

Microbial diversity in a coastal environment with co-existing upwelling and mud-banks along the south west coast of India

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Abstract

Upwelling and mud banks are two prominent oceanographic features in the coastal waters along the south west coast of India during the southwest monsoon (MON) season. The present study investigates the microbial diversity in the coastal environments of Alappuzha, India, where upwelling and mud banks co-exist. Water samples were collected from three stations, M1, M2, and M3, on a weekly basis to estimate the physico-chemical parameters and microbial abundance. Presence of cold waters (<26°C) with high nitrate (6-8 µM) and low dissolved oxygen (5 µM) in the sub surface waters during monsoon confirmed the presence of upwelling at all the three stations. Simultaneously, presence of unusually calm waters was seen at M2 alone during monsoon indicating the formation of mud banks. The microbial diversity was determined from three stations, with distinct oceanographic conditions (M1: coastal reference station with only upwelling, M2: Mud banks + upwelling, and M3: offshore reference station with only upwelling). The water samples were collected during two seasons, pre-monsoon (PRM) (April) and monsoon (M) (July) and analysed using 16S rRNA-based Illumina high-throughput metagenomic sequencing. Proteobacteria was the most dominant phyla, followed by Bacteroidetes, Firmicutes, Cyanobacteria, Actinobacteria, and Verrucomicrobia in order, with variations in their relative abundance spatially and seasonally. Though the microbial abundance increased during monsoon at all the stations, the relative abundance of most of the bacterial phyla except Proteobacteria decreased during monsoon season. Interestingly, most of the sequences at M2 during mud banks were unclassified at the class level indicating the presence of unique microbial populations in this station. Prediction of metabolic activity revealed ammonia oxidation, nitrite reduction, sulphate reduction, xylan degradation, dehalogenation, chitin degradation, etc. as important functions. The metabolic activity throws light on the role of microbes in this environment thereby providing a system-scale perspective of microbial community interactions.

Keywords: Upwelling, bacterial metagenome, mud banks, upwelling, Archaea.

Introduction

Upwelling is a basin-wide oceanographic process occurring at various places around the globe. Upwelling occurs during the south west monsoon (MON) season along the southwest coast of India. It brings cold, nutrient-rich and hypoxic waters to the surface resulting in enhanced biological production in the surface waters. The western continental shelf of India is the only region where the upwelling occurs in conjunction with high fresh water influx (due to monsoonal rains) resulting in strong stratification of the water column. This leads to increased respiration and development of coastal hypoxia [1, 2].

On the contrary, mud banks are clearly demarcated areas of calm littoral waters adjoining the coast during the roughest south west monsoon conditions in the Arabian Sea [3]. The calmness is caused due to the damping of waves by the mud suspensions [4, 5]. The remarkable calmness of mud banks provides conducive environment for fishing, while the surrounding sea is unsuitable for fishing during monsoon due to heavy monsoonal waves. Thus, mud banks have significant socio-economic relevance as they provide a fishing zone for subsistence fishermen and protect the beaches from erosion. It has a semi-circular shape extending up to about 6 km from shore at a water depth of about 7-15 m and about 4 km alongshore. Unlike upwelling, the mud banks are purely coastal processes confined to small stretches occurring at only few places along the south west coast of India [6].

The south west monsoon plays a significant role in the formation of mud banks and upwelling with the maximum observed activity during July-August [7]. Among the several mud banks that occur along the southwest coast of India, the Alappuzha mud banks are unique for their consistency in the annual appearance and popular fishery [3]. The Alappuzha mud banks is a rare place where mud banks and upwelling occur concurrently. It was recently demonstrated that the upwelled waters (nutrient-rich and hypoxic) intrude to the mud bank region [5, 8]. The biology of this region is influenced by upwelling and summer stratification, resulting in eutrophic and oligotrophic conditions, respectively. As a result, a noticeable seasonal shift in plankton biomass is reported along the south eastern Arabian Sea [3]. However, information on microbial players in ecosystems where large-scale oceanographic processes such as upwelling and intermittent phenomena like mud banks co-exist is lacking in the literature. This study forms a part of a multidisciplinary oceanographic program of CSIR-National Institute of Oceanography, India, on Alappuzha mud bank process studies (AMPS, 2014), which was executed to study the formation mechanism of mud banks and also to investigate its biological aspects. This paper describes the microbial abundance and diversity in the coastal waters off Alappuzha, influenced by co-occurring coastal upwelling and mud banks. Apart from that, the methane concentrations of the mud bank region exhibited an abrupt reduction (< 50 nM) during south west

monsoon (against 400 nM during pre-monsoon) [8]. We hypothesize that microbes play important roles in the biogeochemical processes of this unique environment and that the observed changes in methane concentrations could also be linked to microbial processes. This forms the first study to elucidate the microbial diversity and the various biogeochemical roles of microbial communities in an upwelling and mud bank region along the south west coast of India.

Materials and Methods

Site description and sampling

In the present study, an 18 week sample collection was organized at three locations (M1, M2, and M3) in the Alappuzha mud banks from April to September 2014 (Fig.1). Station M1 (7 m) served as a coastal reference point, while station M2 (7 m) was located inside the mud bank. Station M1 was located 7 km north of M2, and M3 served as the offshore reference station at 13 m depth to the west of M2.

Physico-chemical parameters

Temperature and conductivity were measured at each location using a CTD profiler (SBE 19 plus Seabird Scientific, USA) with accuracies of $\pm 0.001^\circ\text{C}$ for temperature and ± 0.0001 psu for salinity. Water samples were collected from 3 depths (surface, mid depth and bottom) at M1 and M2 and from 5 depths (surface, 3m, 6m, 9 m and bottom) at M3 using a 5 L Niskin bottle for estimating dissolved oxygen (DO), methane (CH_4), and inorganic nutrients. DO samples (ml L^{-1}) were immediately fixed by adding 0.5 ml each of Winkler reagents and titrated against standard thiosulphate using starch as the visual end point detector. Dissolved inorganic nutrients (in μM) such as ammonia ($\text{NH}_4\text{-N}$), nitrite ($\text{NO}_2\text{-N}$), nitrate ($\text{NO}_3\text{-N}$), phosphate ($\text{PO}_4\text{-P}$), and silicate ($\text{SiO}_4\text{-Si}$), were analysed spectrophotometrically by following standard procedures using a Spectrophotometer (Shimadzu UV 1800) [9]. The methane samples (measured in nM) were analysed using a Gas Chromatograph following standard protocols [10]. Chlorophyll *a* (Chl *a*) was estimated using standard procedure [11].

Microbial abundance (MA)

For the estimation of microbiological parameters, water samples were transferred to 30 ml sterile acid washed polypropylene bottles, kept in iceboxes and brought to the laboratory. Briefly, 1 ml of formaldehyde-fixed sample was filtered under vacuum (20 Kpa) through a $0.2 \mu\text{m}$ pore-size (25 mm diameter) black nucleopore filter (Whatman USA) backed by $0.8 \mu\text{m}$ cellulose mixed ester membrane (Millipore USA). The filter papers were stained with 100 μl of acridine orange (0.1g/100 ml) and enumerated using epifluorescence microscope. For each filter, 10-20 fields were selected randomly

and a total of >200 microbes were counted at 1000 X using an epifluorescence microscope (Olympus BX 41, Japan) under blue excitation [12].

Sample processing for metagenome analysis

Water samples (10 L) were collected from the surface waters from three stations, M1, M2 and M3 on two occasions, pre-monsoon (PRM) (April), and monsoon (M) (July). Samples were collected three times (considered as triplicates) at weekly intervals in each season. Each 10 L sample was filtered through a 0.22 µm cellulose filter membrane (Millipore, USA). The filter paper was cut into 4 pieces and DNA from a quarter of the filter paper was extracted using the Invitrogen Genomic DNA isolation kit according to the manufacturer's instructions [13]. DNA was stored at -20°C until further downstream processing. The triplicate DNA samples in each season were pooled together, and thus obtained two samples representing each season, PRM and M. The pooled DNA samples were quantified using a nanodrop spectrophotometer (Nanodrop 2000, Thermofischer Scientific, USA), and 25 ng/µL of DNA was used for sequence analysis. The first PCR amplified V3-V4 region of 16S region with specific primers with added Illumina adapter overhang nucleotide sequences (16S Forward Primer 16S_341F, 5'-TCGTCGGCAGCGTCAGATGTGTATAAAGAGACAG CCTACGGGNGGCWGCAG and 16S Reverse Primer, 16S_805R 5'-GTCTCGTGGGCTCGGAGATGTGTATAAAGAGACAGGACTACHVGGGTATCTAATCC). PCR conditions included 95 °C initial denaturation for 3 min, followed by 25 cycles of 95 °C for 30 s, 62.3 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. The attachment of 'tag' sequence that is complementary to Illumina sequence adapters and index primers were performed using the above amplicon PCR product and Nextera XT index primers from the Nextera XT Index kit V2 with an initial DNA concentration of 25 ng/µl. The PCR conditions included 95 °C initial denaturation for 3 min, followed by 8 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min [14]. The library was purified and validated for quality by running an aliquot (1:10 Dilution) on high sensitivity Bioanalyzer Chip (Bioanalyzer, Agilent Technologies, 2100, https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf).

