

## Diversity and seasonal distribution of ammonia-oxidizing archaea in the water column of a tropical estuary along the southeast Arabian Sea

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**Running title:** Diversity and distribution of AOA in a tropical estuary.

### Abstract

Diversity and distribution pattern of ammonia-oxidizing archaea (AOA) were studied across a salinity gradient in the water column of Cochin Estuary (CE), a tropical monsoonal estuary along the southeast Arabian Sea. The water column of CE was found to be nutrient rich with high bacterial ( $3.7 - 6.7 \times 10^8$  cells L<sup>-1</sup>) and archaeal abundance ( $1.9 - 4.5 \times 10^8$  cells L<sup>-1</sup>). Diversity and seasonal variation in the distribution pattern of AOA were studied using clone library analysis and Denaturing Gradient Gel Electrophoresis (DGGE). Clone library analysis of both the *amoA* and 16S rRNA gene sequences showed similar diversity pattern, however the diversity was more clear when the 16S rRNA gene sequences were analyzed. More than 70% of the sequences retrieved were clustered under uncultured Thaumarchaeota group 1 lineage and the major fractions of the remaining sequences were grouped into the *Nitrosopumilus* lineage and *Nitrosopelagicus* lineage. The AOA community in the CE was less adaptable to changing environmental conditions and its distribution showed seasonal variations within the DGGE banding pattern with higher diversity during the pre-monsoon period. The distribution of AOA also showed its preference to intermediate salinity for their higher diversity. Summer monsoon associated runoff and flushing played a critical role in regulating the seasonality of AOA distribution.

**Key words:** Ammonia oxidation, Archaea, Clone library, DGGE, Tropical estuary

## Introduction

Nitrogen (N) acts as a limiting nutrient in the marine ecosystem and its biogeochemical cycling normally functions in close association with the carbon cycle, which in turn determines the productivity and the health of the entire ecosystem (Falkowski 1997). However, in eutrophic environments like estuarine and coastal system, N compounds contribute to a significant portion of the organic matter overloading. The microbial oxidation of ammonia to nitrate via nitrite ( $\text{NH}_3\text{-NO}_2\text{-NO}_3$ ) is a key reaction in the global biogeochemical cycle of N. The microbial nitrification plays a vital role in the biodegradation and bioremediation of anthropogenic N inputs received by the estuarine and coastal environments (Caffrey et al. 2007; Francis et al. 2005). About 30 % of the globally fixed N loss occurs in these environments through coupled nitrification-denitrification or nitrification–anammox (anaerobic oxidation of ammonia) processes (Lam et al. 2007; Seitzinger 1988; Voss et al. 2011). Nitrification also functions as an important oxygen-consuming process in eutrophic environments and significantly contributes to the formation of hypoxic and anoxic conditions (Kemp et al. 2009).

Bacteria were thought to be the sole group of organisms involved in the ammonia oxidation process, the first and the rate-limiting step in microbial nitrification, until the discovery of ammonia oxidizing archaea (AOA) in 2005 (Könneke et al. 2005). The discovery of mesophilic archaea within the phylum of Crenarchaeota with the potential for ammonia oxidation established the important role of archaea in the  $\text{N}_2$  cycle. Although they belong to different domains, both ammonia oxidizing bacteria (AOB) and AOA possess homologous ammonia monooxygenase enzyme, which oxidizes ammonia to hydroxylamine with the addition of oxygen to ammonia. Recent studies using *amoA* gene sequence showed that AOA is phylogenetically more diverse than AOB and based on these sequences analysis, AOA is now re-classified into a newly defined phylum, the Thaumarchaeota lineage (Brochier-Armanet et al. 2008). Currently, AOA has been reported from various environmental conditions extending from nutrient-rich river-estuarine system to highly oligotrophic polar-marine system (De Corte et al. 2009; Wankel et al. 2011).

Higher abundance of AOA over AOB in estuarine and coastal environments were observed in previous quantitative studies (Mincer et al. 2007; Wuchter et al. 2006) and a few studies have also reported increased nitrification rates in association with higher abundance of AOA *amoA* gene (Caffrey et al. 2007). While the recent studies have made it clear that although AOA is higher in number, the significant player in nitrification is AOB when the rate of reaction is considered, at least in the case of eutrophic estuarine system (Li et al. 2015; Vipindas et al. 2015). It has recently been suggested that AOA and AOB niches are delineated by the amount of ammonia available, with AOA

predominating in low-ammonium systems such as open Ocean and low-nutrient estuaries whereas, AOB dominating the eutrophic natural environments (Hou et al. 2013).

Although AOA diversity has been studied in various estuarine environments, most of the studies were concentrated on temperate and subtropical regions. The tropical estuaries, especially those along the southeast Arabian Sea have not been studied for the diversity of AOA. Hence, the current study has been focused in the Cochin Estuary (CE), a monsoonal tropical estuary that opens into the southeast Arabian Sea. The CE is the largest wetland ecosystem along the southwest coast of India, covering an area of ~25600 ha. The nutrient composition of the estuary is greatly influenced by the anthropogenic and terrestrial inputs from seven rivers and the seawater influx from two bar mouths (Menon et al. 2000). A relatively higher nitrification rate has been reported from the CE and the process is being regulated by the influence of monsoon mediated river and land runoff (Miranda et al. 2008; Vipindas et al. 2018). In a previous study though we have reported the significance of AOA, which contributed upto 35 % of ammonia oxidation in the CE, neither their diversity nor ecology were looked into in detail (Vipindas et al. 2015). It is for this reason that in the present study, the diversity and the seasonal pattern of AOA distribution were investigated from the water column of CE along a salinity gradient. The influence of environmental factors on the distribution of AOA was also investigated. To the best of our knowledge, this study forms the first report on the diversity of AOA from a tropical estuary that opens into the Arabian Sea.

