

Microbial community shifts along an estuarine to open ocean continuum

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Abstract

The water bodies along a continuum from estuaries to open ocean display significant changes in their physical, chemical and biological characteristics. In the current study, we report the influence of such changes on the abundance and community structure of bacteria and archaea in a continuum from Cochin Estuary, in the southwest coast of India, to the open ocean of Arabian Sea. The bacterial and archaeal abundances were in the range of 10^4 to 10^6 and 10^3 to 10^5 cells ml⁻¹ respectively in all the stations and showed a clear dependence on nutrients. The bacterial abundance decreased with depth in the open ocean, whereas the archaea showed a similar reduction at both shelf and open ocean stations. The α - Proteobacteria (35 %) and Cyanobacteria (23 %) were dominant in the estuary. More than 70 % of the community of bacteria on the shelf and in the open ocean were similar. The α - and γ -Proteobacteria contributed 37 & 27 % of the OTUs in the clone library of shelf and 20 & 29 % in the open ocean. The archaeal community structure in the open ocean and shelf were similar (90 %) composed mostly of *Nitrosopumilaceae* (70- 75 %), while they were highly diverse in the estuary. In short, our study shows that the archaea are more diverse in the estuary compared to shelf and open ocean, while bacteria are equally diverse in all ecosystems.

Keywords: Upwelling; Archaea; Bacteria; Arabian Sea; Estuary, community structure

1. Introduction

Estuaries and oceans are physically connected ecosystems with distinct chemical and biological features. These distinctions are generally imparted by physical processes such as upwelling, winter cooling, seasonally-reversing monsoonal winds, freshwater discharge and anthropogenic inputs, which in turn influence the diversity and activities of microorganisms through altering the biogeochemical structure and functions of each ecosystem. For example, upwelling exerts strong influences on the functioning of the marine ecosystem through inducing strong changes in the biogeochemical settings (Gupta et al. 2016). The coastal upwelling is initiated by the upward movement of nutrient rich bottom seawater, the intensity and extent of which depends on meteorological factors and continental morphology. The studies from coastal regions of Arabian Sea, Namibia, Chile, Peru and Benguela reported the ecological importance of upwelling in nitrogen loss (Kuypers et al. 2005; Codispoti et al. 2001; Woebken et al. 2007a), increased biological productivity (Gonzalez-Rodriguez 1994; Gonzalez-Rodriguez et al. 1992; Pereira et al. 2009), modulating tertiary production (Cury et al. 2000) and response of microorganisms (Cury et al. 2011; Anas et al. 2018). Extension of oxygen minimum waters creating hypoxic conditions with major changes in biogeochemical characteristics has been reported earlier from coastal regions (Naqvi and Jayakumar 2000). Previous studies focused mainly on the formation mechanisms of upwelling and the biological response at different trophic levels with few reports on bacterial response (Habeebrehman et al. 2008; Malik et al. 2015; Sathish Kumar et al. 2020; Jemi and Hatha 2019).

Estuaries are generally richer in both nutrients and pollutants compared with the open ocean, and the rate of their conversion is different between these ecosystems (Campbell and Kirchman 2013; Judd et al. 2006). Bacteria and archaea play significant roles in orchestrating the biogeochemical cycling of nutrients in any ecosystem, while their community structure may vary depending on environmental conditions. Examples for such environmental influences include the proliferation of anaerobic ammonium-oxidizing bacteria belonging to Planctomycetes in oxygen minimum zones (Jasmin et al. 2017; Galan et al. 2009), anaerobic methane and sulphate oxidizing microorganisms in the seabed (Beulig et al. 2018) and Poribacteria group of bacteria associated with sponges (Jasmin et al. 2015). Understanding of the diversity of bacteria and archaea in the estuarine and marine ecosystem has improved significantly in the past few decades with the aid of molecular phylogenetic tools. An intriguing outcome of these investigations is that cycling of the same nutrient in the different ecosystems is mediated by different groups of microorganisms through contrasting biochemical pathways. The classical example is the oxidation of ammonia through aerobic pathway of Proteobacteria and anaerobic pathway of Planctomycetes in the estuaries and oxygen minimum

zones respectively (Vipindas et al. 2018; Youssef and Elshahed 2014). Although the microbial distribution and community structure in the estuaries and oceans across the globe are reported separately, a straight forward comparison in a continuum of the estuary to open ocean has not been reported till date. During monsoon season, the estuaries are limnetic and oxygenated while the increased freshwater discharge and upwelling creates a stratified water column with oxygenated and nutrient rich surface water and hypoxic bottom in the coastal region. While in open ocean, the influence of fresh water is minimum, the dissolved oxygen and nutrients are controlled by oceanographic processes and productivity. The varying environmental settings along a transect from river mouth to open ocean may influence the distribution and community structure of microorganisms also. Since the changes in microbial community can impact the biogeochemical cycle and food web, any studies on the distribution and community structure of microorganisms under such conditions would be interesting.

The goal of this study was to determine the abundance and community structure of bacteria and archaea in a continuum from an estuary, Cochin Estuary (CE) in the southwest coast of India, to the open ocean (1000 m depth) in the Arabian Sea during monsoon season. The CE is one of the largest monsoonal estuaries, opening to Arabian Sea at Fort Cochin (9° 58'N) and Azhikode (10° 10'N). The major opening of CE is at Fort Cochin through which the estuary flushes nearly forty-three times in a year, with maximum number during the monsoon season (Vinita et al. 2015). The entire estuary behaves like a fresh water ecosystem during monsoon season due to the heavy terrestrial runoff and river water discharge (Vinita et al. 2015; Vipindas et al. 2015). On the other hand, the influence of seawater influx extends up to more than 70 % of the estuary during summer. Such interesting connections between physical and chemical characteristics of estuaries, and coastal and open ocean water, have been reported from different geographical locations. The influence of such connections on the diversity of microorganisms in an estuary to open ocean continuum are significant. The changes can influence the efficiency of biogeochemical cycles and food web dynamics in these ecosystems. In the present study, we monitored the distribution and community structure of archaea and bacteria using Fluorescent *In situ* Hybridisation as well as clone library approach.

2. Materials and methods

2.1. Sample collection and analysis of hydrographic variables

The water samples used in the present study were collected in August 2011. The water samples from Cochin Estuary and Cochin transect of Arabian Sea were collected using a hired

fishing boat and during the cruise No 289 of the research vessel (FORV Sagar Sampada of Centre for Marine Living Resource and Ecology (CMLRE), Ministry of Earth Sciences Govt of India) respectively. The water samples from 50, 100 and 200 m depth stations of Arabian Sea were considered as shelf samples, and those from 500 and 1000 m depth stations were considered as open ocean samples. Details of the vertical sampling profile in each station are given in Table 1. The water samples from the surface (0.5 m below the surface) and bottom (0.5 m above sediment) were collected from five locations in the Cochin Estuary along a salinity gradient from the river mouth to bar mouth (Table 1). The water samples were collected using 5 L capacity Niskin water samplers and transferred to separate bottles for chemical and molecular analysis. The samples of Cochin Estuary were analysed on board for salinity, temperature and pH and the samples for nutrients analysis and dissolved oxygen measurements were preserved and transported to the laboratory. All hydrographic variables were analysed on board in Cochin transect of the Arabian sea using CTD (salinity and temperature), pH meter (pH), an auto analyser (Nitrite, nitrate, silicate and phosphate) and Winkler's titrimetric method (Dissolved oxygen). The water samples for DNA extraction were collected separately in sterile bottles, filtered through 0.2 µm polycarbonate membrane filter and preserved at -80 °C till further analysis.

