

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

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Abstract

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

Keywords: Mudbank; archaea; bacteria; upwelling; step up regression modeling; Arabian Sea

Introduction

The ecosystem at interfaces linking terrestrial and aquatic or aquatic and marine systems undergo drastic seasonal changes in environmental conditions, which may induce changes in community structure and activities of microorganisms(Naiman and Decamps, 1997). Such types of drastic environmental changes are observed in coastal environments, where multiple oceanographic processes happen simultaneously. Upwelling and mud bank formation are two processes that occur simultaneously in coastal areas of southeast Arabian Sea during the southwest monsoon(Rao et al., 1992). An interesting feature of this region is that the upwelling induces dramatic changes in the nutritional characteristics, which in turn influence the functioning of the ecosystem. The coastal upwelling is controlled by the strengthening of the alongshore wind stress towards southward direction and continental morphology. Such systems are characterized by intense primary biomass production in the surface waters after the outcropping of the nutrient-rich bottom water. Studies from other locations of coastal upwellings such as the southwest coast of Africa, have demonstrated the ecological importance of upwelling which is known to cause high nitrification(Codispoti et al., 2001; Kuypers et al., 2005; Woebken et al., 2007), increased biological productivity (Gonzalez-Rodriguez, 1994; Gonzalez-Rodriguez et al., 1992; Pereira et al., 2009),stimulate fishing activities (Cury et al., 2000) and response of microorganisms(Cury et al., 2011). Previous studies have confirmed the existence of a typical upwelling system in the southwest coast of India,with Kerala coast as the location where strongest upwelling occur(Gupta et al., 2016; Rao et al., 1992). During upwelling, the oxygen minimum zones of Arabian Sea extend to the coastal waters, creating hypoxic conditions and causing rapid changes in biogeochemical properties(Helly and Levin, 2004; Naqvi and Jayakumar, 2000). The physical forces inducing the formation of upwelling off the south westcoast of India and its implications for biological processes at different trophic levels are reported earlier, but there have been few reports on bacterial response during the process(Habeebrehman et al., 2008; Malik et al., 2015).

The biological implications of upwelling may become more complex in a mudbank, aunique feature appearing in the coastal waters of Kerala, India, during southwest monsoon(Dinesh Kumar, 2016). The waters above mud banks are characterized by high suspended matter which attenuates the waves to retain the water calm even during roughest monsoon, and copious fish catch.Historically, the mud banks have been reported across about 21 locations along the coastal areas of Kerala, of which Alappuzha is the only location where a mud bank appears every year during the southwest monsoon (Dinesh Kumar, 2016). There are several hypotheses regarding the formation of mudbanks, which includes subterranean passage, water-bearing stratum, river deposition, upwelling, rip current

wave convergence and hydrocarbon explosion. Although there is a lack of clarity over the formation of this phenomenon, mud banks have been accepted as a region of high organic matter content and a potential platform for fishing activities. Previous studies have focused on the physical factors influencing the formation of mud banks, their chemical characteristics and fishery potential (Dinesh Kumar, 2016; Jacob and Qasim, 1974; Noujas et al., 2016; Parvathy et al., 2015). The upwelling-induced high nutrient conditions in a mud bank region may create an oxygen minimum condition which provokes the conversion of microbial processes from aerobic to anaerobic. The influence of upwelling on abundance and community structure of microorganisms in a mud bank region would be important to understand, as microorganisms have central roles in food webs and biogeochemical cycles. Microorganisms with their small size and high growth rate are considered to respond quickly to environmental changes (Tuyet et al., 2015). This study forms the first detailed observation made with the goal of understanding the influence of upwelling on abundance and community structure of archaea and bacteria in a recurring mudbank region along the southwest coast of India.

