (Madhu et al. 2007, Rajaneesh et al. 2015). In the present study, high abundance of *Prochlorococcus* and

Thermosynechococcus were detected. The abundance of *Prochlorococcus marinus* increased between the upstream stations to the inlet. *Synechococcus* has been indicated as an indicator of trophic status in the CE (Rajaneesh et al. 2015).

5. Conclusion

The present study demonstrated that the most dominant bacterial phyla, class and genus using high throughput metagenomic analysis. Additionally, with the help of clustering and multivariate analysis, we showed the impact of different factors on bacterial community structure. Our study showed that seasonal and spatial variations in bacterial community structure is largely brought out by salinity and other inorganic nutrients like nitrite, nitrate, ammonia, phosphate and silicate in the CE. Salinity and nitrite influenced the distribution of dominant classes such Alphaproteobacteria, Gammaproteobacteria, and Cyanobacteria. Various genera indicative of eutrophication, heavy metal pollution and sewage pollution were identified in this study. Our study suggests that monitoring the presence of important bacterial groups could serve as a relevant indicator of ecosystem health and pollution. Our study also identified various genera with bioactive potential. The results of this study provide important targets for future study and may assist in detecting and quantifying imminent changes in estuarine water quality. Future studies should focus on the functional gene profiling of different bacterial communities to understand the ecological roles of bacterial communities in the CE.

Declarations

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Conflicts of interest/Competing interests

Authors declare no conflicting or competing interests

Ethics approval

No need of ethics approval, since this work does not include research on identifiable human material or data.

Consent to participate

All the authors agree to participate in this manuscript

Consent for publication

The manuscript has written consent form all the authors and from the institution.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Credit authorship contribution statement

AP conceptualized the work, made funding arrangements data analysis and prepared the manuscript. MC conceptualisation, data collection and sample analysis, JV analysed samples, performed statistical analysis and prepared the manuscript. NP, NG carried out the Illumina Miseq sequencing.