2.2. The abundance of archaea and bacteria

Fluorescence in situ hybridization (FISH) technique with Cy₃ labelled oligonucleotide probes such as ARCH915 (GTGCTCCCCCGCCAATTCCT) and EUB 338 (GCTGCCTCCCGTAGGAGT) were used to enumerate the archaea and bacteria respectively in the water samples (Vipindas et al. 2015). The probes ARCH915 and EUB338 bind specifically with the highly conserved 16S rRNA gene region of archaea and bacteria respectively. Here the water sample preserved in paraformaldehyde (Final concentration of 4 % v/v) were passed separately through 0.1µm (Millipore VCTP02500) and 0.2 µm (Millipore GTTP2500) white polycarbonate membrane filters for enumerating archaea and bacteria respectively. The membrane filters were immersed for 90 – 120 min in hybridization solution (0.9M NaCl, 20mM Tris-HCl (pH7.4) and 0.01 % SDS), containing 50 ng nucleotide probes and formamide (55 % and 50 % respectively for archaea and bacteria). Subsequently, the filter paper containing cells were counterstained with DAPI to localize the nuclei and distinguish the nonspecific binding. The unbound/excess probes were removed by rinsing the membranes in wash buffer. Fluorescent signals from labelled cells were counted after exciting under an epifluorescence microscope equipped with a 100W Hg lamp and filter sets specific for DAPI and Cy₃. The cell counts from 20 fields each from duplicate filter sets of every sample were counted and the average cell counts were expressed in cells

ml⁻¹. Direct counts of hybridized cells were taken first, as the Cy3 fluorescence fades much rapidly than that of DAPI. Hybridization conditions were optimized with positive and negative samples of *Escherichia coli* cells with and without recombinant plasmids of the respective genes. A filter section of the sample without hybridization was used for subtracting auto-fluorescence from FISH counts.

Bacterial cells ml⁻¹ were calculated using the equation

Bacterial cells ml⁻¹ = (C_f x R)/F_s, where

C_f = mean number of cells per field; R = active area of filter/ area of field counted; and F_s = volume of sample filtered in ml

2.3. DNA extraction

The method of Bostrom et. al was used for the extraction of genomic DNA from polycarbonate filter paper with minor modifications (Boström et al. 2004; Vipindas et al. 2015). Here the small pieces of filter paper were immersed in Tris- EDTA buffer (565µl) in a 1.5 ml capacity centrifuge tube and vortex for 2 min. The reaction mixture was supplemented with lysozyme (4µl of 10 mg ml⁻¹) and incubated for 1 hr at 37°C. The reaction was continued at 55°C for 2.5 hr after adding SDS (60µl of 10 % concentration) and proteinase K (6µl of 20 mg ml⁻¹). The crude DNA sample was extracted with chloroform: isoamyl alcohol (700µl of 24:1 mixture) twice and the aqueous phase containing DNA was retrieved by centrifugation at 10000 rpm for 10 min. The aqueous phase was transferred into a fresh microcentrifuge tube and isopropanol was added to this at a concentration of 0.6 volume, incubated at room temperature for 1 hr to precipitate DNA. The DNA pelleted at 10000 rpm for 10 min, washed two times with 70 % ethanol and dried at room temperature for 30 to 40 min. The DNA was dissolved in Milli Q water (30 µl) and the quality and quantity were confirmed by agarose gel electrophoresis and Nanodrop (Thermofisher, USA) respectively.

2.4. Construction of 16S rRNA gene libraries and sequencing

The 16S rRNA gene of archaea and bacteria were amplified separately in 25 µl reaction volume containing 1 µl DNA sample (50–100 ng), 1 µl each of primers (10 picomoles µl⁻¹), 2.5 µl of 10X Taq polymerase buffer, 1 µl of Taq DNA polymerase (0.5 units ml⁻¹) and 2 µl of dNTPs mix (10 µM each) (NEB, Canada). The 16S rRNA gene of archaea was amplified using universal primers [ARCH 21F: TTCCGGTTGATCCYGCCRG and ARCH 958R: TCCGGCGTTGAMTCCAATT], while that of bacteria was amplified using universal primers [27F: AGAGTTTGATC(AC)TGGCTCAG and 1492R: GGTTACCTTGTTACGACTT]. The cycling conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 30 cycles of

denaturation at 94°C for 40 sec, annealing at 55 °C for 60 sec for archaea/ 40 sec for bacteria, extension at 72°C for 1.5 min and a final extension for 10 min at 72°C. The PCR products were purified using Nucleopore Genetix brand Sure Extract-PCR clean-up/Gel extraction kit (Genetix Biotech, India) and cloned into a pJET 1.2 blunt vector using CloneJET PCR cloning kit (Thermo Scientific), following the instruction manual. The recombinant plasmids were purified from a library of 700 recombinant *Escherichia coli* (DH5 α) cells using Sure Spin Plasmid Mini Kit (Genetix Biotech Asia Pvt. Ltd). The quality and quantity of plasmids were checked using agarose gel electrophoresis and microplate spectrophotometer (SYNERGY MX microplate reader) respectively. Recombinant plasmids were sequenced using vector-specific primers (pJET 1.2 F or pJET1.2 R) available in the pJET 1.2 blunt vectors (Thermo Scientific). The sequencing PCR was done with ABI PRISM BigDye terminator ready reaction mix (Life Technologies, USA). The cycle extension products were purified following ethanol/ EDTA/ sodium acetate precipitation. The products were analysed on an Applied Biosystems ABI 3730xl DNA analyser.

2.5. Data analysis

The sequences were quality-checked and trimmed using the software Sequencher V4.10.1 (GeneCodes Corporation, Ann Arbor, MI USA) and vector sequences and chimeric sequences were removed from the dataset using Vecscreen program of NCBI and DECIPHER online (<https://decipher.sanger.ac.uk/>). The quality of sequences was further confirmed in the NGS analysis pipeline of SILVA (<https://www.arb-silva.de/ngs/>). Here, the ambiguous reads and the reads that are not of the required rRNA gene type were removed based on the alignment score and alignment identity. Further, the remaining sequences were dereplicated, clustered and classified into OTUs. Sequences from representative OTUs were searched in NCBI using BLASTn tool and nearest neighbours were selected. Together, these sequences were multiple aligned using clustalW and neighbor-joining Phylogenetic trees were constructed using MEGA 5.2 version. Bootstrap tests were performed 1000 times using MEGA 5.2. Rarefaction was performed to estimate OTU richness as a function of the number of clones sequenced and to determine whether the total diversity in the samples was well represented by the number of clone sequenced in each library. Rarefaction curves were plotted using Origin 7.5 (Origin Lab Corporation, USA). The differences in microbial community structure in the estuary, shelf and Open Ocean were compared using library compare tool of RDP (<http://rdp.cme.msu.edu>). Transformed sequence data were aggregated to family level and used to perform principal component analysis (PCA), multivariate cluster analysis and similarity percentage analysis (SIMPER) using PRIMER v.6 software package (Plymouth Marine Laboratory, UK) to understand the influence of environmental conditions on structuring microbial diversity in the

estuary, coast and Open Ocean. Bray-Curtis similarity was used to construct a similarity matrix on square root transformation data. The stations were classified by employing group-average linkage.

The spatial variations in hydrographic variables were plotted using tools in Surfer and Microsoft Excel. One-way ANOVA was performed to establish the significance of spatiotemporal variation of both environmental variables and biological variables. Karl Pearson's correlation was used for understanding the relationship between water quality parameters, bacterial and archaeal abundance and distribution of AOA.

2.6. Nucleotide accession numbers

The accession numbers for the sequences submitted to NCBI from this study are bacteria: - KX609628 - KX609684 (estuary), KX890245 - KX890303 (shelf) and KX581246 - KX581304 (open ocean). Archaea: - KX581305 - KX581360 (estuary), KX648300 -KX648356 (shelf) and KX423567 - KX423542 (open ocean).

3. Results

3.1. Hydrographic settings of the study area

There was a significant difference between the hydrographic settings of the estuary compared with marine (shelf and open ocean) water samples. The estuary was more oxygenated in the freshwater region (i.e. St 1 and St 2), while the bottom waters were less oxygenated in stations near to the bar mouth (St 3 and St 4) (Fig. 1). The surface waters were oxygenated in the shelf and open ocean samples also. The hypoxic conditions were prevalent at 50 m and down in all the stations in shelf and open ocean. Similar differences were also found in the level of nitrite, nitrate and phosphate also (Fig. 2). The nitrite concentrations in the estuary were comparable with the sub-surface waters (up to 100 m depth) of the shelf, while a sharp decline was observed after 50 m in the 500 and 1000 m stations in the Open Ocean. The nitrate concentration was higher in the upper region (St 1) of the estuary, it declined significantly in the stations down to the bar mouth. Comparatively low concentrations of nitrate were found in the surface waters, which showed a significant increase with depth. A sharp decline in the nitrate was observed between 50-100 m in the 200 m depth station in the shelf and between 100-200 m in the 500 m station in the open ocean. The phosphate concentration was comparatively higher in the first three station (St 1, 2 and 3) of the estuary. The phosphate was lower in the fourth station in the estuary and surface waters of stations in the shelf and open ocean. The phosphate level showed a steady increase in the subsequent depths in the 50m and 100 m stations in the shelf and 1000 m station in the open ocean. A sharp decline in the phosphate

levels was observed at 75 m in the 200 m station of shelf and at 150 m in the 500 m station of open ocean.