1. Materials and Methods

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

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

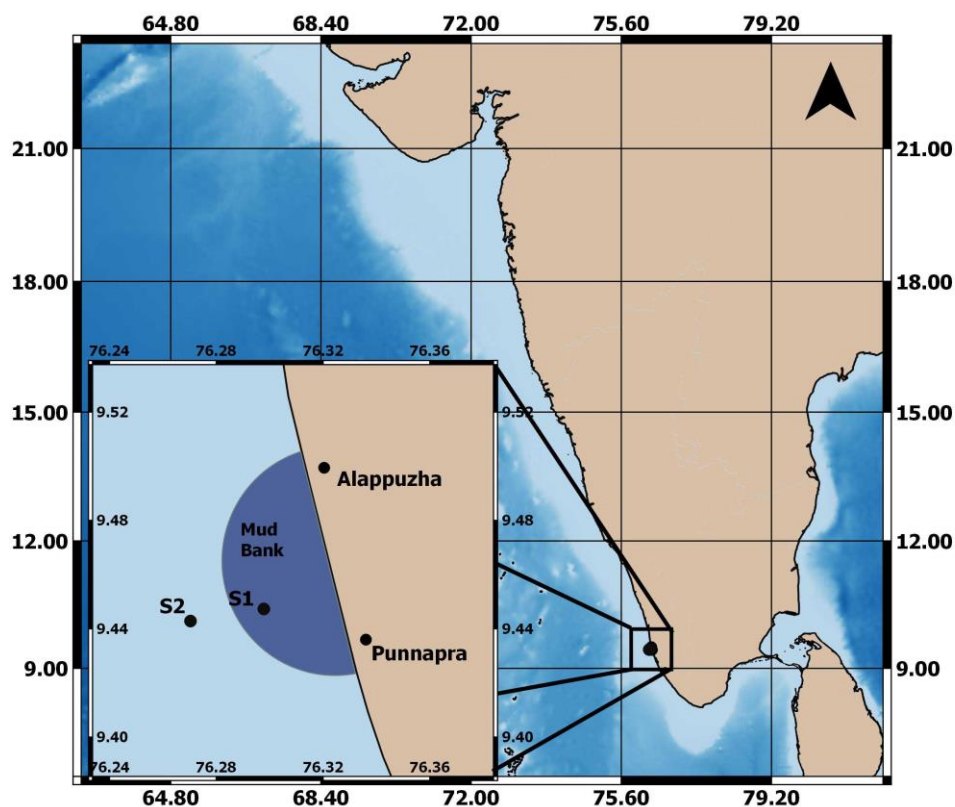


Figure 1. Map showing study area and sampling locations

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

2.2 Abundance of Archaea and Bacteria

Abundance of archaea and bacteria were quantified using fluorescent *in situ* hybridization (FISH), following the protocol of Glöckner et al., (1999) with Cy₃ labeled oligonucleotide probes such as ARCH 915 (GTGCTCCCCGCCAATTCCT) (Stahl and Amann, 1991) and EUB 338 (GCTGCCTCCCGTAGGAGT) (Amann et al., 1990) respectively. Briefly, water samples preserved in paraformaldehyde (final concentration 4% v/v) were passed through 0.2 μm white polycarbonate membrane filter (Millipore GTTP2500) for bacteria and through 0.1 μm white polycarbonate membrane filter (Millipore VCTP02500) for archaea. The membrane filters were hybridized for 90-120 min in a hybridization solution (0.9 M NaCl, 20 mM Tris-HCl (pH 7.4) and 0.01 % SDS), containing 50 ng nucleotide probes and formamide (55 and 50% respectively for archaea and bacteria). The cells were counterstained with DAPI to localize the nuclei and distinguish nonspecific bindings. Fluorescent signals from labeled cells were counted after exciting under an epifluorescence

microscope equipped with a 100 W Hg lamp and filter sets specific for DAPI and Cy₃. The cell counts from 20 fields each from duplicate filter sets of each sample were counted and the average cell counts were expressed in cells ml⁻¹. Hybridization conditions were optimized with positive and negative samples of *E. coli* cells with and without recombinant plasmids of the respective genes. A filter section of the sample without hybridization was used for subtracting auto-fluorescence from FISH counts.

2.3 Community Structure of Archaea and Bacteria

2.3.1 Extraction of DNA from water samples

Genomic DNA from water samples was extracted following Boström et al., (2004) with slight modification. Briefly, 1 to 2 L of water sample was passed through 0.2 µm polycarbonate membrane filter (Millipore, GTTP2500). The membrane filter was incubated at 37°C for 1 hr in 600 µl lysis buffer (NaCl 400 mM, Sucrose 750 mM, EDTA 20 mM and Tris-HCl 50 mM), containing 1 mg ml⁻¹ lysozyme. Subsequently, SDS (1%) and proteinase K (100 µg ml⁻¹) were added to the solution and incubation continued for 5 hr at 55°C. A 360 µl of isopropanol was added and DNA was precipitated by keeping at -20°C for 60 min. The DNA pellet was washed with 70% ethanol, dissolved in TE buffer and stored at -20°C until used.

2.3.2 PCR and Denaturing Gradient Gel Electrophoresis (DGGE)

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

PCR reaction mixture (50 μ l) was subjected to thermal conditions mentioned above with different annealing conditions for archaea and bacteria.

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

2.4 Statistical Analysis

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

2. Results

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

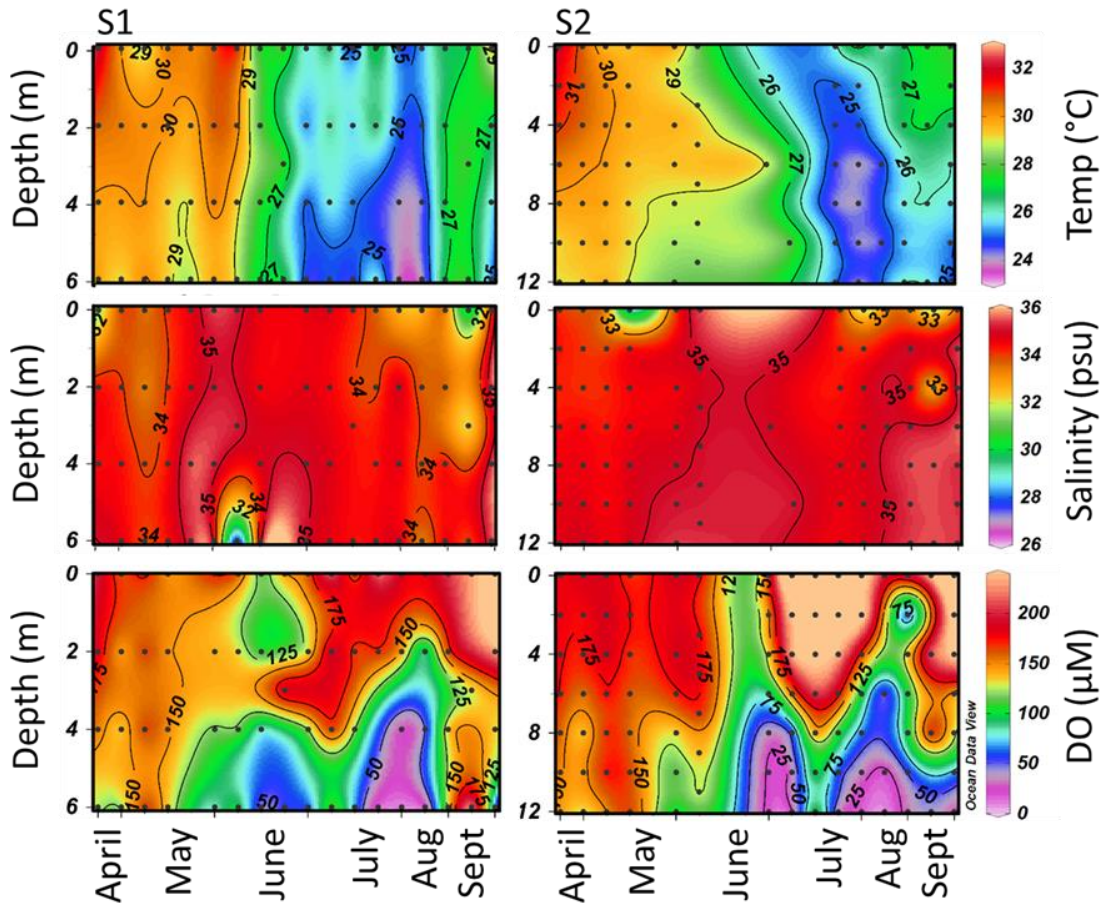


Figure 2. Profile of temperature, salinity and dissolved oxygen in S1 and S2 during the study period