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Figures

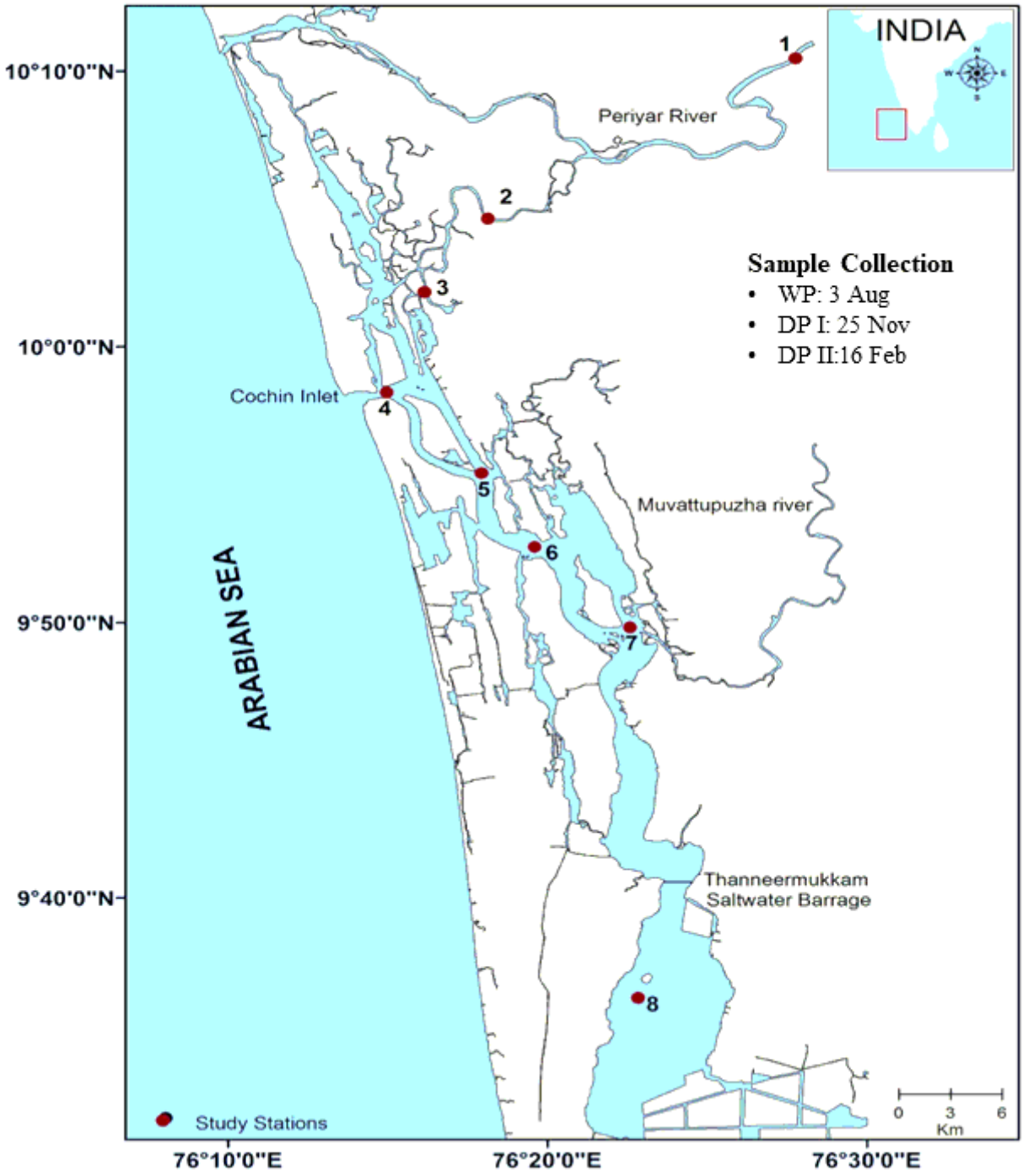

Figure 1

Station locations in the Cochin estuary (CE). A total of 8 stations were sampled. Stations S1 to S3 lies in the north of the inlet, S4 and S5 to S8 lies toward the upstream (south) of the estuary. S8 lies beyond the Thaneermukham barrage, which protects the paddy fields from salinity incursion during dry months.