3.2. The abundance of bacteria and archaea

Bacteria and archaea were found to be equally abundant in the estuary, while the abundance of the former was one log higher in the water column of the shelf and open ocean (Fig. 3). The bacterial and archaeal abundance in the estuary was in the range of 4 to 6 x10⁵ cells ml⁻¹ and 2 to 3 x10⁵ cells ml⁻¹ respectively. The bacterial abundance didn't follow any trend in the shelf region, while it decreased with depth in the open ocean. The archaeal abundance followed a decreasing trend in both shelf and open ocean water column. The PCA analysis showed a clear dependence of abundance on the nutrient composition of the water column (Fig. 4). The principle component analysis (PCA) was employed using PRIMER 6 software on environmental data to detect trends in the variation of environmental characteristics and species abundance across the study area. The PCA plot uses the principle components which explain maximum variation in the data as the axes (PC axes) and visualizes the orientation of sampled sites around PC axes so that sites having similar environmental conditions fall close together. The abundance of archaea and bacteria of each station was superimposed as bubbles onto the environmental PCA, to visualize their preference to environmental conditions. The measured environmental variable such as depth, dissolved oxygen, nutrients (Nitrite, Nitrate, Phosphate) and species abundance matrix was used for the analysis.

The pattern of variation in the abundance of bacteria and archaea in relation to depth and location was made clear in PCA ordination. The analysis produced a total of five canonical axes, three of which explained 77 % of the total variance between the stations. We plotted the PCA ordination using PC1 against PC2 which could explain nearly 63 % of the total variance. The PC1 contributed to the 41 % of the variance (eigen value 3.26) among which depth and DO were the major contributing variables. Based on the ordination of PC1, the shallow stations (< 5 m depth) with high oxygen (> 3 ml L⁻¹) levels (estuary and surface stations of shelf and open ocean) formed a cluster towards the left side while stations in the deeper regions (> 15 m) with low oxygen (< 2 ml L⁻¹) in the shelf and open ocean formed a different cluster towards the right side. The PC2 could explain 22 % of the variance (eigen value 1.76) contributed mainly by pH, nitrite and phosphate. On this basis, the surface stations of the shelf and open ocean with low levels of nitrite (< 0.2 μM) and phosphate (< 0.8 μM) were ordinated towards the upper side of the plot while remaining stations were in the lower side.

3.3. Community structure of bacteria and archaea

The community structure of bacteria and archaea also were different in the estuary, shelf and open ocean. The bacterial community structure in the clone library prepared from the estuary was dominated by α -Proteobacteria (35.1 %) followed by Cyanobacteria (22.8 %), Flavobacteria (14 %) and Actinobacteria (5.3 %) (Fig. 5). The members of α -Proteobacteria (37.3 %) were dominant in the clone library generated from the water column of the shelf, followed by γ -Proteobacteria (27.1 %), Flavobacteria (8.5 %), unidentified bacteria (8.5 %), Actinobacteria (6.8 %) and Cyanobacteria (5.1 %). The order of dominance in the water column of open ocean was in the order of γ -Proteobacteria (28.8 %) followed by Flavobacteria (25.4 %), α -Proteobacteria (20.3 %), Cyanobacteria (10.2 %), unidentified bacteria (5.1 %) and Actinobacteria (1.7 %). Members of Physcisphaerae (1.7 %) were also found in the water column of the open ocean. The α - and γ -Proteobacteria were less diverse in the estuary compared to the water column of the shelf and Open Ocean (Fig. 6). The α -Proteobacteria in the estuary were represented largely by Rhodobacteraceae (90 %), followed by members of SAR 11 (5 %) and unidentified ones. In the shelf region, the α -Proteobacteria were more diverse, with members from SAR 11 (45.46 %), followed by Rhodobacteraceae (36.36 %), unidentified ones and hyphomonadaceae (4.5 %). In the open ocean, the α -Proteobacteria was represented by a large number of unidentified ones followed by SAR11 (33.33 %), Rhodobacteraceae (16.67 %), hyphomonadaceae (8.33 %) and Rhodospirillae (8.33 %). The γ -Proteobacteria in the water column of the estuary was represented by members of *Vibrionaceae*, whereas *Moraxellaceae*, *Altermonadaceae* and unidentified ones were found on the shelf and members of *Moraxellaceae*, *Altermonadaceae*, *Halomonadaceae* and *Enterobacteriaceae* were found in the open ocean. The Flavobacteria (Fig. 6c) were more diverse in the estuary, represented by *Cryomorphaceae*, *Flavobacteriaceae* and unidentified ones, while only members of *Flavobacteria* could be found in the clone library from the shelf. The *Flavobacteria* was also dominant in the open ocean followed by unidentified ones and *Cryomorphaceae*. A comparison of the clone library showed that the bacterial community structure in the shelf and open ocean were 70 % similar (Fig. 8), while the bacterial community structure in the estuary showed a nearly 60 % similarity with that of the shelf and open ocean. The bacterial community structure of the estuary differs from that of the shelf, in terms of the composition of γ -Proteobacteria (27 %), Cyanobacteria (23 %) and unidentified bacteria (11 %), and from that of the open ocean for γ -Proteobacteria (26 %) α -Proteobacteria (16 %), Flavobacteria (14 %) and Cyanobacteria (12 %). The difference in the composition of α -Proteobacteria (26 %), Flavobacteria (24 %) and Actinobacteria (16 %) significantly contributed to the dissimilarity in the bacterial community structure between shelf and open ocean. The community

structure of the shelf and open ocean formed a single cluster with 69 %, whereas that of the estuary formed a different lineage with less than 60 % similarity to the other cluster (Fig. 8). The dissimilarity in community structure between shelf and open ocean was contributed by γ -Proteobacteria (~28 %), α -Proteobacteria (~16 %), Flavobacteriia (~14 %) and Chloroplast (~14 %). The α -Proteobacteria (~27 %) and Flavobacteriia (~27 %) were the major contributors in the dissimilarity in bacterial community structure of estuary with shelf-open ocean cluster.

The community structure of archaea at the family level is represented in Fig. 7. The archaea were more diverse in the estuary and the majority of them were unclassified (~80 %), followed by representatives from Nitrosopumilaceae (~15 %) and remaining were Nitrososphaeraceae, Desulfurococcaceae, Methanomassiliicoccaceae, Halobacteriaceae and Haloferacaceae. The archaeal representatives in the shelf and open ocean were largely from Nitrosopumilaceae (73.68 % and 71.7 % respectively) followed with unclassified archaea (1.75 %). The members of Haloferacaceae were also found in the water column of the shelf. The clone library comparison showed a 90 % similarity between the shelf and open ocean while both of them shared only less than 50 % similarity with that of the estuary. The significant dissimilarity of the community structure in the estuary with shelf and open ocean were contributed by unclassified archaea (~50 %) and Nitrosopumilaceae (~20 %). The Unclassified archaea (49 %), Haloferacaceae (35 %) and Nitrosopumilaceae (11 %) contributed towards the negligible dissimilarity in archaeal community structure found between the shelf and Open Ocean.