Illumina sequencing

PCR products were purified using PureLink PCR Purification kit (Invitrogen, CA, USA). The purified, pooled sample was diluted to 4 nM final concentration using resuspension buffer (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 the number of samples pooled in each sub-library. Pooled libraries were further diluted down to a final 15 pM library 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*250 mode on an Illumina MiSeq next generation sequencer (Illumina, CA, USA) at Genotypic Technology Pvt. Ltd (Bangalore, India) [13]

(<https://www.illumina.com/content/dam/illumina-marketing/documents/products/datasheets/hiseq-3000-4000-specification-sheet-770-2014-057.pdf>).

Initial processing of sequence reads

Demultiplexing was completed using bcl2fastq conversion software embedded in MiSeq. The automated FASTQC (QIIME) Tool Kit application on Illumina Base Space Labs was used to filter out quality reads, in which quality reads above Q30 were kept for downstream analysis. Within the application, the TagCleaner software was used to remove the adapter sequences (https://www.illumina.com/documents/products/technotes/technote_Q-Scores.pdf)

Analysis of sequence data

The raw sequence data was analysed using the QIIME pre-processing [15] application (Illumina BaseSpace, Version 1.0.0). Pre-processing included demultiplexing, quality filtering, OUT picking, and taxonomic assignment using Greengenes database (version 13.5). A BIOM file was created using the QIIME outputs, and a multivariate analysis technique, known as principle coordinate analysis (PCoA), was applied to the dataset using R Studio, which made it easier to visualize the data. PCoA was generated using classical MDS on a Pearson covariance distance matrix [16].

Statistical analysis

Goods coverage, an alpha diversity metric was calculated from the biome file (QIIME, alpha_diversity.py). Coverage indices provide information on what fraction of OTUs has been picked up more than once. Richness and diversity was calculated using Shannon-Weaver and Simpson diversity index. These diversity indexes and richness estimators are useful statistical tools for comparing the relative complexity of communities and for estimating the completeness of sampling. ANOSIM analysis was performed to confirm significant differences between the stations and seasons (pre-monsoon-PRM and monsoon-M). Comparative metagenomic studies was performed using a user friendly, web-based analytical pipeline, METAGENassist (<http://www.metagenassist.ca/METAGENassist/faces/Home.jsp>) [17].

MetaGeneMark prediction software was used to access to gene prediction in metagenomes, utilizing metagenome parameters and gene prediction program [18].

Nucleotide sequence accession number

The Illumina sequencing data were deposited into the NCBI Sequence Read Archive (<https://submit.ncbi.nlm.nih.gov/subs/>) under the bioproject number, PRJNA402275, and SRA number SUB3020743.

Results

Physico-chemical parameters

The observed changes in the physico-chemical parameters in the three locations during the 18-week study period are presented (Table 1). The first indication of upwelling (lowering of temperature and dissolved oxygen) was observed in the 2nd week of May. The water temperature started to decrease from 29°C to 25°C from mid-May to mid-June, indicating the presence of intense upwelling. Upwelling persisted at all the stations till 2nd week of August. The water temperature became warmer (>26°C) from the 3rd week of August till the end of September. The water column was well oxygenated up to the 2nd week of May and presence of less oxygenated-upwelled waters was noticed with the onset of monsoonal rains (Supplementary Fig. 1). Interestingly, during the peak upwelling period, hypoxic waters intruded in to the entire study area (Supplementary Fig. 1). The AMPS report on the presence of hypoxic water (5 µM DO) in the shallow regions very close to shore. In order to avoid hypoxic waters, fishes probably aggregate at the surface waters during monsoon. Additionally, high concentration of nitrate (6-8 µM) in the cold (< 26°C) subsurface waters was noticed over the entire study region during monsoon indicating upwelling (Table 1). Coinciding with the peak upwelling period, the Alappuzha mud banks was formed (unusually calm waters in a small area during the rough monsoon season) by mid-June at M2 and persisted there till the end of monsoon (September 2014). Interestingly, there was a considerable reduction in the dissolved methane levels from pre-monsoon (400 nM in April) to monsoon (< 50 nM). This anomaly (maximum methane concentration in oxic conditions and minimum in hypoxic conditions) was noticed at M1 and M3, whereas at M2, methane concentrations were irregular. This methane anomaly could be attributed to some microbial processes associated with hypoxic conditions. Further, the substantial nitrogen deficit (N* up to – 15 µM) obtained from the present study and the reduction in the methane levels indicated mud banks as active sites of denitrification (Supplementary Fig. 1).

Microbial abundance

The total microbial abundance during the 18-week study period ranged from $6.6 - 15.5 \times 10^5$ cells mL⁻¹ at M1 (Fig. 2 a), $7.5 - 20.4 \times 10^5$ cells mL⁻¹ at M2 (Fig. 2b) and from $5.9 - 19.4 \times 10^5$ cells mL⁻¹ at M3 (Fig. 2c). The microbial abundance decreased with increasing depth at all the three sampling locations. The microbial abundance was significantly ($p < 0.001$) high in M (monsoon) samples compared to PRM (pre-monsoon) irrespective of the sampling locations. The highest microbial abundance was observed during June and July, when the upwelling and the mud bank activity was at its peak. With the onset of monsoon, the microbial abundance increased by 22%, 18%, and 20% at M1, M2, and M3 respectively.

Taxonomic richness and diversity of the microbial community

Sequence tags based on a BLASTN search in RefHVR_V3 database were clustered into groups. The number of valid reads were significantly higher during pre-monsoon at M1 (254,649), M2 (201,785) and M3 (184,201) when compared to monsoon (151,996 in M1 and 68,655, in M2 and 128,493 in M3) (Table 2). The number of species was highest in the upwelled waters (805 species at M3) when compared to M2 where upwelling and mud banks co-existed (407 species). The unidentified species were unusually high at species level (42.83-93.67%) at M2. The overall species coverage was $\geq 44.31\%$ for M1 and 28.33% for M2 and 44.84 % in M3.

The Good's coverage value (0.99) indicated that most of the microbial diversity was detected in the samples. However, Shannon diversity indices representing the abundance and evenness of the species, decreased during upwelling, both at M1 and M3. The diversity was lowest at M2 during MON where upwelling and mud banks coexisted (Table 2). The results indicated that the bacterial community diversity during pre-monsoon period were richer than the upwelling period. Principal coordinate analysis (PCoA) showed that the M2 during monsoon is significantly different from M1 and M3, which clustered together, but were separated from M1 BM and M3 BM which formed another cluster (Fig. 3). Similarly, UPGMA clustering results showed that the bacterial communities in offshore (M3) and coastal station (M1) with upwelling alone were quite similar compared to M2, where mud banks and upwelling coexisted (Fig. 3). Comparative sequence analysis showed shared OTUs between different water samples, of which, 22 sequences were shared by all the six samples (Fig. 3c). At all stations the dominant phyla such as Proteobacteria, Bacteroidetes, Firmicutes, Cyanobacteria, Actinobacteria, Verrucomicrobia were represented, but their relative abundances varied spatially (stations) and temporally (with season). ANOSIM analysis demonstrated that the differences between the three stations (spatial) and two seasons (temporal) at phylum level were significant (stations: $R =$

0.45, $p < 0.01$, seasons: $R = 0.89$, $p < 0.01$). Heat map of the top 29 taxa at phylum level indicated significant spatial (stations) and temporal (with season) differences in microbial composition ($p < 0.01$) (Supplementary Fig. 2).

Taxonomic composition of the microbial community.