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

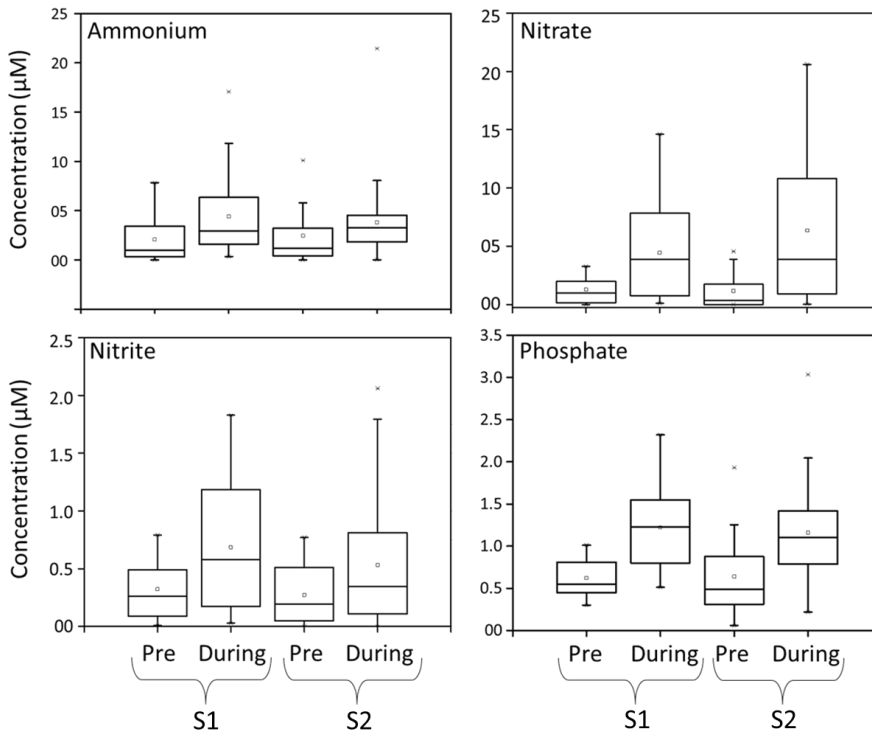


Figure 3. Box-Whisker plots showing the quartile deviation in the level of ammonium, nitrite, nitrate, and phosphate in the stations S1 and S2 at pre- and during upwelling conditions

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

3.2 Abundance and community structure of archaea and bacteria

Box-Whisker plot showing the variation in the abundance of archaea and bacteria are given in Figure 4. Archaeal abundance in S1 ranged from $1 - 16 \times 10^4$ cells ml^{-1} , at both pre- and during upwelling conditions, with an average cell count of 5.3×10^4 and 6.6×10^4 cells ml^{-1} respectively. In S2 also, the archaeal abundance ranged from $1 - 16 \times 10^4$ cells ml^{-1} , during both pre-upwelling and during upwelling conditions, with an average cell count of 5.4×10^4 and 6.2×10^4 cells ml^{-1} respectively ($P > 0.05$, Table 1). The bacterial abundance showed a six- and four-fold increase respectively for S1 and S2 during upwelling compared with that of pre-upwelling condition ($F_{(5,62)} = 4.81$, $P < 0.05$, Table 1). In S1, the bacterial abundance during pre-upwelling condition ranged from $0.5 - 4.5 \times 10^5$ cells ml^{-1} with an average of 2.97×10^5 cells ml^{-1} . These values showed significant increase at during upwelling ($F_{(3,44)} = 3.17$, $P < 0.05$, Table 1), with bacterial abundance

ranging from 0.7 to 72×10^5 cells ml^{-1} with an average of 16.5×10^5 cells ml^{-1} ($F_{(3,44)}=3.01$, $P<0.05$, Table 1). At S2, the range in abundance of bacteria increased from 0.7 to 6.0×10^5 cells ml^{-1} during pre-upwelling to 0.5 to 72×10^5 cells ml^{-1} at during upwelling condition, and the average increased from 2.3×10^5 to 9.4×10^5 cells ml^{-1} .

Variables	F _S	F _{SA}	R	F _{S'}
Station S1				
Temperature	1.89 (2,44) ^{NS}	49.41(3,44)* *	977.7	0.04(2,2) ^{NS}
Salinity	1.14 (2,44) ^{NS}	1.06 (3,44) ^{NS}	20.9	1.07(2,2) ^{NS}
DO	2.81 (2,42) ^{NS}	1.42(3,42) ^{NS}	16.97	1.97 (2,2) ^{NS}
Ammonium	0.18 (2,44) ^{NS}	2.88 (3,44) *	56.95	0.06 (2,2) ^{NS}
Nitrite	0.89 (2,44) ^{NS}	4.24 (3,42) *	83.98	0.20 (2,2) ^{NS}
Nitrate	3.03(2,44) ^{NS}	6.04 (3,44) **	119.61	0.48 (2,2) ^{NS}
Phosphate	0.18 (2,44) ^{NS}	12.18 (3,44) **	241.014	0.01 (2,2) ^{NS}
Archaea	1.83(2,44) ^{NS}	3.17 (3,44) *	75.52	0.56 (2,2) ^{NS}
Bacteria	1.10(2,49) ^{NS}	3.01(3,44) *	62.51	0.35(2,2) ^{NS}
Station S2				
Temperature	1.79 (4,47) ^{NS}	19.67 (5,47)**	9975.2	0.09 (4,4) ^{NS}
Salinity	7.37 (4,47)**	0.24(5,47) ^{NS}	121.71	30.70(4,4) ^{NS}
DO	28.54(4,61) **	8.89(5,61) **	126.18	3.01(4,4) ^{NS}
Ammonium	1.03(4,60) ^{NS}	1.20(5,60) ^{NS}	14.65	0.84(4,4) ^{NS}
Nitrite	6.17 94,60) **	2.73 (5,60) *	41.76	2.16(4,4) ^{NS}
Nitrate	3.39 (4,61) *	7.33 (5,61) **	104.10	0.43(4,4) ^{NS}
Phosphate	3.7294,58) **	3.54(5,61) **	50.19	0.99(4,4) ^{NS}
Archaea	1.33(4,58) ^{NS}	1.07(5,58) ^{NS}	14.39	1.24(4,4) ^{NS}
Bacteria	0.71(4,62) ^{NS}	4.81(5,62) **	45.75	0.14(4,4) ^{NS}