4. Discussion

The present study discusses the changes in the distribution pattern and community structure of archaea and bacteria along a continuum from the estuary to open ocean. The hydrographic settings showed the existence of upwelling in the Cochin transect of the Arabian Sea during the period of the study. This was indicated by the occurrence of low oxygenated nitrate rich subsurface waters from open ocean to bar mouth in the CE. The seasonal fertilization due to upwelling and subsequent increase in productivity have been reported extensively from the coastal upwelling regimes located along the eastern boundaries of Pacific and Atlantic ocean basins (Capone and Hutchins 2013; Antonio Cuevas et al. 2004; Vergas et al. 2007; Anabalon et al. 2014). The occurrence of upwelling along a broad region parallel to the southwest coast of Arabian Sea during May to October and its influence on hydrographic setting and biological productivity have been reported (Gupta et al. 2016; Habeebrehman et al. 2008; Malik et al. 2015; Sathish Kumar et al. 2020; Jemi and Hatha 2019; Shah et al. 2019). Higher nitrate concentrations in the subsurface waters of shelf and open ocean are due to the horizontal advection of nitrate rich waters from the oxygen minimum zones and benthic zones,

where it is produced via remineralisation of organic matter. The time series analysis shows a sharp decline of nitrate levels in the study area with the withdrawal of upwelling in the October (Gupta et al. 2016). The low oxygen concentrations in the bottom waters in the stations near to the bar mouth of the estuary could be due to the upwelling. The advection of upwelled water into the adjacent estuaries have been reported from the northwest coast of Pacific coast of North and South America and western India (Roesgner et al. 2011). The nitrate levels were lower in the downstream of the estuary, while the observed values in the upstream (st 1) were comparable with those found in the upwelled waters in coastal and open ocean. The higher nitrate concentrations in the upstream of CE have been attributed to the disposal of industrial effluents through river discharge and terrestrial flow (Martin et al. 2008; Shivaprasad et al. 2013).

Further, we investigated the distribution and diversity of bacteria and archaea in the study area. The bacterial abundance was higher than that of archaea in the corresponding stations in the estuary, shelf and open ocean. The horizontal distribution of bacteria in the estuary, shelf and open ocean were comparable, while a gradual decrease was observed with bathymetry in the open ocean. A similar reduction in bacterial abundance with respect to bathymetry has been reported from stations at 500 and 1000 m in the Arabian Sea during the summer monsoon (Ramaiah et al. 2000). This difference was attributed to the variation in the availability of transparent exopolymer particles and nutrients with respect to the depth (Ramaiah et al. 2000). The labile organic substrates available in the upper water column promote the proliferation of copiotrophic bacteria while the refractory fraction sinking to the bottom is less amenable to the microorganisms (Hansell and Carlson 1998). The copiotrophic bacteria experience rapid growth on encountering high substrate supply (e.g. during phytoplankton blooms or on detrital aggregates) whereas they persist in low abundances under severe substrate limitation (Overmann and Lepleux 2016). The higher concentrations of organic substrates in the continental shelves (<150 m water depth) support the proliferation of copiotrophic bacteria, while specialized groups of microorganisms such as anammox, n-Damo etc and their adaptations towards substrate supply are increasingly reported from the deep sea (Chen et al. 2014; Cory et al. 2016; Shen et al. 2015). Higher abundance of bacteria in response to nutrient enrichment due to upwelling has been reported from the coastal waters of Northeast Atlantic Ocean (Vaqué et al. 2013) and Arabian Sea (Anas et al. 2018).

The archaea were more abundant in the estuary compared to other regions in the study area and a reduction with depth was evident in the shelf and open ocean. Although the abundance of archaea in the estuary and open ocean were reported in separate studies, a direct comparison between these systems was not reported. Archaea have been recorded as the significant component of total

microbial communities (20 -30 %) of the coastal regions of California (Karner et al., 2001) and sediments of Mediterranean lagoon, Sacca di Goro (Pala et al. 2018). Their abundances have been found to be lower compared with bacteria in the water column of CE (Vipindas et al. 2015). The dominance of different groups of archaea at different depths has been reported in the literature. For example, the dominance of Marine group II (euryarchaeota) and group I (thaumarchaeota) Archaea were reported from the surface to 200 m depth in the coastal waters off Anvers Island, Antarctica (Delong et al. 1999; Ramon et al. 1997). Although we also found that the archaea are equally abundant as bacteria in bottom waters of 1000 m depth, the factors explaining such shifts in the relative abundance of archaea and bacteria are still largely unknown (Roberto et al. 2016).

Differences were also found in the community structure of bacteria and archaea and the latter was more diverse in the estuary compared with the shelf and open ocean. The community structure presented in the current study were made based on the analysis of 178 (derived from 265 clones) and 167 OTUs (derived from 297 clones) of bacteria (Estuary: 57, Shelf: 61 and open ocean: 60) and archaea (Estuary: 57, Shelf: 57 and open ocean: 53) respectively. The rarefaction curves were (Supplementary figure) steeper in all the cases indicating that the community would be even more diverse if analysed further using recent techniques like NGS 16s Amplicon sequencing. The NGS techniques have high throughput abilities which can provide overall community structure of microorganisms at higher taxonomic level, such as phylum, class order and family. Although clone library approach is much inferior to NGS in terms of its high throughput, the abilities to retrieve comparatively long sequences (700 - 1000bp) with high accuracy is still valid to clarify the predominant microorganisms in any study area, in detail (i.e up to genus and species level)(Fukuda et al. 2016). Conventional clone library approach was followed in the current work as the higher read length would support analysing the diversity at the genus and species level. This is important as there were limited datasets available on the community structure of bacteria and archaea available from this region. A diverse group of bacteria were found in the estuary (Shannon Wiener index 1.902, Chao 1: 119) with representatives in the order of α -Proteobacteria (35.1 %) > Cyanobacteria (22.8 %) > Flavobacteria (14 %) > γ -Proteobacteria (7 %), while it was in the order of α -Proteobacteria (37 %) > γ -Proteobacteria (27 %) > Flavobacteria and unclassified bacteria (9 % each) and γ -Proteobacteria (29 %) > Flavobacteria (25 %) > α -Proteobacteria (20 %) > Cyanobacteria (10 %) in the shelf (Shannon Wiener index 1.763, Chao 1: 155) and open ocean (Shannon Wiener index 1.773 Chao 1: 242) respectively.

Examination of taxonomic groups in the clone libraries showed the dominance of Proteobacteria in the Cochin Estuary (50.9 %), adjoining shelf (64.4 %) and open ocean (50.8 %).

Proteobacteria are regularly found in different estuarine, pelagic and marine environments (Bandeekar et al. 2018; Giovannoni and Rappe 2000; Sachithanandam et al. 2020), and they contributed up to 90 % of the clone library of bacterial 16S rRNA genes generated from the deep sea water of East Pacific Rise (Santelli et al. 2008). Dominance of proteobacteria was also reported in the metagenomic library from the coastal sediments of Arabian Sea and water samples of Northern Indian Ocean (Fernandes et al. 2020; Sachithanandam et al. 2020). The global ocean sampling and sequencing efforts have shown that the microbial community recorded more diversity in open ocean than any other habitat (Biers et al. 2009). The α - and γ -proteobacteria were the major classes of proteobacteria that were more diverse in the shelf and open ocean. More than 90 % of the clones of α - and γ -proteobacteria in the estuary were affiliated to the species of *Rhodobacteriaceae* and *Vibrionaceae* respectively. A diverse group of members of *Rhodobacteriaceae* has been reported from the estuarine waters across the globe, and members of this family are known for their participation in the biogeochemical cycling of sulfur and carbon (Simon et al. 2017). *Vibrionaceae* are the native inhabitants of estuarine and marine waters, and their roles as pathogens of aquatic animals to remineralization of large polymers of chitin and laminarin are well documented in the estuarine and marine systems. The dominance of vibrios has been reported in association with algal blooms, light organs of squids, the chitinous shell of crustaceans etc in the estuarine and marine waters. A significant number of α - proteobacteria in the shelf and open ocean belonged to SAR 11 group, which are the most abundant oligotrophic facultative photochemotroph planktons found in marine systems (Morris et al. 2002). They account for ~ 25 % of prokaryotic cells in the global seawater, and half of the total 2.4×10^{28} cells of them are found in euphotic zones (Morris et al. 2002). They play a significant role in the global carbon cycle (Morris et al. 2002). Interestingly, 10 to 40% of the clones of α - proteobacteria were classified as unidentified when compared with the library of NCBI.