Bacteria were the most abundant prokaryotic domain (99%), whereas archaeal reads showed a relative paucity (1%). At the phylum level, all OTUs could be classified and was found to be dominated by Proteobacteria (58.46 to 85.91% of the total amplicons), followed by Bacteroidetes ($14.40 \pm 5.14\%$), Firmicutes ($1.4 \pm 4.91\%$) Cyanobacteria ($0.89 \pm 4.77\%$), Actinobacteria ($2.36 \pm 1.88\%$), and Verrucomicrobia ($0.93 \pm 1.93\%$) in order (Fig. 4). Interestingly at M2, where upwelling and mud banks co-occurred, the proteobacterial percentage was as high as 80.91%. However, at M2, 83.97 % of sequences were unclassified at class level. Though sequencing of this sample was repeated, it yielded more or less same results (80.20% unknown sequences).

In M1, the relative abundance of Proteobacteria increased from pre-monsoon (58.42%) to monsoon (70.16%) (Fig. 4). However, the percentage abundance of Bacteroidetes did not show much variation with the onset of monsoon (10.38% during pre-monsoon and 10.911% during monsoon). But, the percentage abundance of other phyla decreased with the onset of monsoon, viz, Actinobacteria (PRM-5.02%, M-3.07%), Cyanobacteria (PRM-4.77%, M-2.75%), Firmicutes (PRM-4.91%, M-2.45%), Verrucomicrobia (PRM-4.88%, M-0.30%), Chloroflexi (PRM-1.55%, M-0.46%), and Planctomycetes (PRM-1.09%, M-0.12%). The percentage abundances of Proteobacteria showed the same trend at M2 and M3, with marginal increase (76.48 % to 85.92% at M2 and from 68.21% to 71.63 % at M3) in their abundance during monsoon compared to pre-monsoon (Fig. 4). The relative abundance of Bacteroidetes and Firmicutes decreased during mud bank period at M2, whereas, cyanobacterial abundance increased during monsoon. Bacteroidetes and Cyanobacterial abundance decreased at M3 during monsoon compared to pre-monsoon period, while abundance of Firmicutes increased during monsoon. The relative abundance of dominant class Alphaproteobacteria increased at all the stations during monsoon period. However, the percentage abundance of Gammaproteobacteria increased only in M1 and M2, whereas their abundance decreased at 10.22%. The class Betaproteobacteria, Deltaproteobacteria and Epsilonproteobacteria were represented at all the stations and their abundance was marginally higher during monsoon (Fig. 5).

Among Archaea, methanobacteria, methanomicrobia and halobacteria, were present at M1, M2 and M3, respectively before upwelling followed by a decline in their abundance during upwelling (Fig. 6a, and 6b). The phylum *Crenarchaeota* was represented by Thaumarchaeota and Thermoprotei (Fig.

6c). The diversity of archaea was significantly high during pre-monsoon especially at M1. Methane levels were high (400 nM) during PRM at M1 (Fig. 6d). Methanogenic archaea like *Methanolinea*, *Methanosphaera*, *Methanosaeta*, *Methanosarcina* dominated M1, which could be one of the reasons for high methane levels at M1 during pre-monsoon (Fig. 6e). The diversity of Archaea was significantly high during premonsoon especially at M1 (Fig. 6e). Members of the species *Paracoccus sulphuroxidans* are metabolically versatile and are widely distributed group also detected from the mud bank region.

Functional prediction of dominant microbial communities

Comparative metagenomic studies indicated that bacterial populations in the mud banks were involved in various ecological activities such as xylan degradation, ammonia oxidation, dehalogenation, nitrite reduction, sulphate reduction, and sulphide oxidization. The relative contribution of the metagenome to metabolic functions included major activities like dehalogenation (22.08%), sulfate reduction (21.38%), nitrate reduction (20.54%), ammonia oxidation (19.64%), and sulphide oxidation (8.77%) (Fig. 7). The other functions were lignin degradation (0.95%), chitin degradation (2.92%), hydrocarbon degradation (2.36%), xylan degradation (3.03%), atrazine degradation (1%) etc.

Discussion

This study forms the first report on the distribution and diversity of microbes from coastal waters along the south west coast of India influenced by both upwelling and mud banks. Upwelling signals were observed at all the locations, but mud banks was visible only at M2 during monsoon. There were significant variations in the physico-chemical conditions during the two seasons (pre-monsoon and monsoon). The nutrient concentrations were comparatively low during the pre-monsoon period. In general, the nutrient concentrations is low in the south eastern Arabian Sea and is biologically less productive during PRM. Clear upwelling signals were observed with a significant drop in temperature ($p < 0.01$) and dissolved oxygen levels ($p < 0.05$) in the sub surface waters during the peak south west monsoon season. High nutrient concentrations synchronized with the coastal upwelling in the study region. Upwelling, being a basin scale phenomenon, its immediate biological implication is the enhanced biological production and fish stock, along the entire south-western continental shelf [19]. The APMS studies report the presence of upwelled waters ($< 5 \mu\text{M}$) closer to the shore [5, 8]. However, there were no significant ($p < 0.05$) spatial variations (at M1, M2 and M3) in physico-chemical parameters, but temporal variations (pre-monsoon and monsoon) were significant ($p < 0.05$).

The microbial abundance was low during the pre-monsoon season probably due to low nutrient concentrations in the coastal waters during the PRM. However, there was an increase in microbial abundance with the onset of monsoon throughout the study area. The south west coast of India is characterized by numerous rivers (>50 numbers) that bring in allochthonous nutrients, with their maximum flux during the monsoon period. The nutrient rich waters brought in by upwelling in this region support high Chl *a* concentrations (7-8 mg m⁻³) during monsoon against 3 mg m⁻³ during pre-monsoon season [20]. High nutrient concentrations during monsoon support high primary and secondary production. In coastal upwelling ecosystems, the biological community thrives in an environment of high availability of organic molecules derived from high primary production rates, thus enhancing microbial secondary production [21]. Bacterial communities shift over time scales of several weeks during the upwelling period due to physical structuring of microbial communities and/or biotic interactions between the bacteria and the phytoplankton. Hence, the increase in microbial abundance could be due to the combined effect of coastal upwelling and riverine influx during monsoon season.

This study forms the first metagenomics study in an ecosystem with co-existing upwelling and mud banks. The most dominant phylotypes were found to be Proteobacteria, Bacteroidetes, Cyanobacteria, Firmicutes and Actinobacteria during both the seasons (PRM & M). The presence of bacterial phylotypes are critical as they vary according to the biogeochemistry of a particular environment [22]. Hence, knowledge on the microbial diversity is imperative particularly because of their significant biogeochemical roles in the coastal environs. The phylum Proteobacteria was the most dominant phyla irrespective of seasons, though their relative abundance varied across the sampling locations. The abundance patterns were similar along the stations indicating similar lifestyles, but the temporal variations in their percentage abundance suggests that different taxonomic groups could be influenced by different growth-controlling factors [23]. Oceanographic processes like upwelling induce spatial variability in the structure of the bacterial assemblages in marine systems [24-26]. The dominance of these taxonomic groups was in accordance with previous studies in the eastern Atlantic Ocean and northern Benguela upwelling [27-29]. The taxonomic composition in the M1, M2 and M3 were also comparable with the microbial communities in many marine and freshwater ecosystems such as Shark Bay in Western Australia [30,31], Highbourne Cay in Bahamas [32, 33], and Pavilion Lake [34].

Among Phylum Proteobacteria, Alphaproteobacteria was the most dominant class at M1, whereas Gammaproteobacteria dominated at M2 and M3. Alpha- and Gammaproteobacteria are actively involved in the carbon fixation pathway through the Calvin Benson Bassham (CBB) cycle

[35]. The Betaproteobacteria which is typically considered as 'freshwater' groups observed high percentage in the coastal station, M1 compared to other stations. The high abundance of Gammaproteobacteria, Roseobacter and Bacteroidetes were comparable with African upwelling region [36]. High abundance of Gammaproteobacteria had been reported to be associated with upwelled nutrients [27, 37, 39] and Roseobacter with high phytoplankton biomass [39]. Among Gammaproteobacteria, members of the family Alteromonadaceae were the most dominant at M2 in mud banks during monsoon. In pelagic environments, they contribute up to 30% of bacterial communities [40]. Studies in the North Sea and north coast of Crete revealed habitat preferences for Alteromonadales, which varies according to the substrates available in the marine ecosystems or in response to changing environmental conditions [41]. Another dominant phylum, Bacteroidetes have been frequently reported from regions of high nutrient inputs, algal blooms and upwelling systems [27, 42-44]. Verrucomicrobia is commonly abundant in marine bacterial communities [45]. Recent studies provide evidence for high polysaccharide activity of Verrucomicrobia, in which polymer degradation may be more efficient than Bacteroidetes [28, 46]. Consequently, Bacteroidetes are involved in xylan degradation and hence, these polysaccharide-degrading bacteria are copious in organic-rich environments. Interestingly, Xylan degradation is one of the major functions of the microbial communities in the metagenome obtained in this study (Fig 7).