Table 1: Nested ANOVA analysis of the significance of variation in hydrographic variables and abundance of archaea and bacteria in stations S1 and S2. The variables were tested for different depths and between pre- and during upwelling conditions. The values in bracket

indicate degrees of freedom of Snedecor's F statistic, NS means not significant, the level of significance is indicated as *significant ($P < 0.05$) and **highly significant ($P < 0.01$). R indicates constant determining Gaylor and Hopper condition. F_S indicates F statistic among depths and F_S' is F statistic among depths calculated when Gaylor and Hopper's condition is satisfied. F_{SA} is the F statistic calculated among pre- and during upwelling conditions

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

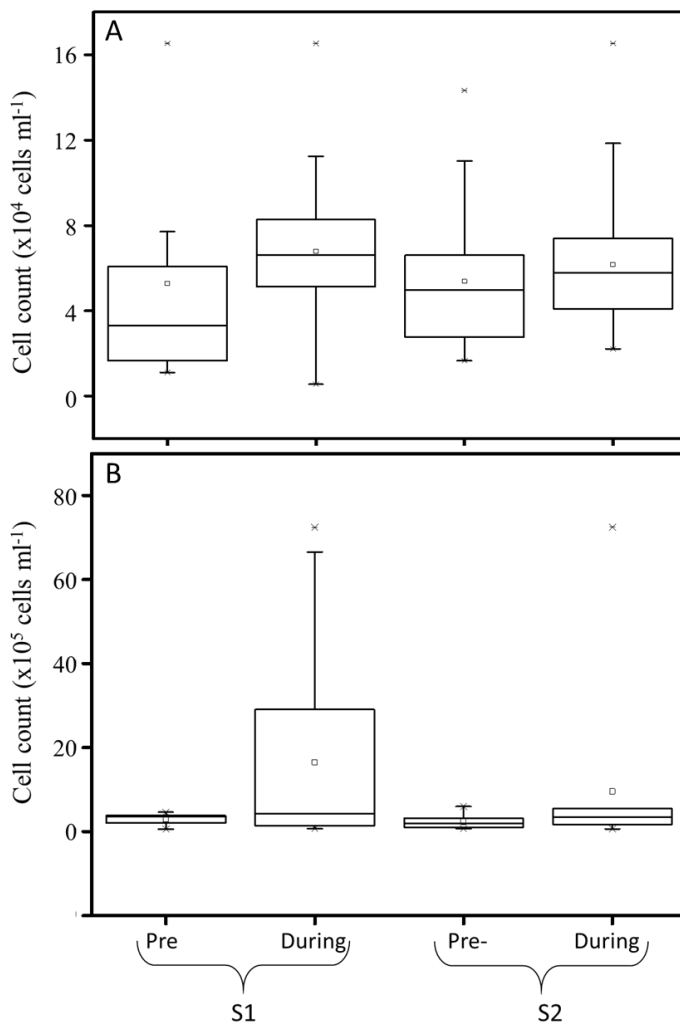


Figure 4. Box-Whisker plot showing the quartile deviation in the abundance of archaea (A) and bacteria (B) in the stations S1 and S2 at pre- and during upwelling conditions

The community structure of archaea and bacteria, at pre- and during upwelling conditions was analyzed using the PCR-DGGE technique. Since there was no statistically significant difference in the abundance of bacteria between samples from the surface to bottom ($P > 0.05$, Table 1), we pooled

the samples. The community structure of archaea and bacteria were analyzed in samples collected during May, June, July, August, and September. About four to six bands were present in DGGE gel of archaea (Supplementary figure 2), which did not show significant variations between pre-upwelling and during upwelling conditions (Fig. 5). Shannon-Wiener diversity index for archaea ranged from 1.1 to 1.5 in both S1 and S2. Nine to fourteen bands were observed in the bacterial DGGE of S1 and S2.

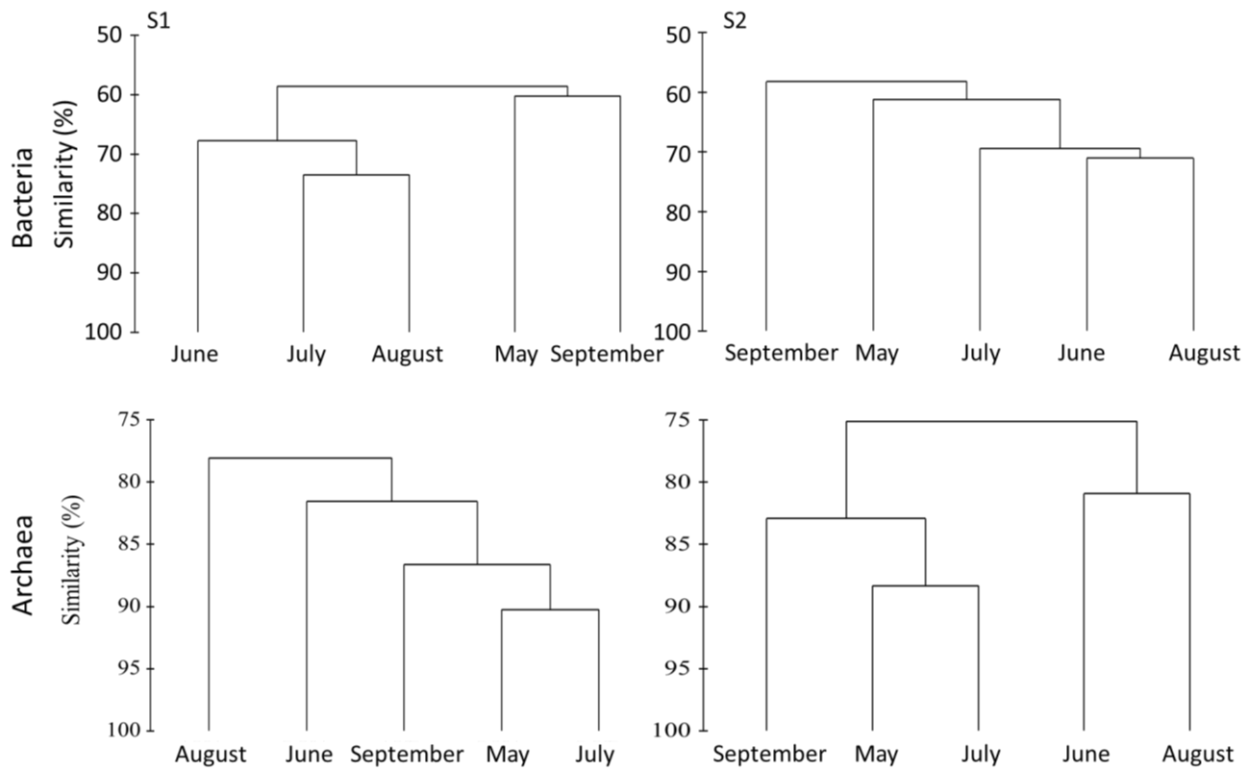


Figure 5. Brey-Curtis similarity cluster based on DGGE pattern of archaea and bacteria in S1 and S2 during pre- (May) and during upwelling (June- September) conditions