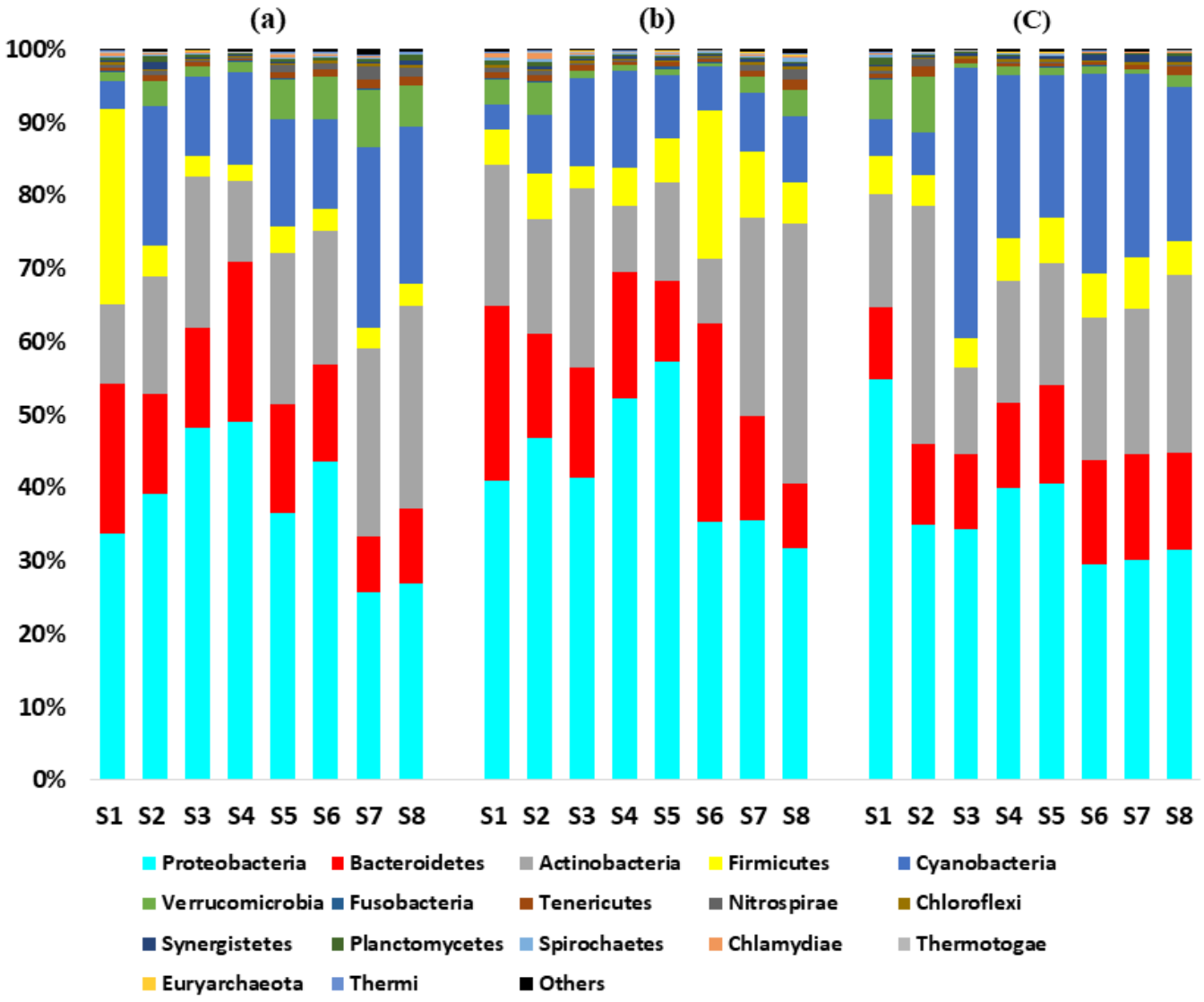

Figure 2

Phylum level taxonomic distribution of major and minor bacterial communities from eight sampling locations during a) Wet period (b) Dry period I and (c) Dry period II, in the Cochin estuary (CE).