The microbial abundance increased during monsoon at all the stations, but the relative abundance of most of the phyla except Proteobacteria decreased during monsoon season. This could be due to increase in turbidity during monsoon due to increased wave action, riverine influx, mud banks and high biological production. Increase in turbidity would increase the bulk of particle-associated microbial assemblages thereby reducing the free-living bacteria. A portion of the particle-associated bacterial groups might have got attached to particles and might have got removed in the prefiltration process in the diversity analysis in the present study. The turbidity of the water column during the study period was higher during monsoon when compared pre-monsoon [4, 5]. The turbidity was highest at the coastal station M2 (PRM 3.55 ± 0.55 , M- 5.15 ± 1.95) when compared to M1 (PRM 2.7 ± 0.5 , M- 3.55 ± 0.9), and M3 (PRM 1.15 ± 0.45 , M- 2.56 ± 1.6). A study from Columbia River metagenome analysis revealed that Bacteroidetes were found to be involved in water column particle colonization [47]. This could be the reason for decrease in percentage abundance of Bacteroidetes during monsoon in the present study. There are several studies suggesting that the bulk of heterotrophic production has often been found to be associated with particle-attached bacteria rather than with their free-living counterparts [48, 49].

Though contributed only to 1% of the total prokaryotic assemblage, Archaea belonging to Euryarchaeota and Crenarchaeota, involved in methanogenesis were also represented in this study. Archaea belonging to both Euryarchaeota and Crenarchaeota has been reported to contribute considerably to carbon and nitrogen cycles in coastal waters by methanogenesis [50]. Euryarchaeota usually dominate the coastal planktonic community with high particle and nutrient load [51], whereas, Crenarchaeota is reported to be more abundant in deeper waters [52-54].

Prediction of metabolic activity revealed ammonia oxidation, nitrite reduction, sulphate reduction, dehalogenation, xylan degradation, and chitin degradation. The metabolic activities recognized in this study were common metabolic activity of the marine microbial communities [55]. In fact, several reports suggest that upwelling conditions corresponded to increase in pathways of nitrate reduction (denitrification, nitrite reduction, nitrite and nitrate ammonification), coupled with ammonia assimilation, and even nitrogen fixation [56, 57]. Previous reports showed that Actinobacteria and Proteobacteria are especially capable of conducting carbon metabolism, membrane transport, and the stress response regulatory system [58]. However, the *in situ* activity rates, metabolic characteristics and their biogeochemical constraints in this study area need to be clarified because microbial diversity, distribution and abundance depends on different physical and chemical conditions, biogeography and geological history of the environment [59,60].

Conclusion

The present study reveals the microbial diversity in the coastal environments of Alappuzha, India, characterised by unique oceanographic phenomena of co-existing mud banks and upwelling. The region was characterised by dominance of prokaryotic communities belonging to Proteobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia, Actinobacteria etc. The microbial communities in stations, M1 and M3 (with only upwelling) were similar and were significantly different from M2 with both mud banks and upwelling. More than 82% of the sequences were unclassified at the class level in M2 even after repeated sequencing experiments. It is possible that the microbial assemblages are unique in this region. Most important predicted metabolic activities included ammonia oxidation, nitrite reduction, sulphate reduction, dehalogenation, xylan degradation, and chitin degradation. To conclude, our study gives valuable insights on the diversity and biogeochemical roles of bacterial communities in the upwelling and mud bank regions.

Abbreviations

CTD-conductivity temperature depth profiler, DO-dissolved oxygen, CH₄- methane, BA-Bacterial abundance, TVC- Total viable counts, PRM- pre-monsoon, M-Monsoon, EtBr- Ethidium Bromide,

PCoA- Principal coordinate analysis, OTUs- Operational Taxonomic Units, UPMA-Unweighted pair group method with arithmetic averages, DOC-dissolved organic carbon, AOA-Ammonia-oxidizing Archaea and AOB-ammonia-oxidizing Bacteria.

Competing interests

The authors declares that they have no competing interests.

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Legends

Fig. 1 Study area indicating the sampling locations, M1, M2 and M3. Location M2 represent mud bank, M1 and M3 represent coastal and offshore references respectively. Mud bank region is indicated as a semicircle. Station map was created with the tool QGIS (version 2.18, Quantum GIS Development Team, 2017. Quantum GIS Geographic Information System. Open Source Geospatial Foundation Project. "<http://qgis.osgeo.org>").

Fig. 2 Figure showing the total microbial abundance on a fortnightly basis at a) M1 b) M2 and c) M3 from April to September, 2014. The sampling dates are marked in the X-axis depth in the Y-axis and bacterial abundance in the Z-axis. The microbial abundance is expressed in 10^6 cells mL^{-1} .

Fig. 3 Comparison of microbial metagenome at the three sampling stations during pre-monsoon (PRM) and monsoon (M) (a) Nonmetric Multidimensional Scaling (NMDS) of Bray–Curtis similarity index Sampling locations (M1, M2 and M3) during pre-monsoon (PRM) and monsoon (M), b) Dendrogram based on Bray–Curtis similarity between stations during pre-monsoon (PRM) and monsoon (M). (c) Venn diagram depicting the uniqueness and sharing at species level among the stations during pre-monsoon (PRM) and monsoon (M)

Fig. 4 Phylum level classification at the sampling locations, M1, M2 and M3 during pre-monsoon (PRM) and monsoon (M)

Fig. 5 Class level distribution of microbial communities at M1, M2 and M3 during pre-monsoon (PRM) and monsoon (M) a) Proteobacteria b) Bacteroidetes c) Cyanobacteria

Fig. 6 Classification of Archaea during pre-monsoon (PRM) and monsoon (M) at three stations (M1, M2, and M3) a) Archaeal diversity b) Euryarchaeota and c) Crenarchaeota d) methane distribution and e) species level distribution at M1, M2, and M3 during pre-monsoon (PRM) and monsoon (M)

Fig. 7 Figure showing prominent metabolic predictions of the bacterial communities during monsoon

Parameters	M1	M2	M3
MA (10 ⁶ /mL)	3.49-14.44	2.73-16.27	2.82-14.98
Chl <i>a</i> (mg/m ³)	0.66-20.29	0.49-19.17	0.24-26.14
DO (ml/L)	3.01-251.92	6.30-242.05	3.10-355.78
pH	7.30-8.38	7.69-8.30	7.39-8.36
NO ₂ (μM)	0.00-2.19	0.01-1.70	0.00-1.79
NO ₃ (μM)	0.08-8.75	0.01-10.74	0.02-20.60
NH ₄ (μM)	0.26-39.07	0.06-11.83	0.02-21.44
PO ₄ (μM)	0.14-2.14	0.13-2.02	0.05-3.04
SiO ₄ (μM)	1.64-26.34	0.83-32.31	0.09-24.27
CH ₄ (nM)	30-275	25-225	12-100
Salinity	26.98-35.59	27.52-35.41	27.84-38.02

Table 1. The range of biological and physicochemical parameters. MA- Microbial abundance, Chl *a* -Chlorophyll *a*, Sal-Salinity, DO – Dissolved Oxygen, NO₂ – Nitrite, NO₃ – Nitrate, NH₄- Ammonia, PO₄ - Phosphate, SiO₄ – Silicate and CH₄-Methane.