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

3.3 Environmental influence on abundance of archaea and bacteria

Linear multiple regression models were employed to explain the role of hydrographic variables and their first-order interaction effects on the distribution of archaea and bacteria at S1 and S2. During pre-upwelling condition, the distribution of archaea in mudbank station S1 was influenced by temperature ($r=0.253$), salinity ($r=-0.566$), DO ($r=-0.458$), nitrate ($r=0.0026$) and ammonium ($r=-0.278$). The individual effect of these hydrographic variables along with nitrite ($r=-0.366$) and phosphate ($r=-0.468$) could explain the variation in archaeal distribution at 32.91% confidence. Further, the variation in archaeal distribution could be explained at 66.5% confidence by considering the interaction effect of the above five hydrographic variables ($F_{(15,5)}=3.6478, P<0.05, N=21$) among which interacting effect of salinity & nitrate (26.73%), temperature & nitrate (25.76%) and nitrate & ammonium (3.56%) are the significant ones. Archaeal abundance in S1 during upwelling conditions were also influenced by individual and interaction effects of temperature, salinity, DO, nitrite, nitrate, ammonium, and phosphate. On an individual scale, the archaeal abundance was negatively influenced by temperature ($r=-0.2530$), DO ($r=-0.3273$) and nitrate ($r=-0.0904$), while salinity ($r=0.2610$), nitrite ($r=0.2169$), ammonium ($r=0.115$) and phosphate ($r=0.0745$) showed positive influence. About 67.01% of the temporal variability of archaea in S1 at during upwelling condition could be explained by modeling using the individual and interaction effect of the seven hydrographic variables listed above ($F_{(28,2)}=3.1764, P<0.05, n=31$). The major interactions which contributed to the distribution of archaea in S1 were: DO & phosphate (8.77%), nitrite & phosphate (8.13%), temperature & nitrite (7.98%), salinity & phosphate (5.83%), salinity & nitrite (5.3%) and temperature & ammonium (4.1%).

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

When the individual contributions were considered, only 22.9% of the temporal variability of bacterial distribution ($F_{(7,13)}=1.8484, P<0.16, n=21$) in S1 station at pre-upwelling conditions were controlled by temperature ($r=0.34$), salinity ($r=-0.28$), DO ($r=-0.053$), nitrite ($r=0.086$), nitrate ($r=-0.046$), ammonium ($r=-.35$) and phosphate ($r=-0.29$). On the other hand, 96.46% of the

temporal variability in bacterial distribution ($F_{(15,5)}=37.6634, p<0.01, n=21$) at S1 could be explained with the step up multiple regression model. The major contributing interaction effects include nitrite&ammonium(31.62%), DO&nitrite (3.34%) and salinity&ammonium (3.23%). The hydrographic variables such as salinity, DO, nitrite, nitrate, ammonium, phosphate and their first order interaction effects could explain only 41.4% of the temporal variability of bacteria ($F_{(21,9)}=2.01, P<0.05, n=31$) in S1 during-upwelling condition. The hydrographic variables phosphate ($r= -0.547$), nitrite ($r= -0.467$) and salinity ($r=-0.121$) influenced the bacterial distribution negatively while temperature ($r=0.369$) and DO ($r=.3354$) imparted positive influence.

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

3. Discussion

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

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

During upwelling, nutrient-rich waters are physically transported to the surface, which initiates a cascade of energy transfer process from microorganisms to other organisms in a higher level of food web through phytoplankton and zooplankton (Postel and Packard, 2014). However, our understanding of the influence of upwelling on abundance and community structure of microorganisms are limited. We observed a significant increase in the abundance of bacteria during upwelling. The bacterial abundance found in the study area at pre-upwelling and during upwelling conditions were in agreement with previous reports from elsewhere on the Kerala coast (Malik et al., 2015) and other upwelling waters (Barbosa et al., 2001). In a two-year data sets, Tsai et al., (2010) observed the higher abundance of bacteria during upwelling and suggested the more pronounced influence of available nutrients than the temperature in subtropical Ilan Bay in Taiwan. A coupling between available nutrients and temperature in determining the bacterial distribution in Cabo Frio upwelling region in Brazilian coast was also suggested by Coelho-Souza et al., (2015). An increase in the abundance and growth rate of prokaryotes in response to temperature has been reported earlier from coastal waters of the temperate and subtropical zone (Tuyet et al., 2015). Chen et al., (2012) reported a unimodal relationship between bacterial abundance and temperature in the South China Sea using generalized additive models. Although many studies reported the individual effect of

nutrients on the distribution of microorganisms in the marine environment, the interaction effects are rarely discussed (Sneha et al., 2016). The linear stepup multiple regression models used in the present study indicates that the interaction effects of nutrients have a more pronounced influence on the distribution of archaea and bacteria in the system than the individual nutrients. For example, the confidence level of explaining bacterial distribution in S1 station at pre-upwelling condition increased from 22.9 to 96.5 %, when the individual influence of hydrographic variables was replaced with their interaction effect. However, the step up regression modeling using interaction effects of hydrographic variables monitored in the study were also not successful in explaining the distribution of archaea or bacteria at during upwelling condition to more than 70 % confidence. The percentage of the temporal variation in bacteria distribution at station S1 that can be explained by the regression model decreased from 96.5 % in pre-upwelling conditions to 41.4 % during upwelling conditions. This clearly indicates the existence of complex characteristics of the system during upwelling. The major limitation of the present model is that we didn't include the bacterivory and viral lysis, which can also play a significant role in regulating bacterial distribution (Chen et al., 2012). A coupled physical-biogeochemical model would give a more realistic view of the complex interactions taking place during upwelling. Interestingly, the abundance of archaea remained unchanged before (pre-upwelling) and during upwelling condition in S2, while it shifted slightly during upwelling in S1. Also, the diversity of archaea was found to be less compared to bacteria, which is in agreement with the observations from tropical upwelling regions of the Brazilian coast (Cury et al., 2011).