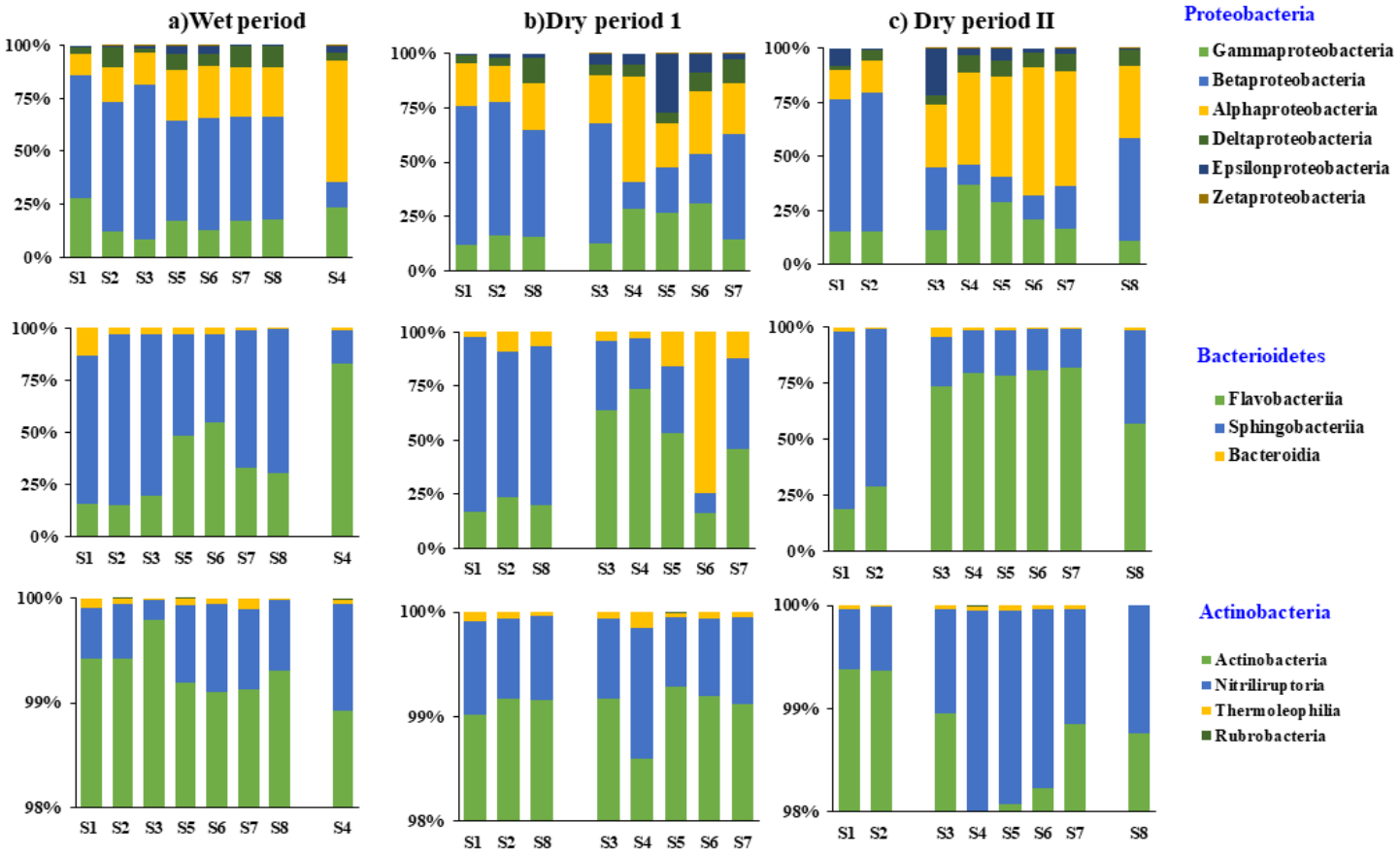

Figure 3

Class level distribution of dominant bacterial phyla, Proteobacteria, Bacteriodetes and Actinobacteria from eight sampling locations during a) Wet period (b) Dry period I and (c) Dry period II, in the Cochin estuary (CE).

Figure 4

Genus level distribution of dominant bacterial communities, from eight sampling locations during a) Wet period (b) Dry period I and (c) Dry period II, in the Cochin estuary (CE).