Parameters	M1		M2		M3	
	PRM	M	PRM	M	PRM	M
Total reads	254,649	151,996	201,785	68,655	184,201	128,493
Unclassified reads	2.47 %	5.90 %	3.12 %	83.98 %	1.82 %	2.74 %
Shannon Species Diversity	2.502	2.304	2.399	4.60	2.146	2.615
% reads classified to Genus	59.74%	62.97%	58.50%	57.22%	55.67%	67.45%
Number of species Identified	1539	1452	1261	1744	1239	1349

Table 2. Summary of the Illumina sequencing: The number of raw and valid reads using Illumina Miseq sequencing is indicated for all the samples. Abbreviations used, PRM-pre-monsoon and M-monsoon. M1, M2 and M3 are the three sampling stations

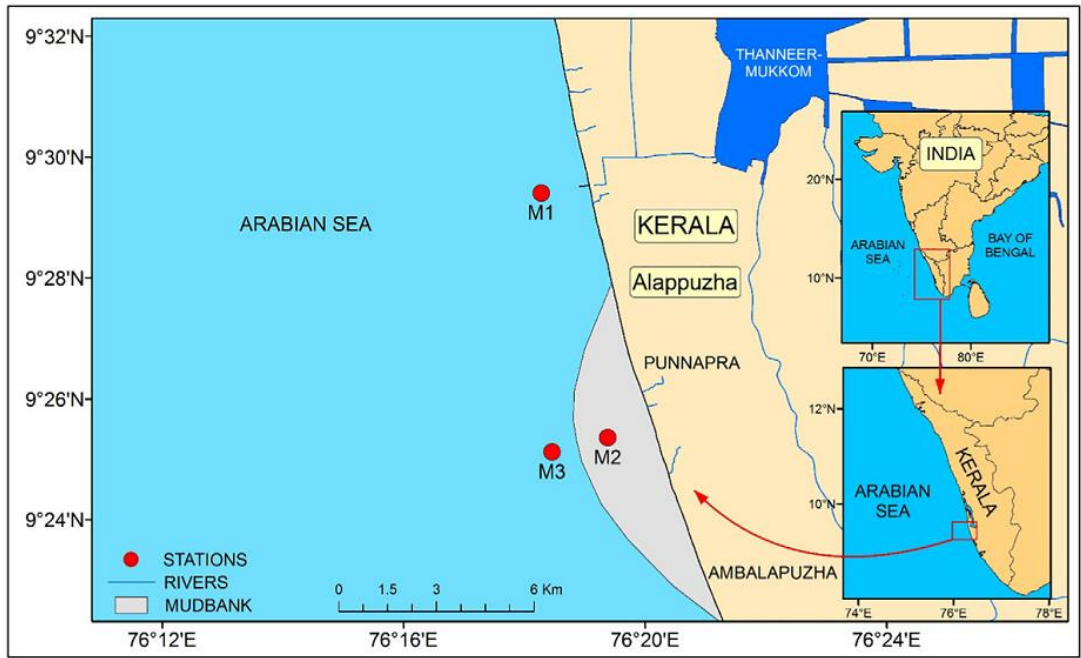


Fig. 1

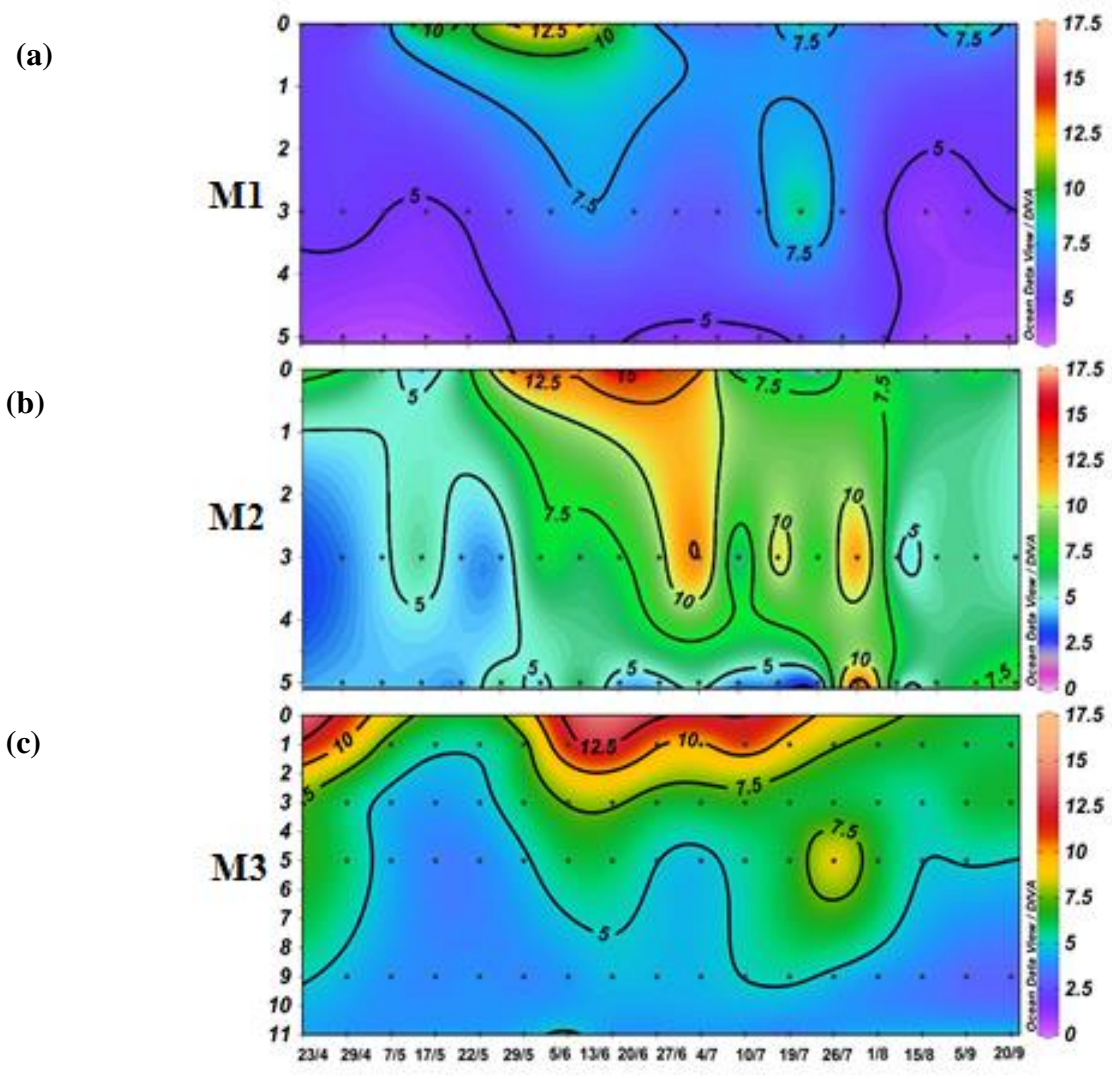
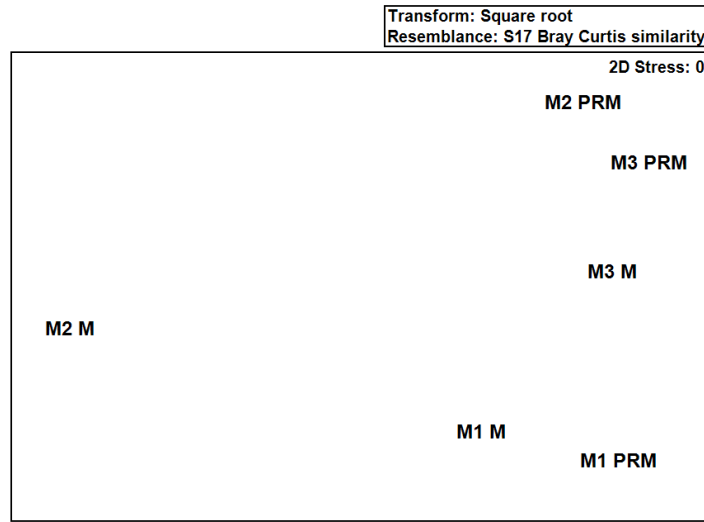
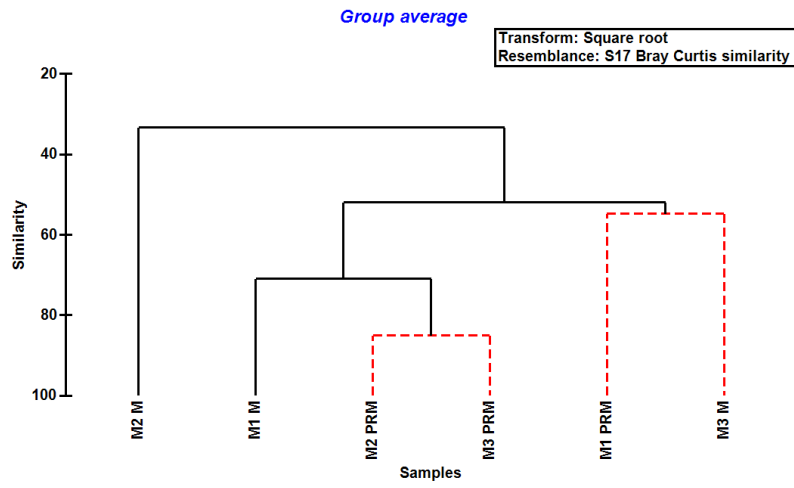