The mud banks, being calm and hypoxic water bodies, may function as the bioreactor for the mineralization of organic matter brought up by upwelling. The pileup of organic matter was indicated by high levels of Chlorophyll-a during upwelling (7-8 mg m⁻³) compared to pre-upwelling (3-4 mg m⁻³) condition (Madhu et al. 2015). The increase in substrate availability enhances the abundance and activity of microorganism (Antonio Cuevas et al., 2004). It was perceived earlier that the conditions such as high organic load and upwelling induced suboxic/anoxic conditions across the coastal waters of India are conducive for the biogenic production of methane (Jayakumar et al., 2001). However, an abrupt reduction in methane during upwelling was reported recently in the mud banksof Alappuzha (Gireeshkumar et. al., 2017). The recurrence of hypoxic water with the accumulation of nitrite and ammonium in highly productive systems like mud banks sets a favorable condition forenriching anaerobic microorganisms. This may be discussed in line with the observed shift in the community structure of archaea and bacteria during upwelling. The two clusters of the bacterial community, i.e cluster 1 (May and September) and cluster 2 (June, July, and August), strongly suggest the existence of a transient population of bacteria during upwelling which participates actively in degradation of detrital organic matter. The community structure returns to the

native stage after the process, and accordingly the bacterial community structure in May and September clustered with nearly 60 % similarity. A community shift of bacteria due to upwelling was also reported from the Brazilian coast (Cury et al., 2011). It is evident in the literature that different groups of bacteria thrive in different depths and nutrient conditions in the sea (Cury et al., 2011; DeLong et al., 2006; Massana et al., 1997; Murray et al., 1998). Under the hypoxic conditions prevailing over a mudbank, it is possible that the oxygen-dependent conversion of ammonium and methane could be shifted to a nitrite dependent anaerobic methane (n-damo) and ammonium (anammox) oxidation. It is proved in closed system experiments that the anaerobic reactions are more efficient compared to oxygen-dependent reactions (Ni et al., 2013) and releases fewer amounts of greenhouse gases. This could be a possible reason for reduced methane over a mud bank during upwelling observed by Gireeshkumar et al., (2017), and if so mud banks could function as a sink of greenhouse gases like methane.

4. Conclusions

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

Acknowledgement

The authors thank the Director, CSIR-National Institute of Oceanography, Goa and the Scientist-in-Charge, CSIR-NIO Regional Centre Kochi, for extending all required support. The laboratory facility was extended by MMRF of CSIR-NIO, RC, Kochi, funded by Ministry of Earth Sciences, New Delhi. This is a CSIR-NIO contribution No. XXX

Conflict of interest

All authors declare no conflicts of interest

Reference

- Amann, R., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.* 56, 1919 - 1925.
- Anabalon, V., Aristegui, J., Morales, C.E., Andrade, I., Benavides, M., Correa-Ramirez, M.A., Espino, M., Ettahiri, O., Hormazabal, S., Makaoui, A., Montero, M.F., Orbi, A., 2014. The

structure of planktonic communities under variable coastal upwelling conditions off Cape Ghir (31°N) in the Canary current system (NW Africa). *Prog. Oceanogr.* 120, 320 - 339.

Antonio Cuevas, L., Daneri, G., Jacob, B., Montero, P., 2004. Microbial abundance and activity in the seasonal upwelling area off Concepcion (~36 °S), central Chile: a comparison of upwelling and non-upwelling conditions. *Deep-Sea Res. II.* 51, 2427 - 2440.

Barbosa, A.B., Galvao, H.M., Mendes, P.A., Alvarez-Salgado, X.A., Figueiras, F.G., Joint, I., 2001. Short-term variability of heterotrophic bacterioplankton during upwelling off the NW Iberian margin. *Prog. Oceanogr.* 51, 339 - 359.

Boström, K.H., Simu, K., Hagström, Å., Riemann, L., 2004. Optimization of DNA extraction for quantitative marine bacterioplankton community analysis. *Limnol. Oceanogr. Methods* 2. 365-373.

Capone, D.G., Hutchins, D.A., 2013. Microbial biogeochemistry of coastal upwelling regimes in a changing ocean. *Nature Geosc.* 6, 711 - 717.

Chen, B., Liu, H., Huang, B., 2012. Environmental controlling mechanisms on bacterial abundance in the South China Sea inferred from generalized additive models (GAMs). *J. Sea Res.* 72, 69 - 76.

Codispoti, L.A., Brandes, J.A., Christensen, J.P., Devol, A.H., Naqvi, S.W.A., *et.al*, 2001. The oceanic fixed nitrogen and nitrous oxide budgets: moving targets as we enter the Anthropocene? *Sci. Mar.* 65, 85 - 105.

Coelho-Souza, S.A., V.Araujo, F., Cury, J.C., Jesus, H.E., Pereira, G.C., Guimaraes, J.R.D., Peixoto, R.S., Davila, A.M.R., Rosado, A.S., 2015. Bacterial and Archaeal communities variability associated with upwelling and anthropogenic pressure in the protection area of Arraial do Cabo (Cabo Frio region-RJ). *An. Acad. Bras. Cienc.* 87, 1737 - 1750.

Cury, J.C., Araujo, F.V., Coelho-Souza, S.A., Peixoto, R.S., Oliveira, J.A.L., Santos, H.F., Davila, A.M.R., Rosado, A.S., 2011. Microbial diversity of a Brazilian coastal region influenced by an upwelling system and anthropogenic activity. *PLoS ONE* 6, e16553.

Cury, P., Bakun, A., Crawford, R.J.M., Jarre, A., Quinones, R.A., *al e.*, 2000. Small pelagics in upwelling systems: patterns of interaction and structural changes in "wasp-waist" ecosystems. *ICES J. Mar. Sci.* 57, 613 - 618.

DeLong, E.F., Preston, C.M., Mincer, T., Rich, V., Hallam, S.J., *et.al*, 2006. Community genomics among stratified microbial assemblages in the ocean's interior. *Science* 311, 496 - 503.

Dinesh Kumar, P.K., 2016. Mudbanks of Kerala: Mystery to be unveiled. *Sci. Rep.* 53, 38 - 41.

Gaylor, G.W., Hopper, F.N., 1969. Estimating the degrees of freedom for linear combinations of mean squares by Scatterhwaite's formula. *Technometrics* 11, 695 - 705.