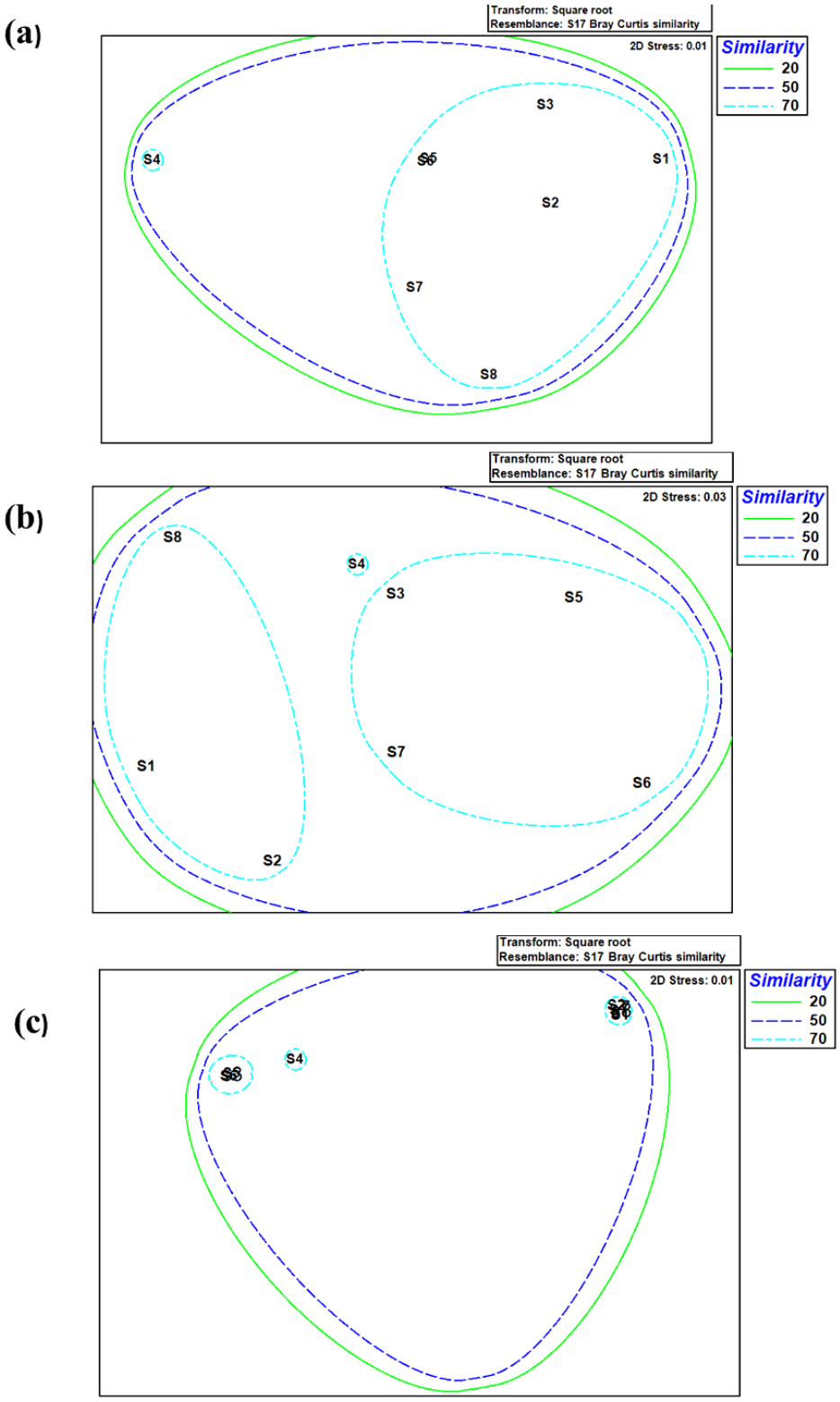

Figure 5

Non- metric multidimensional scaling (NMDS) analysis showing different clusters of bacterial communities during a) Wet period (b) Dry period I and (c) Dry period II, in the Cochin estuary (CE).

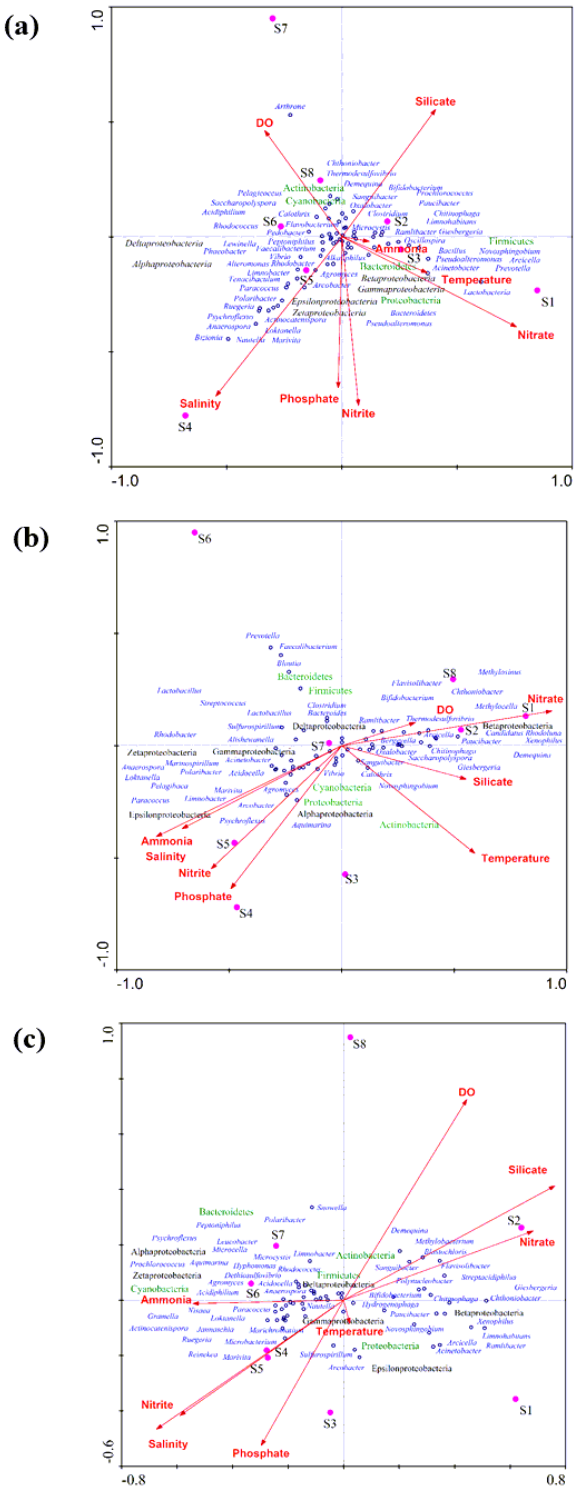

Figure 6

Canonical Correspondence Analysis (CCA) representing multiple correlation among all environmental and biological parameters. The sampling stations are marked in magenta filled circles and environmental parameters are represented as red arrows. Major bacterial phyla, class and genus are marked in black, green and blue colors, during a) Wet period (b) Dry period I and (c) Dry period II, in the Cochin estuary (CE).