Fig. 2

(a)



(b)



(c)

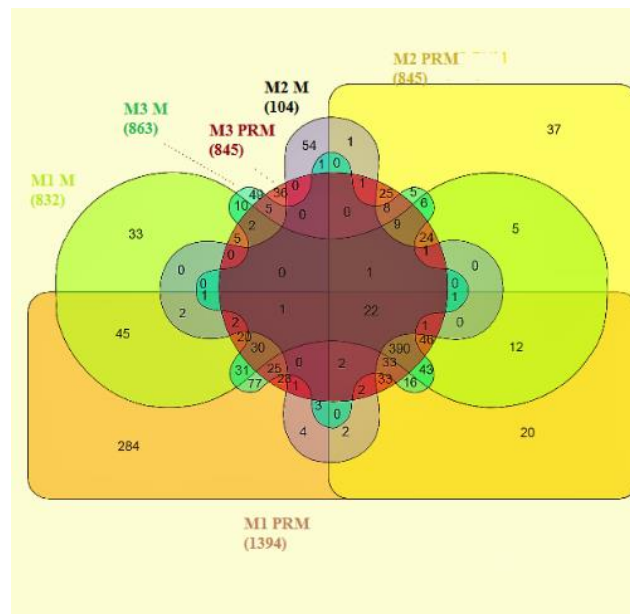


Fig. 3

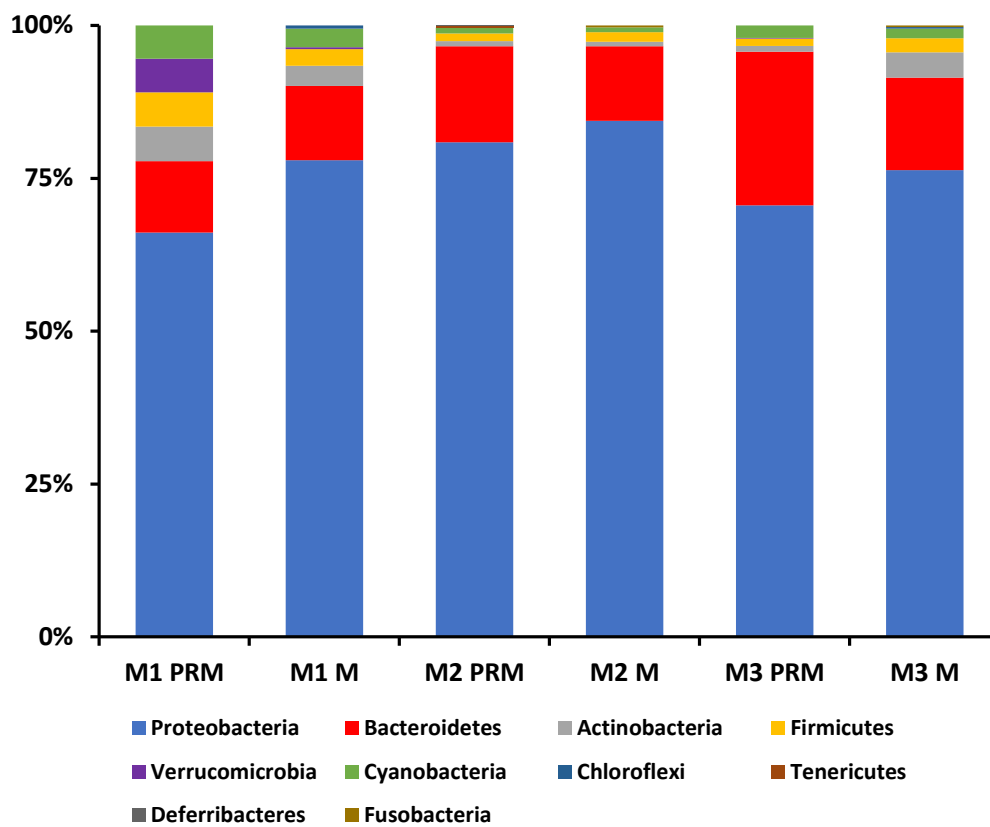


Fig. 4

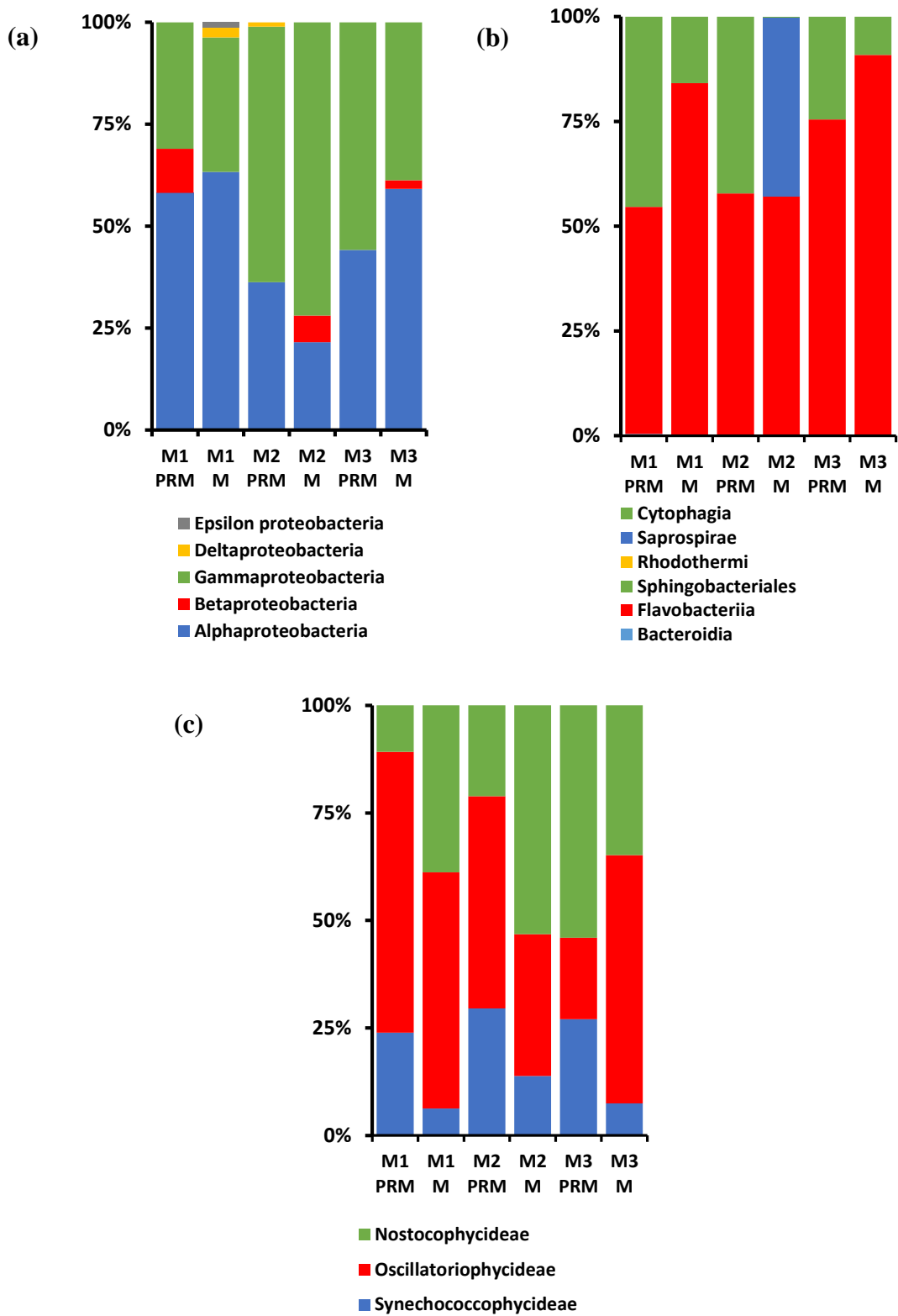


Fig. 5