Gireeshkumar, T.R., Mathew, D., Prathihary, A.K., Naik, H., Narvekar, K.U., Araujo, J., Balachandran, K.K., Muraleedharan, K.R., Thorat, B., Nair, M., Naqwi, S.W.A., 2017. Influence of upwelling induced nearshore hypoxia on the Alappuzha mud banks, southwest coast of India. *Cont. Shelf Res.* 139, 1-8

- Glöckner, F.O., Fuchs, B.M., Amann, R., 1999. Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. *Appl. Environ. Microbiol.* 65, 3721-3726.
- Gonzalez-Rodriguez, E., 1994. Yearly variation in primary productivity of marine phytoplankton from Cabo Frio (RJ, Brazil) region. *Hydrobiologia* 294, 145 - 156.
- Gonzalez-Rodriguez, E., Valentin, J.L., Andre, D.L., Jacob, S.A., 1992. Upwelling and downwelling at Cabo Frio (Brazil): comparison of biomass and primary production responses. *J. Plankton Res.* 14, 289 - 306.
- Grasshoff, K., Ehrhardt, M., Kremling, K., 1983. *Methods of seawater analyses.* Verlag Chemie. Weinheim, Germany 511, 342-355.
- Gupta, G.V.M., Sudheesh, V., Sudharma, K.V., Saravanane, N., Dhanya, V., Dhanya, K.R., Lakshmi, G., Sudhakar, M., Naqwi, S.W.A., 2015. Evolution to decay of upwelling and associated biogeochemistry over the southeastern Arabian Sea shelf. *J. Geophys. Res.: Biogeosci.* 121, 159 - 175.
- Gupta, G.V.M., Sudheesh, V., Sudharma, K.V., Saravanane, N., Dhanya, V., Dhanya, K.R., Lakshmi, G., Sudhakar, M., Naqwi, S.W.A., 2016. Evolution to decay of upwelling and associated biogeochemistry over the southeastern Arabian Sea shelf. *J. Geophys. Res.: Biogeosci.* 121, 159 - 175.
- Habeebrehman, H., Prabhakaran, M.P., Jacob, J., Sabu, P., Jayalakshmi, K.J., achuthankutty, C.T., Revichandran, C., 2008. Variability in biological responses influenced by upwelling events in the Eastern Arabian Sea. *J. Mar. Sys.* 74, 545 - 560.
- Helly, J.J., Levin, L.A., 2004. Global distribution of naturally occurring marine hypoxia on continental margins. *Deep-Sea Res. I* 51, 1159 - 1168.
- Jacob, P.G., Qasim, S.Z., 1974. Mud of a mudbank in Kerala, South-West coast of India. *Indian J. Mar. Sci.* 3, 115 - 119.
- Jayakumar, D.A., Naqwi, S.W.A., Narvekar, P.V., George, M.D., 2001. Methane in coastal and offshore waters of the Arabian Sea. *Mar. Chem.* 74:1 - 13
- Kuypers, M.M.M., Lavik, G., Woebken, D., Schmid, M., Fuchs, B., *al., e.*, 2005. Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation. *Proc. Nat. Acad. Sci., USA* 102, 6478 - 6483.
- Madhu, N.V., Ullas, N., Ashwini, R., Jyothibabu, R., Muraleedharan, K.R., *et.al.*, 2015. Upwelling induced shifts in the phytoplankton community in the Alleppey mudbanks, southwest coast of India. *Ocean Society of India Conference (OSICON)*, 22 - 25 March 2015, Goa, India.
- Malik, A., Fernandes, C.E.G., Gonsalves, M.-J.B.D., Subina, N.S., Mamatha, S.S., Krishna, K., Varik, S., Kumari, R., Gauns, M., Cejocice, R.P., Pandey, S.S., Jineesh, V.K., Kamaleson, A.S., Vijayan, V., Mukherjee, I., Subramanyan, S., Nair, S., Ingole, B., LokaBharathi, P.A., 2015. Interactions between trophic levels in upwelling and non-upwelling regions during summer-monsoon. *J. Sea Res.* 95, 56 - 69.

- Massana, R., Murray, A.E., Preston, C.M., DeLong, E.F., 1997. Vertical distribution and phylogenetic characterization of marine planktonic Archaea in the Santa Barbara Channel. *Appl. Environ. Microbiol.* 63, 50 - 56.
- Murray, A.E., Preston, C.M., Massana, R., Taylor, L.T., Blakis, A., al, e., 1998. Seasonal and spatial variability of bacterial and archaeal assemblages in the coastal waters near Anvers Island, Antarctica. *Appl. Environ. Microbiol.* 64, 2585 - 2595.
- Naiman, R.J., Decamps, H., 1997. The ecology of Interfaces: Riparian Zones. *Ann. Rev. Ecol. Syst.* 28, 621 - 658.
- Naqvi, S.W.A., Jayakumar, D.A., 2000. Ocean biogeochemistry and atmospheric composition: Significance of the Arabian Sea. *Curr. Sci.* 78, 289 - 299.
- Ni, B-J., Smets, B.F., Yuan, Z., Pellicer-Nàcher, C., 2013. Model-based evaluation of the role of Anammox on nitric oxide and nitrous oxide productions in membrane aerated biofilm reactor. *J.Membr. Sci.* 446, 332-340.
- Noujas, V., Thomas, K.V., Badarees, K.O., 2016. Shoreline management plan for a mudbank dominated coast. *Ocean Eng.* 112, 47 - 65.
- Parvathy, K.G., Noujas, V., Thomas, K.V., Ramesh, H., 2015. International conference on water resources, coastal and ocean engineering (ICWRCOE'15) Impact of Mudbanks on Coastal Dynamics. *Aqua. Proc.* 4, 1514-1521.
- Pereira, G.C., Evsukoff, A.G., Ebecken, N.F.F., 2009. Fuzzy modeling of chlorophyll production in Brazilian upwelling system. *Ecol. Model.* 220, 1506 - 1512.
- Postel, L., Packard, T.T., 2014. Upwelling and successive ecosystem response in the Northern Benguela Region - an in situ experiment. *J. Mar. Sys.* 140, 73 - 81.
- Rao, D.S., Ramamirtham, C.P., Murty, A.V.S., Muthusamy, S., Kunhikrishnan, N.P., Khambadkar, L.R., 1992. Oceanography of the Arabian Sea with particular reference to the southwest monsoon. *Bulletin of Central Marine Fisheries Research Institute* 45, 4 - 8.
- Rohlf, R.R., James Sokal, F., 1981. *Biometry, the principles and practice of statistics in biological research*, 2nd ed. W.H. Freeman & Company.
- Sneha, K.G., Anas, A., Jayalakshmy, K.V., Jasmin, C., Vipin Das, P.V., Somnath Pai, S., Pappu, S., Nair, M., Muraleedharan, K.R., Sudheesh, K., Nair, S., 2016. Distribution of multiple antibiotic resistant *Vibrio* spp across Palk Bay. *Reg. Stud. Mar. Sci.* 3, 242 - 250.
- Stahl, D.A., Amann, R., 1991. *Development and application of nucleic acid probes*. John Wiley & Sons Ltd, Chichester.
- Tsai, A.-Y., Gong, G.-C., Sanders, R.W., Chao, C.-F., Chang, K.-P., 2010. Microbial dynamics in an oligotrophic bay of the western subtropical Pacific: Impact of short term heavy freshwater runoff and upwelling. *J. Oceanogr.* 66, 873 - 883.
- Tuyet, D.T.A., Tanaka, T., Sohrin, R., Hao, D.M., Nagaosa, K., Kato, K., 2015. Effects of warming on microbial communities in the coastal waters of temperate and subtropical zones in the Northern hemisphere, with a focus on *Gammaproteobacteria*. *J. Oceanogr.* 71, 91 - 103.