We found a significant number of Cyanobacteria in the clone library of CE (22.8 %), dominated by Chloroplast and Family II group. The cyanobacteria were relatively less abundant in the clone library of the shelf (5.1 %) and Open Ocean (10.2 %) and were restricted to Family II in this region. The presence of *Prochlorococcus* and *Synechococcus* has been reported from the Arabian sea, where the *Synechococcus* sp were often found restricted to the upper part of the euphotic zone (Bandeekar et al. 2018). The blooming of *Synechococcus* sp with abundant species diversity and different niche adaptations were also reported from the Pearl River Estuary (Li et al. 2019). Cyanobacteria are the major picoplankton distributed in the estuarine and marine environment, where they occupy a key position at the base of the food web and play a central role in carbon, nutrient and oxygen cycling (Day et al. 2012). The cyanobacteria, *Prochlorococcus* and

Synechococcus alone are responsible for an estimated 20 – 40 % of global carbon fixation, although they account for only one per cent of photosynthetic biomass (Blindauer 2008).

A distinct factor of open ocean was the increase in the number of OTUs classified within the Flavobacteriia. The bacteria belonging to this class are known for their abilities to mineralize particulate organic matter (Allen et al. 2012) and their higher prevalence in upwelled waters of California Bight and California current (Allen et al. 2012). The number of OTUs of Flavobacteriia/Cytophagia/Sphingobacteriia in the estuary, shelf and open ocean were in the order of 14.0, 1.8 and 1.8 % respectively in the present study. Although not very significant in terms of the number of OTUs in the clone library, the members of class *Phycisphaerae* of phylum Planctomycetes also were found in the open ocean. These organisms are widely distributed in the sediments of estuarine and marine environments and oxygen minimum zones, where they are proposed to exhibit unique biogeochemical properties such as anaerobic ammonium oxidation (Neumann et al. 2013) methane oxidation (Sharp et al. 2013) and participation in carbon recycling (Woebken et al. 2007b). They are reported to be the dominant group in oxygen minimum zones, where 30 – 50 % nitrogen loss takes place in the ocean (Lam and Kuypers 2011). Previously we reported the distribution of different genera of Planctomycetes, such *Blastopirellula*, *Rhodopirellula*, *Candidatus scalindua*, *Candidatus brocadia* and a significant number of unclassified ones in the sediments of Arabian Sea (Jasmin et al. 2017).

Most of the previous studies on archaeal diversity in estuaries and marine environment have been related to the nitrogen cycle. The community structure of ammonium oxidizing archaea from CE (Vipindas et al. 2015; Vipindas et al. 2018) and total archaea from the OMZ of Arabian Sea (Bandeekar et al. 2018) are discussed earlier. The phylogenetic comparison of 16S rRNA gene sequence of archaea showed clear differences in their community structure in the estuary, shelf and open ocean. The archaea were more diverse in the CE compared with the adjoining shelf and open ocean. A large diversity pool of archaea composed of 12 phyla has been reported from estuaries and the predominant groups were Bathyarchaeota, Euryarchaeota and Thaumarchaeota (Liu et al. 2018). The diverse population in the CE was comprised of a large number of unidentified archaea (21.43 %) and representatives of Thaumarchaeota (Nitrososphaeraceae, Nitrosopumilacea), Crenarchaeota (Desulfurococcaceae), and Euryarchaeota (Methanomassiliicoccaceae, Haloferacaceae and Halobacteriaceae). More than 70 % of the archaeal OTUs in the shelf and open ocean were restricted to Thaumarchaeota (Nitrososphaeraceae) and the remainder were unidentified archaea (~30 %) and a small number of OTUs in the shelf region belonged to Euryarchaeota (Halobacteriaceae). The current study indicates that the archaeal diversity decreases from estuary to open ocean. The archaeal

population in the water column of shelf and Open Ocean showed nearly 90 % similarity and was contributed by *Nitrosopumilaceae* species which accounted for 88.99 %. Haloferacaceae (35 %) and unclassified Thermoplasmata (35 %) were identified as the major contributor of dissimilarity between shelf and open ocean. The average dissimilarity of Archaeal community of estuary compared with shelf region is 51 % and with open ocean is 52 %.

Earlier studies proposed archaea as the residents of extreme environments such as hydrothermal vents, oligotrophic marine waters etc., while subsequent studies revealed their ubiquitous presence in diverse ecosystems of estuaries, marine and waste treatment systems etc (Zhang et al. 2016). Thaumarchaeota and euryarchaeota are known to be present in marine water columns, and the presence of their signature lipid tetra ethers has been detected from different depths (Lincoln et al. 2014). It has been estimated based on available studies that the *Crenarchaeota* contribute approximately 20 % of all picoplankton cells in the world ocean (Karner et al. 2001). The spatial differences in the community structure of archaea have been reported from stratified water columns of marine ecosystems, examples include the dominance of *Euryarchaeotain* in the upper 200 m and *Crenarchaeota* in the layer below the thermocline (Lonescu et al. 2009). The *Crenarchaeota* and Euryarchaeota also have been reported from the Gulf of Aquaba in the northern Red Sea (Lonescu et al. 2009) and oxygen minimum zones of Arabian Sea (Bandeekar et al. 2018). The dominance of crenarchaeota has been reported from the sediments of White Oak River estuary in USA (Lazar et al. 2015). The high number of unidentified archaea in the CE indicates the need for further studies to understand the archaeal community in the system and the factors controlling their distribution.

In short, the current study forms the first attempt to understand the distribution and community structure of bacteria and archaea in a continuum from an estuary to open ocean in a tropical system. The abundance of bacteria and archaea in the estuary to open ocean continuum is controlled by the variation in the nutrient characteristics brought in by oceanographic processes and anthropogenic inputs. On the other hand, the variation in the community structure along the continuum is intriguing and needs further investigations. It is estimated that archaea and bacteria are more diverse in the estuary compared with the adjoining shelf and the open ocean. Based on the results of the current study, along with the literature, it could be stated that no archaea are specific to a particular ecosystem: they could grow anywhere depending on the hydrographic settings. More detailed investigations are necessary to puzzle out the interactive effect of these controlling factors on the community structure of archaea in each environment.

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Author credit statement

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Tables and Figures

Tables

Table 1: The geographic locations of the stations from where water samples were collected

	Station	Location		Sample details
	No	Latitude	Longitude	
Estuary	St.1	76°16'25.12"E	10°04'21.93"N	Surface, Bottom
	St. 2	76°16'03.8"E	10°02'38.0"N	Surface, Bottom
	St. 3	76°14'37.57"E	9°58'13.57"N	Surface, Bottom
	St. 4	76°13'47.78"E	9°57'56.66"N	Surface, Bottom
Shelf	50 M	75°50'1.12"E	9°58'6.42"N	Surface, 15M, 50M
	100M	75°38'14.5"E	9°58'6.4"N	Surface, 25M, 50M, 500M
	200 M	75°34'37.1"E	9°58'6.42"N	Surface, 25M,75M, 150M, 200M
Open Ocean	500M	75°32'31.1"E	9°58'6.42"N	Surface, 25M, 75M, 150M,500M
	1000M	75°29'3.2"E	9°58'6.42"N	Surface, 75M, 150M, 250M, 1000M

Figures

Fig.1. Dissolved oxygen profile in different stations in the Estuary (table), Shelf and Open Ocean. Different locations in the Shelf are marked with black (50 m), red (100 m) and blue line (200 m). The stations in Open Ocean are marked with black (500 m) and red line (1000 m)

Estuary Stations	Dissolved oxygen mL ⁻¹
St 1 Surface	4.84
St 1 Bottom	4.79
St 2 Surface	7.47
St 2 Bottom	5.85
St 3 Surface	3.19
St 3 Bottom	2.16
St 4 Surface	7.48
St 4 Bottom	3.76