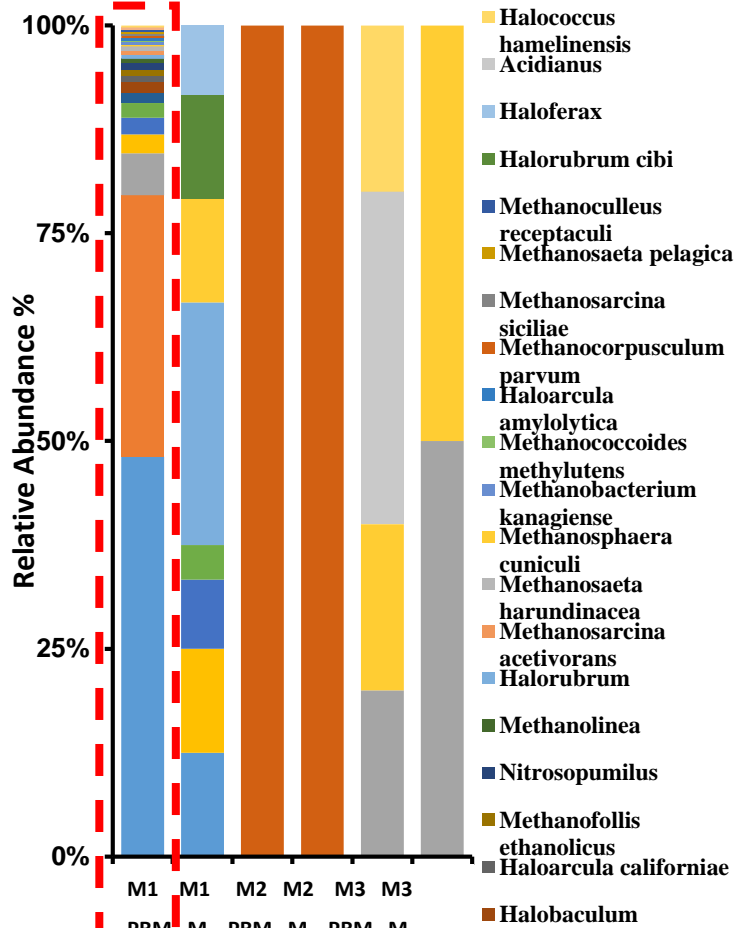
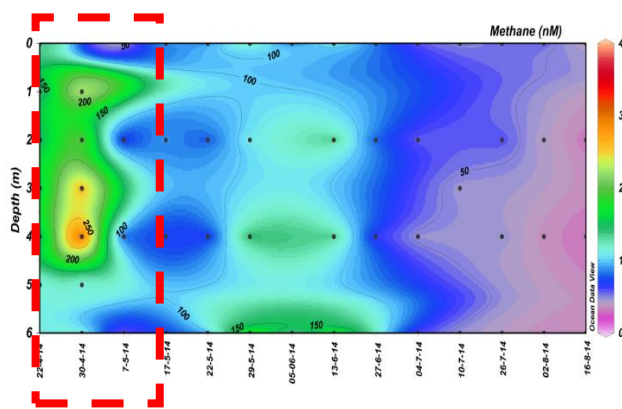
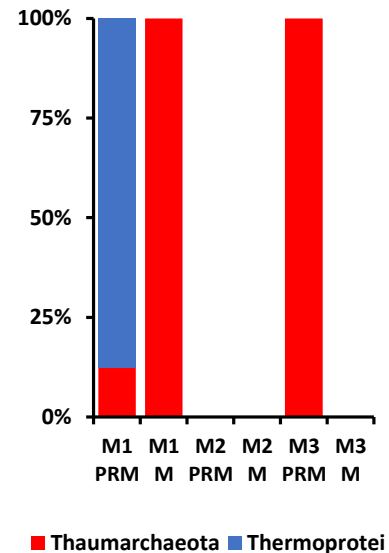
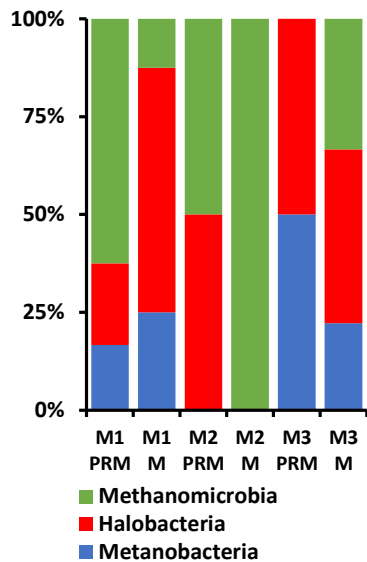
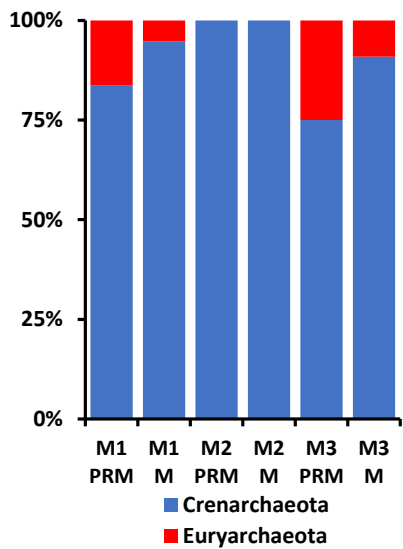