Vergas, C.A., Martinez, R.A., Antonio Cuevas, L., Pavez, M.A., Cartes, C., Gonzalez, u.E., Escribano, R., Daneri, G., 2007. Relative importance of microbial and classical food webs in a highly productive coastal upwelling area. *Limnol. Oceanogr.* 52, 1495 - 1510.

Woebken, D., Fuchs, B., Kuypers, M.M.M., Amann, R., 2007. Potential interactions of particle-associated anammox bacteria with bacterial and archaeal partners in the Namibian upwelling system. *Appl. Environ. Microbiol.* 73, 4648 - 4657.

Figure legends

Figure 1. Map showing study area and sampling locations

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

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

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

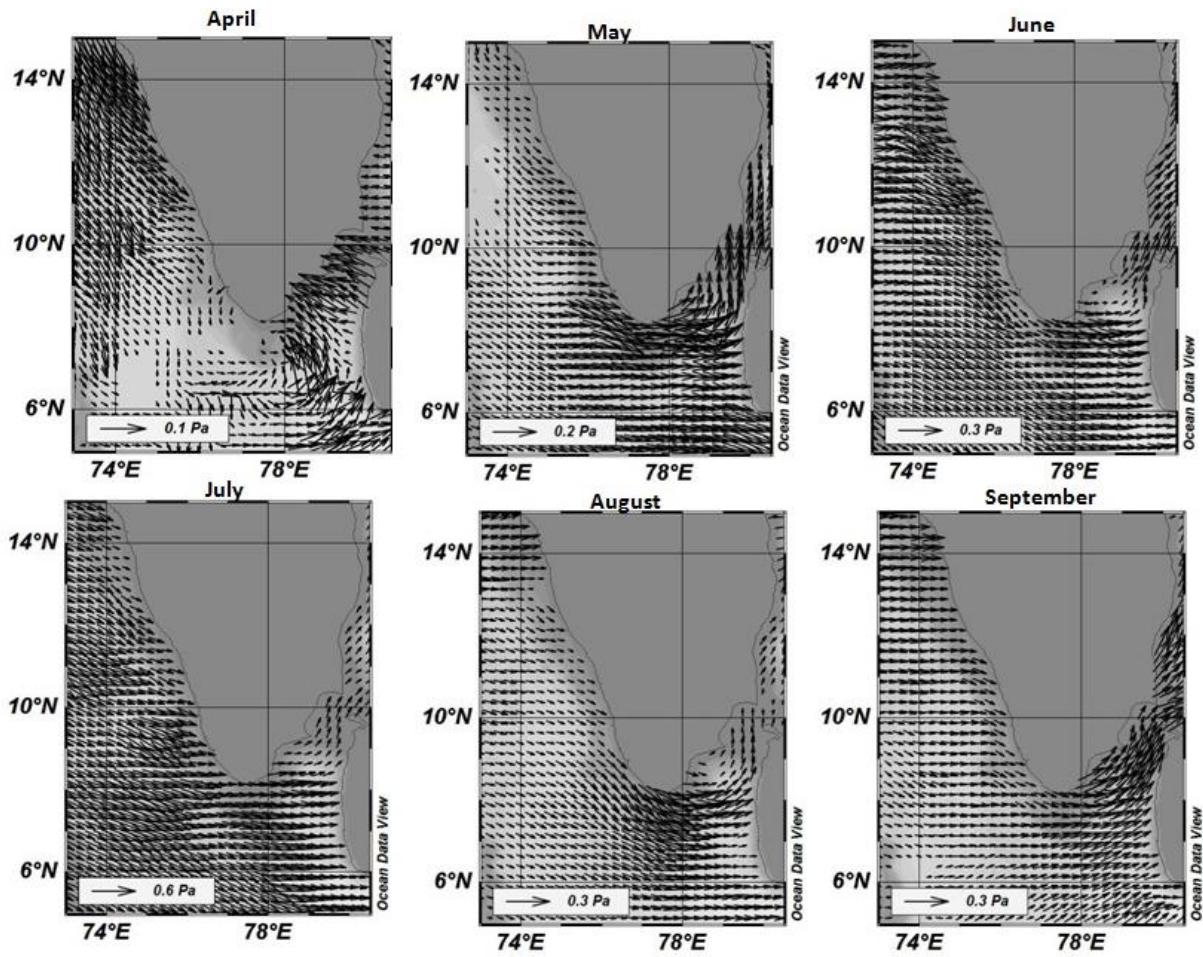
Figure 5. Bray-Curtis similarity cluster based on DGGE pattern of archaea and bacteria in S1 and S2 during pre- (May) and during-upwelling (June- September) conditions

Supplementary Figure

Supplementary Figure 1: Monthly variation in wind pattern and speed in the study area from April to September

Supplementary Figure 2. DGGE band pattern of archaea and bacteria in S1 and S2 during study period

Supplementary Figure 1: Monthly variation in wind pattern and speed in the study area from April to September



Supplementary Figure 2. DGGE band pattern of archaea and bacteria in S1 and S2 during study period