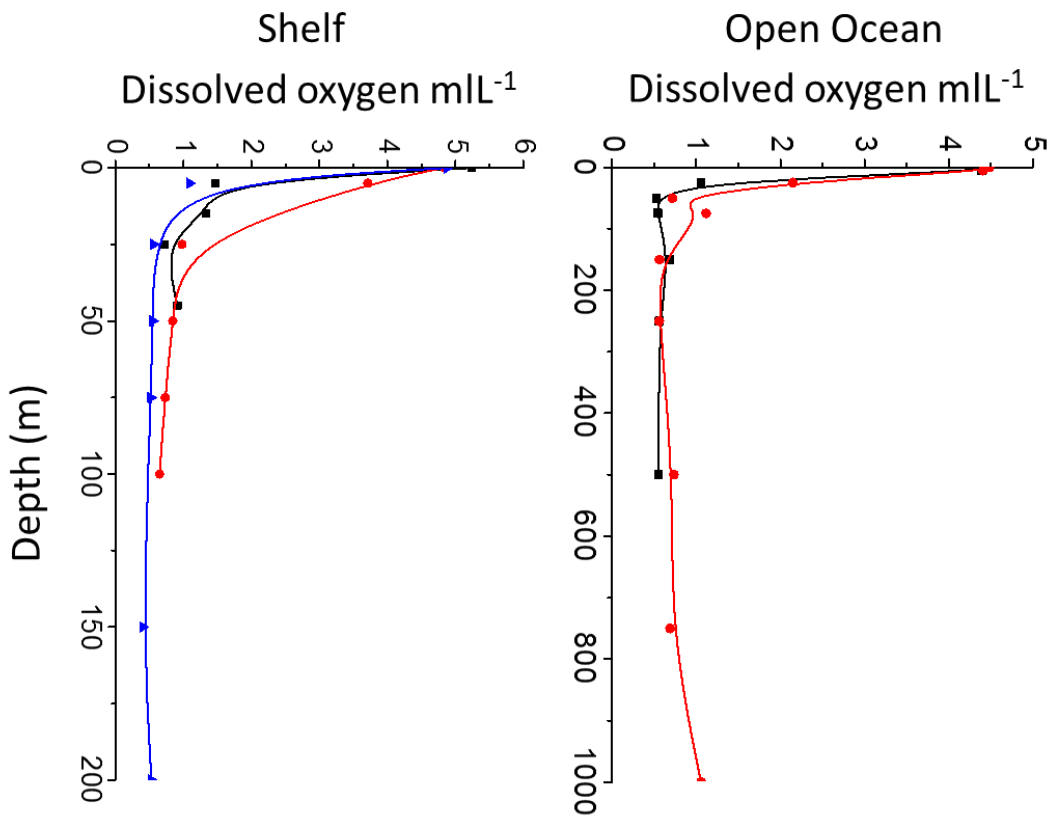


Fig. 2. Profile of nitrite, nitrate and phosphate in different stations in the Estuary (table), Shelf and Open Ocean. Different locations in the Shelf are marked with black (50 m), red (100 m) and blue line (200 m). The stations in Open Ocean are marked with black (500 m) and red line (1000 m)

Estuary Stations	Nitrite (μM)	Nitrate (μM)	Phosphate (μM)
St 1	0.39	21.83	1.42
St 2	0.40	5.15	1.87
St 3	0.83	2.03	1.53
St 4	0.34	3.73	0.69

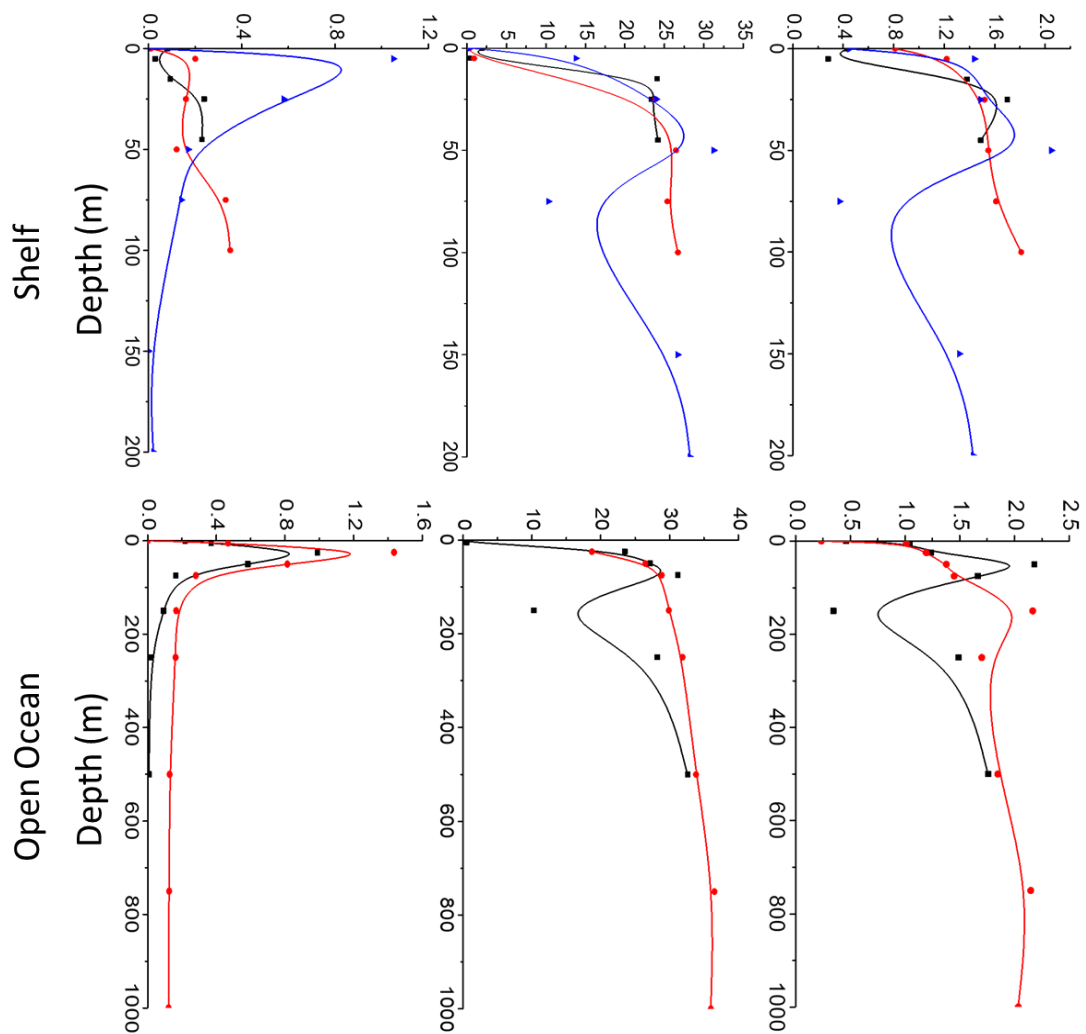


Fig. 3. Abundance of bacteria and archaea in different stations in the Estuary (table), Shelf and Open Ocean. Different locations in the Shelf are marked with black (50 m), red (100 m) and blue line (200 m). The stations in Open Ocean are marked with black (500 m) and red line (1000 m)

Estuary Stations	Abundance of	
	Bacteria ($\times 10^5$ cells ml^{-1})	Archaea ($\times 10^4$ cells ml^{-1})
St 1	5.91	33.05
St 2	6.47	32.28
St 3	5.40	32.47
St 4	4.13	21.74