Fig. 6

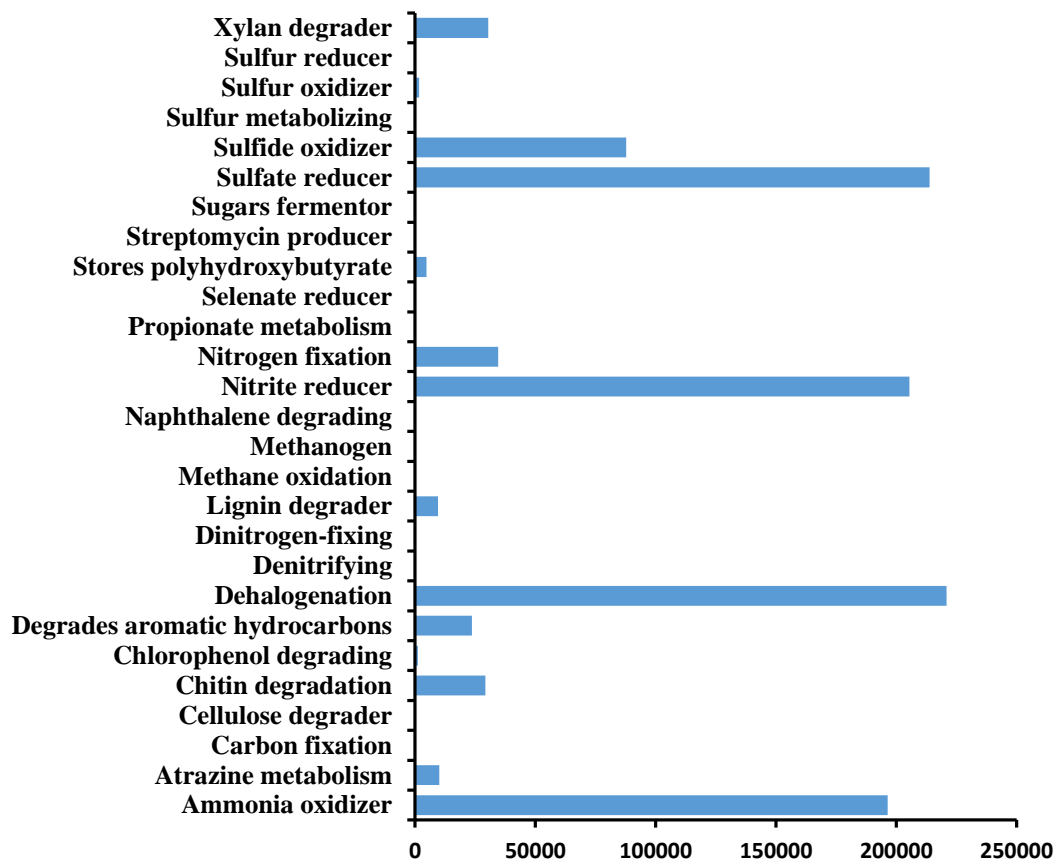
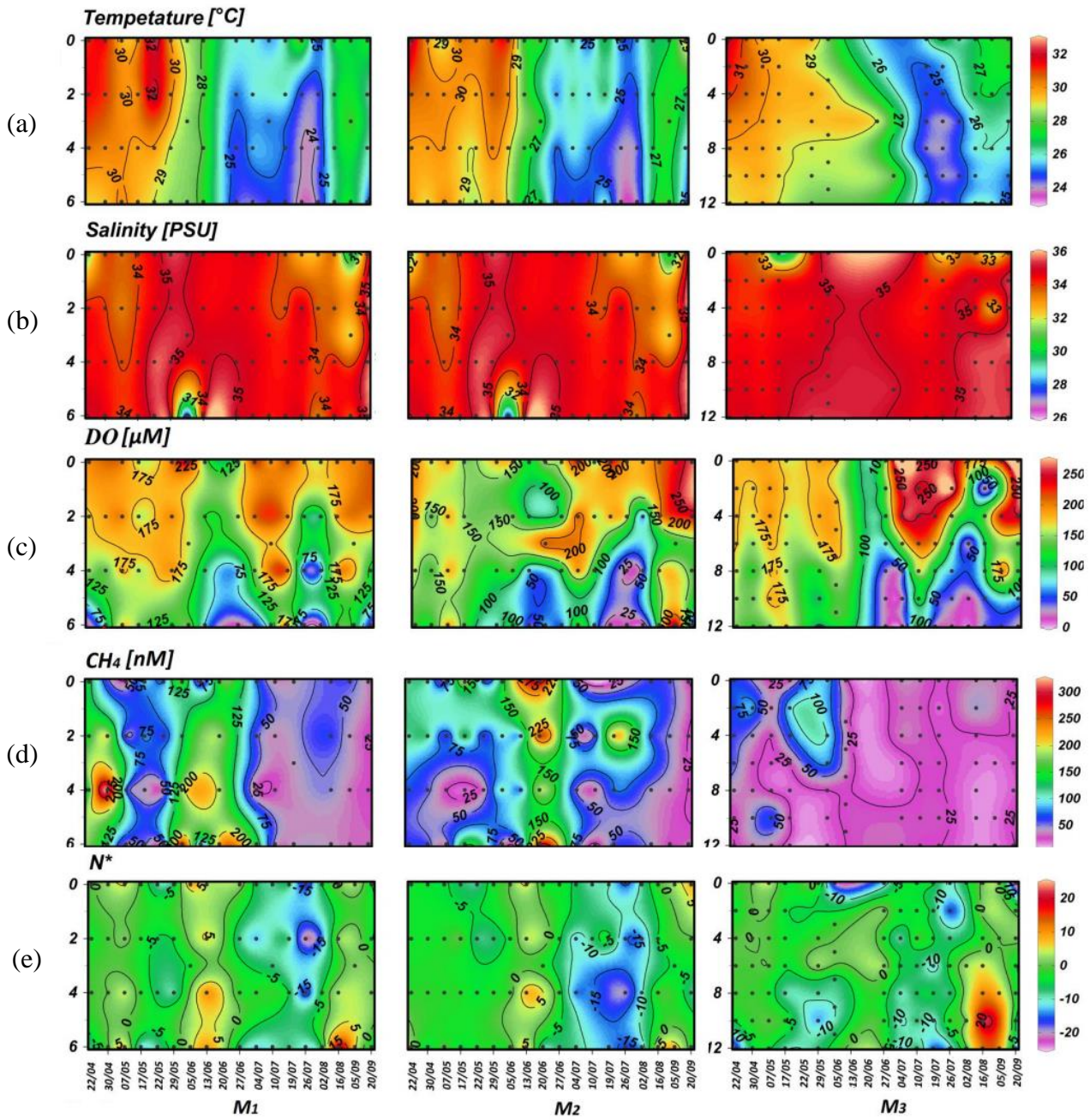
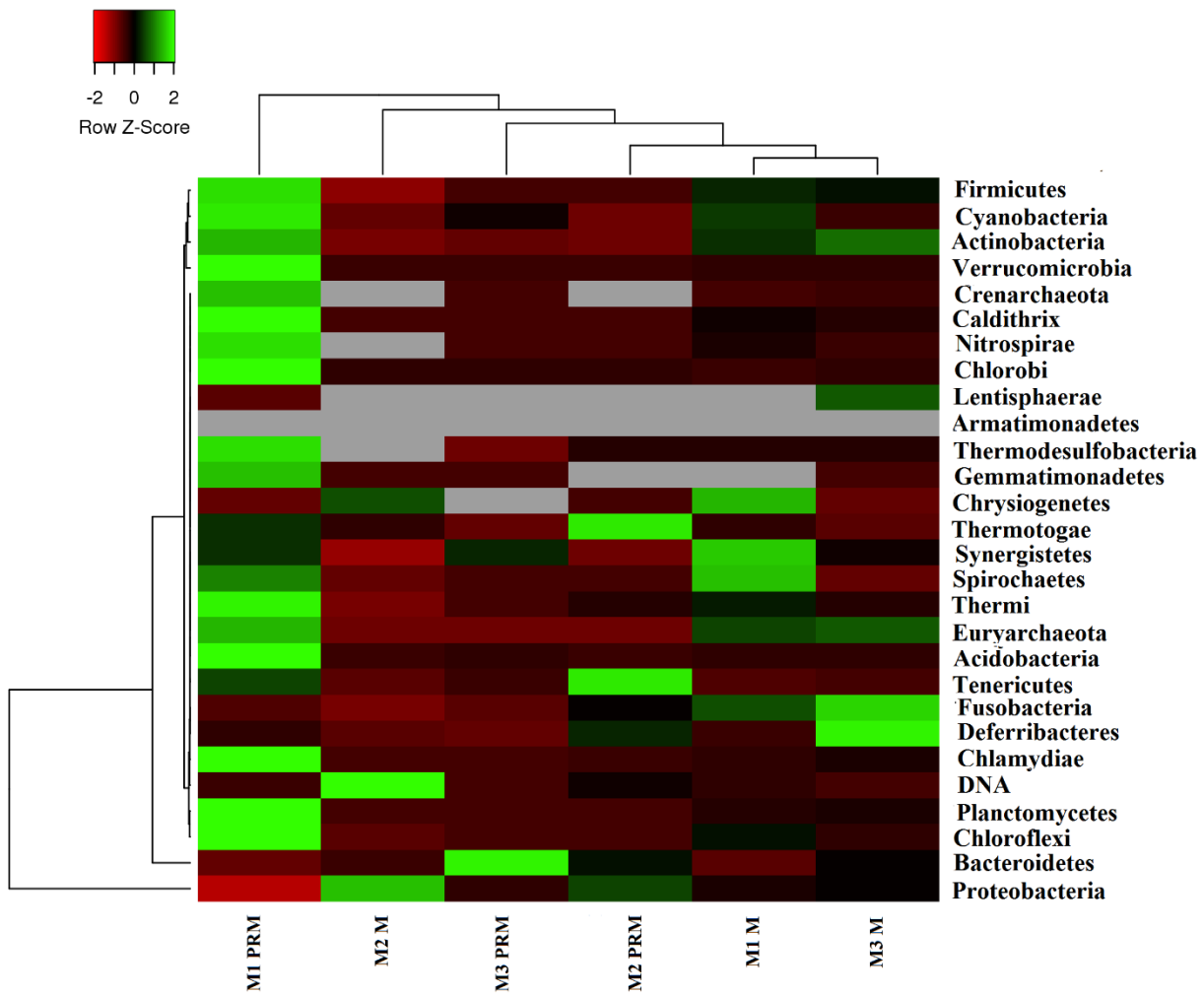


Fig. 7



Supplementary Fig. 1 Distribution of a) temperature, (b) salinity, (c) DO, (d) methane, and (e) Nitrogen deficit at the three sampling locations, M1, M2 and M3.



Supplementary Fig. 2 Generalized heat map with dendrogram representing the 16S rRNA gene sequences of Class level distribution at the sampling locations, M1, M2 and M3 during premonsoon (PRM) and monsoon (M). The color key represents centred and scaled counts of each taxon.