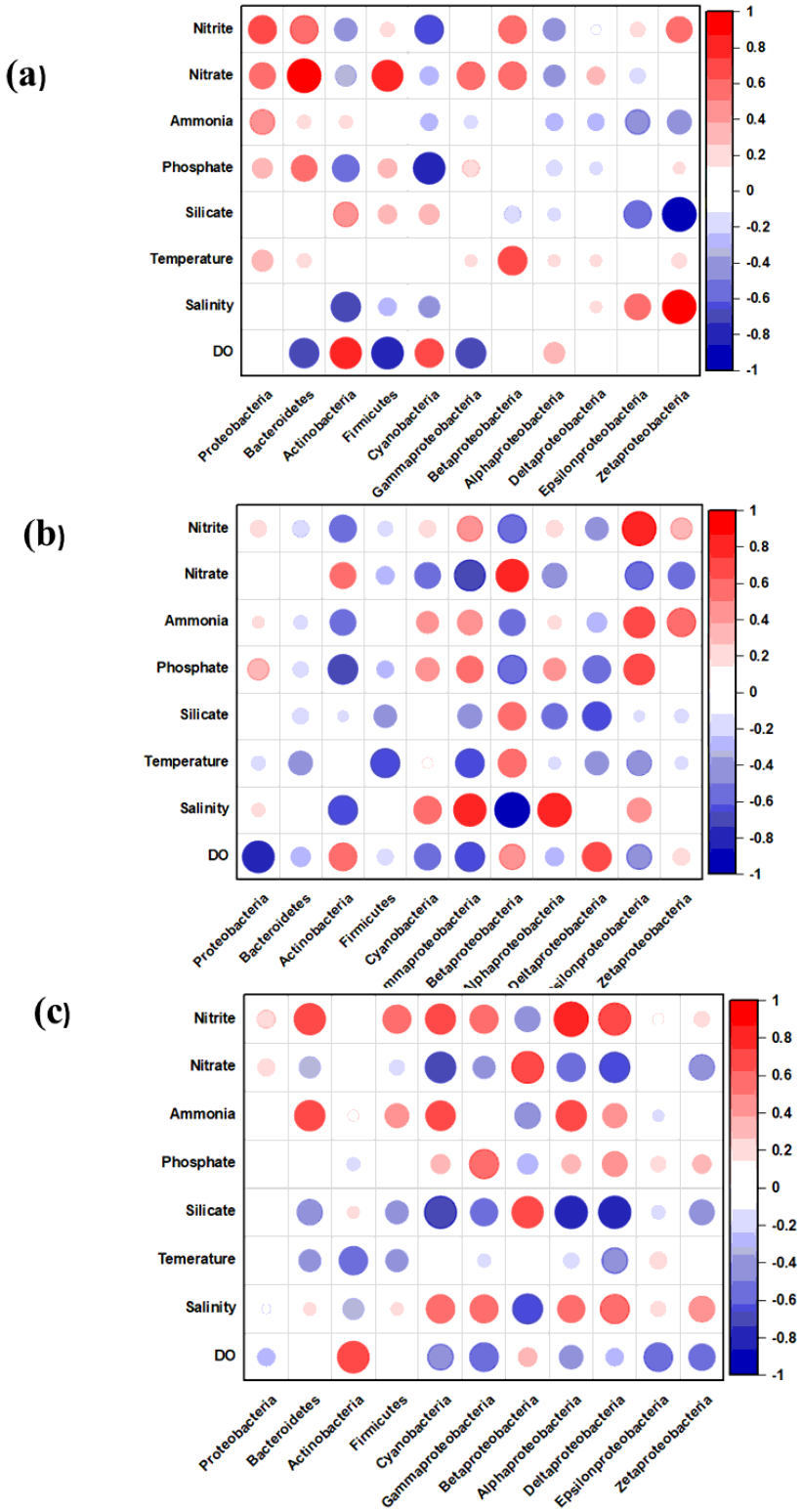

Figure 7

Correlogram showing the correlations between the dominant phylum and class with environmental variables during a) Wet period (b) Dry period I and (c) Dry period II, in the Cochin estuary (CE). The Pearson correlation coefficients in the correlogram plot are colored based on the value and on the degree of association among the variables. Red and blue colors represent significant negative correlations and positive correlations. Darker color represents stronger correlations