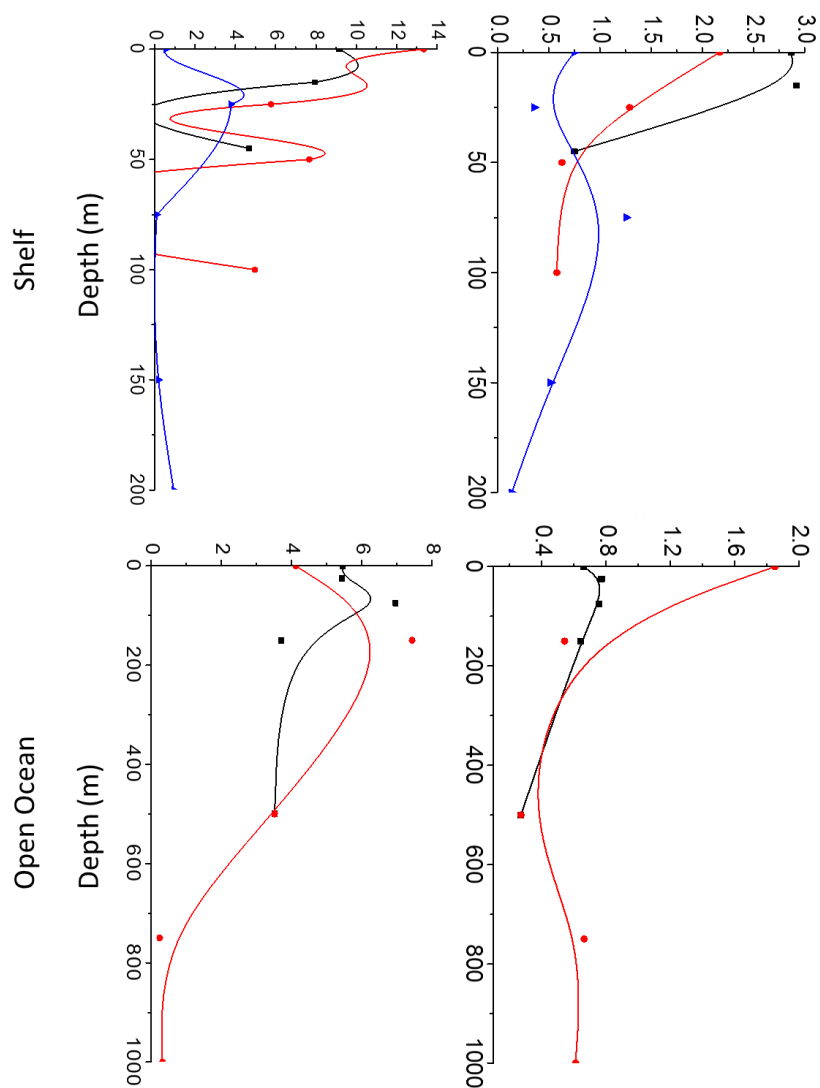


Fig. 4. PCA analysis showing the influence on environmental factors on distribution of Bacteria and Archaea. The different size bubbles given in scale on the right side of each image indicates the abundance of bacteria ($\times 10^5$ cells ml^{-1}) and archaea ($\times 10^4$ cells ml^{-1}). Different stations in Estuary are marked as E1 (station 1), E2 (station 2), E3 (station 3) and E4 (station 4), Shelf are marked as S1 (50 m), S2 (100 m), S3 (200 m) and open ocean are marked as O1 (500 m) and O2 (1000 m). Sample collection depths in each station are marked as surface (S), 5 m(B), 15 m(C), 25 m(D), 45 m (E), 50 m(F), 75 m (G), 100 m(H), 150 m(I), 200 m(J), 500 m (K), 750 m (L) and 1000 m(M)

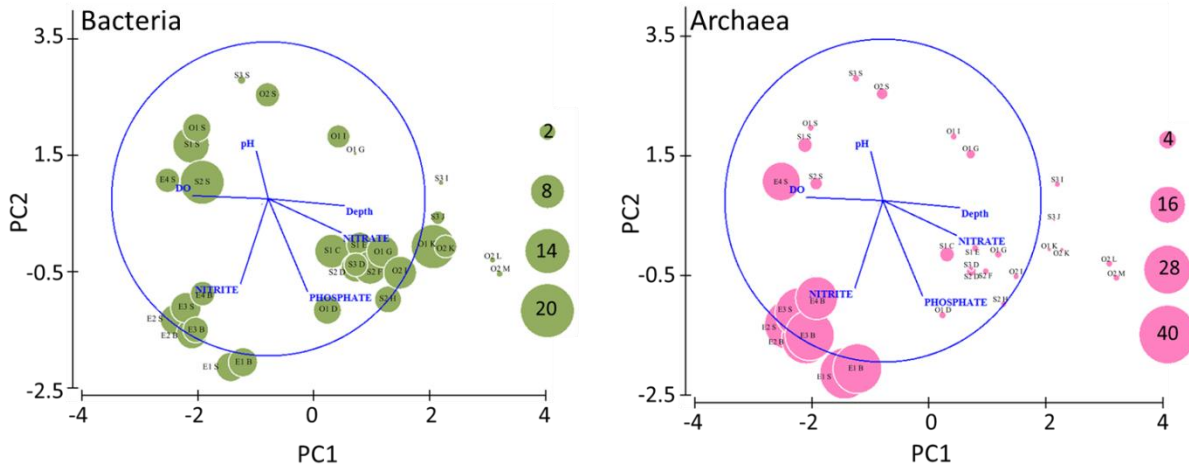


Fig. 5. Community structure of bacteria at class level in the estuary, shelf and open ocean

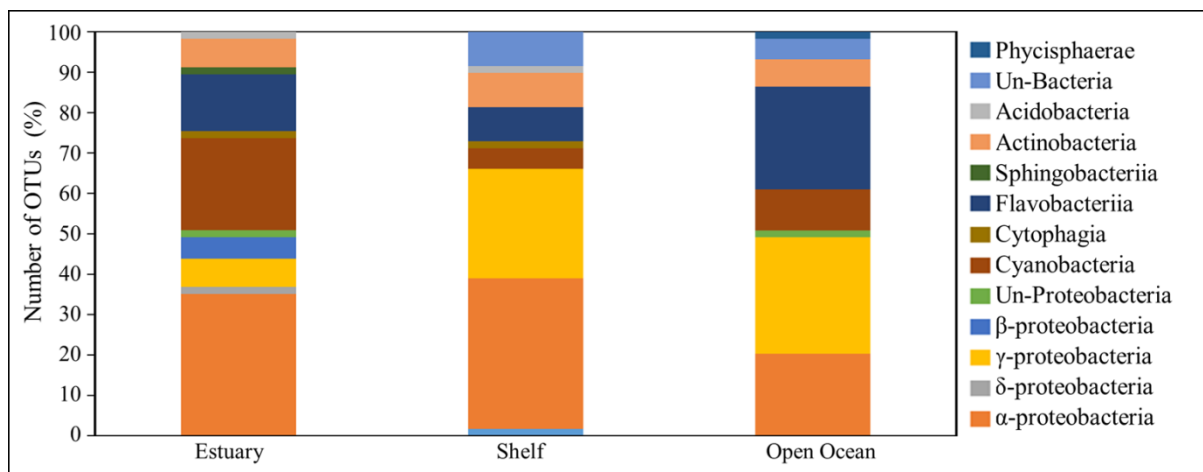


Fig. 6. Species level distribution of alpha (a) and gamma (b) proteobacteria and *Flavobacteria* (c)