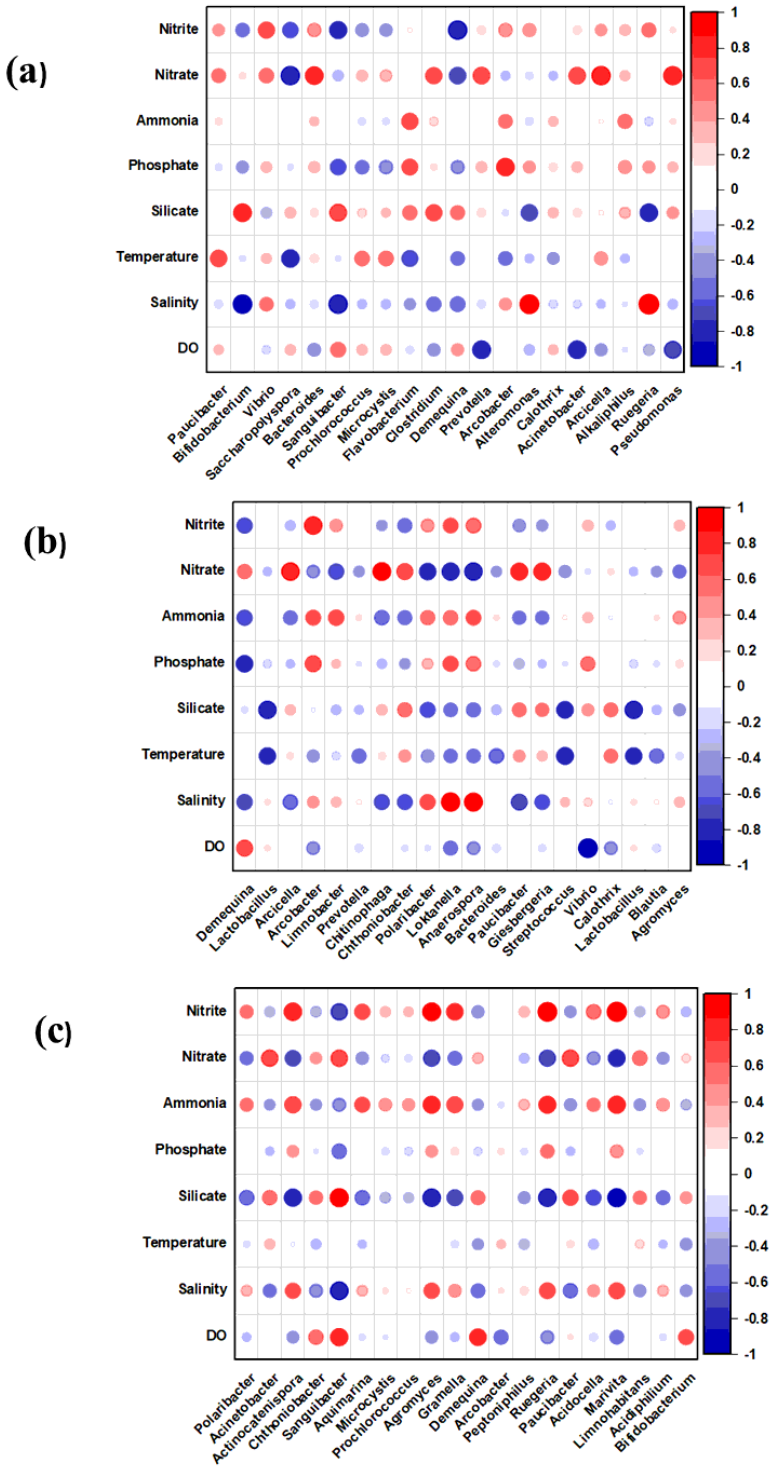

Figure 8

Correlogram showing the correlations between of most dominant 20 genera with environmental variables during a) Wet period (b) Dry period I and (c) Dry period II, in the Cochin estuary (CE). The Pearson correlation coefficients in the correlogram plot are colored based on the value and on the degree of association among the variables. Red and blue colors represent significant negative correlations and positive correlations. Darker color represents stronger correlations

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