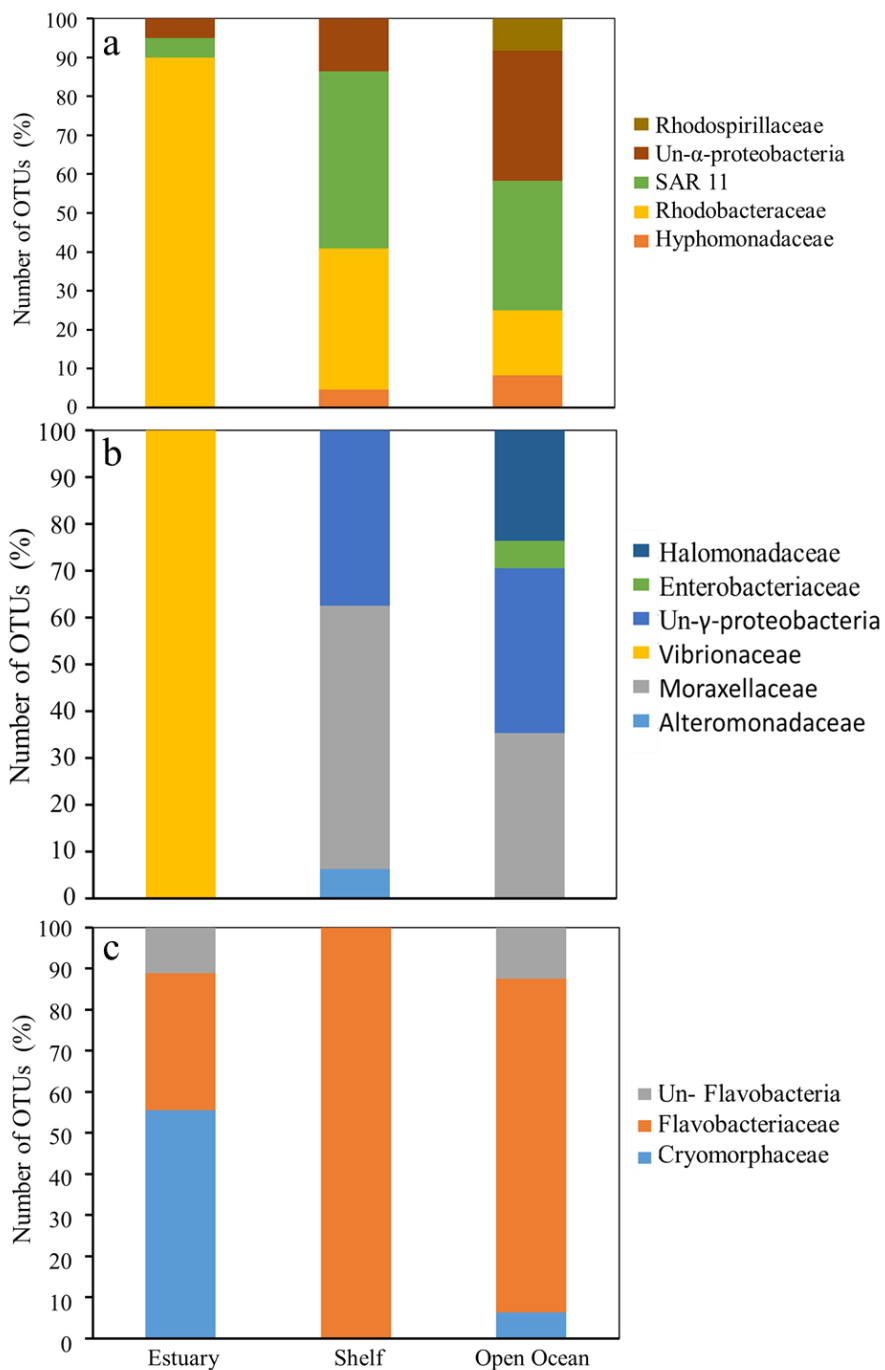


Fig. 7. Community structure of archaea at family level in the estuary, shelf and open ocean

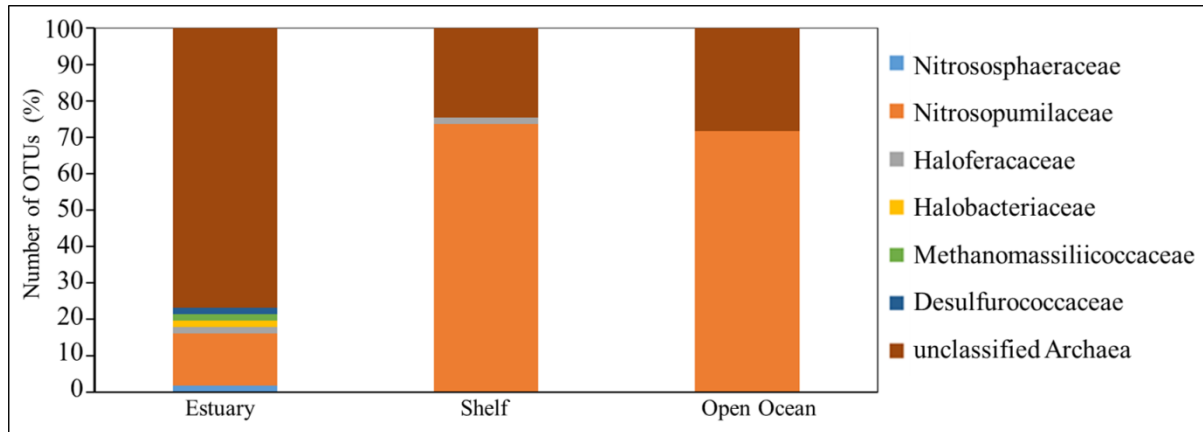
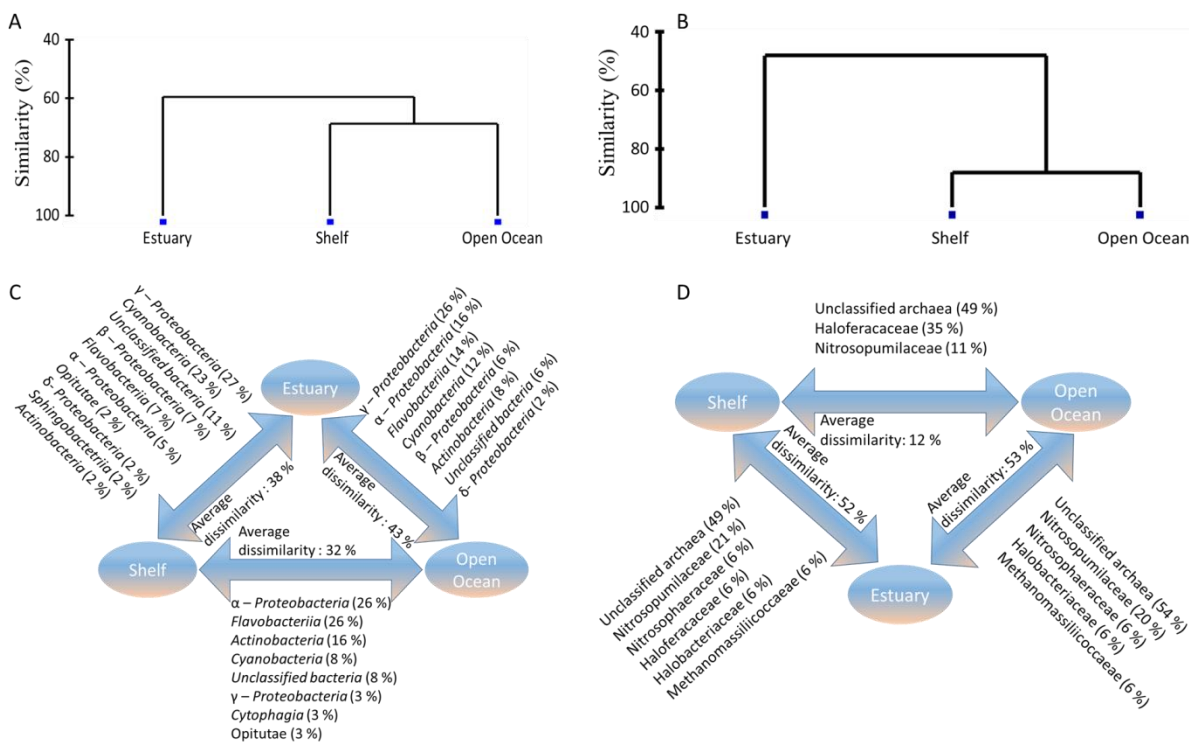
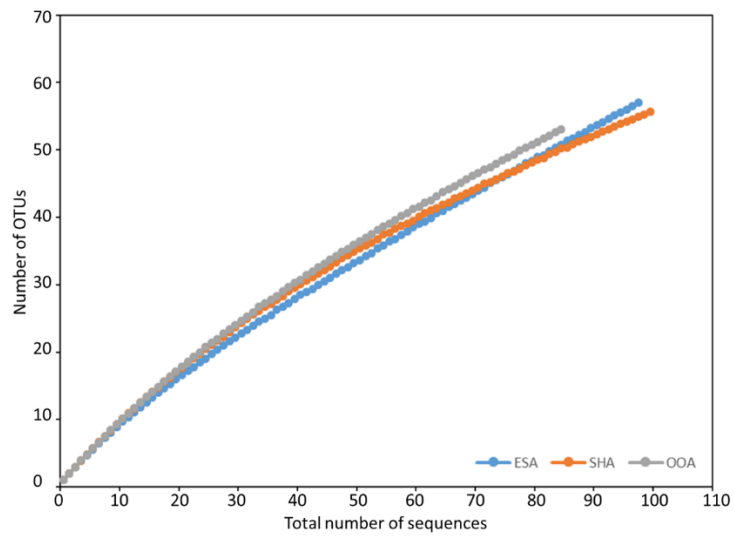


Fig. 8. Dendrogram (A & B) and schematic representation of similarity % analysis (C & D) of bacteria (A & C) and archaea (B & D) in estuary, shelf and open ocean



Supplementary Figure 1: Rarefaction curve of clone library of archae (A) and bacteria (B) from Estuary (ESA/ESB), Shelf (SHA/SHB) and Open Ocean (OOA/OOB)

A



B