## **Materials and Methods**

### **Sample collection**

Subsurface (ca 1m below the surface) and near-bottom water samples were collected from four stations in the CE (Fig. 1), using Niskin sampler (5 L capacity). Sampling was carried out during 2011, covering three seasons, viz. pre-monsoon (March), monsoon (July) and post-monsoon (November). Station 1 (Stn.1) was low saline (salinity range 0-10 PSU) with high freshwater influx while stations 2 and 3 were intermediate saline (salinity range 5 -15 PSU) and station 4 (Stn.4) was located 20 km away from station 3 with the least influence of freshwater hence considered as a high saline (salinity range 25- 35 PSU) station.

### **Analysis of environmental parameters**

Environmental variables were measured in triplicate following standard protocols. Salinity was determined using a Digi Auto Salinometer (Model TSK, accuracy  $\pm 0.001$ ) and pH was measured using an ELICO LI 610 pH meter. Dissolved oxygen (DO) concentration was determined following Winkler's titration method (Grasshoff et al. 1983). Samples for nutrients (ammonium, nitrite, nitrate,

phosphate, and silicate) were filtered through Whatman No 1 filter paper and estimated spectrophotometrically within six hours of sampling using the protocol of Grasshoff et al. (1983). The sum of ammonium, nitrite and nitrate is being represented as Dissolved Inorganic Nitrogen (DIN). Suspended Particulate Matter (SPM) was determined gravimetrically on Millipore membrane filters (nominal pore size, 0.45  $\mu\text{m}$ ) after drying at 70 °C for 6-8 hr to reduce water content before weighing.

## **Microbial abundance**

### **Total microbial abundance**

Total microbial abundance was determined by DAPI (4,6-Diamidino-2-Phenylindole) staining described by Porter and Feig (1980). Samples for total microbial counts were fixed with buffered formalin (final concentration 4 % v/v) and stored at 4°C. These samples were stained with DAPI (Fluka) (final concentration 0.01% w/v) for five minutes before filtering it through 0.2- $\mu\text{m}$  polycarbonate nucleopore filter (Millipore, USA). Samples were enumerated under the 100X magnification of an Olympus epifluorescence microscope, and at least 20 fields of >30 cells per field were counted. Total prokaryotic abundance was expressed as cells L<sup>-1</sup>.

### **Abundance of bacteria and archaea**

The abundance of total bacteria and archaea in the water column were estimated using FISH following the protocol of Glöckner et al. (1999) with oligonucleotide probes labeled with fluorescent dye (Cy<sub>3</sub>) that target the 16S rRNA gene of bacteria and archaea (total bacteria and total archaea). Briefly, the paraformaldehyde (final concentration 4 % v/v) preserved water samples were passed through 0.2  $\mu\text{m}$  white polycarbonate membrane filter (Millipore GTTP2500) for bacteria and through 0.1  $\mu\text{m}$  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 (EUB 338- GCTGCCTCCCGTAGGAGT for bacteria and ARCH 915-GTGCTCCCCCGCCAATTCCT for archaea) and formamide (60 % for bacteria and 40 % for archaea). The cells were counterstained with DAPI. 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 Cy<sub>3</sub>. The cell counts from 20 fields each from duplicate filter sets of each sample were counted and the average cell counts are expressed in cells L<sup>-1</sup>.

## Diversity and seasonal distribution of AOA

### DNA extraction

Genomic DNA was extracted from water samples following the protocol of Boström et al. (2004) with minimum modification. Briefly, 1 to 2 L of water sample was passed through 0.2  $\mu$  polycarbonate membrane filter (Millipore; GTTP2500), followed by incubation at 37 °C for 1 hr in lysis buffer containing 400 mM NaCl, 750 mM Sucrose, 20 mM EDTA and 50 mM Tris HCl, in addition to 1 mg ml<sup>-1</sup> lysozyme. Afterward, 1 % SDS and 100  $\mu$ g ml<sup>-1</sup> proteinase K were added to the solution and incubated again for 5 hr at 55 °C. Further 0.6 volume of isopropanol was added and DNA was precipitated. DNA pellet was washed copiously with 70 % ethanol, dissolved in TE buffer and stored at -20 °C until used. DNA concentration was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific).

### Clone library preparation

DNA extracted from each station during the three seasons including surface and bottom samples were pooled and PCR reactions were performed with two sets of primers: (1) targeting the ammonia monooxygenase gene (*amoA*) of AOA and (2) targeting the 16s rRNA gene of total archaea. The primer sets and thermal programs for amplification of gene fragments are shown in Table 1. Each PCR reaction was performed separately in 50  $\mu$ l which contained, 50 ng of DNA sample as the template, 2  $\mu$ l each of primers (10 picomoles  $\mu$ l<sup>-1</sup>), 5  $\mu$ l 10X Taq polymerase buffer (Sigma Aldrich), 1.5 U Taq DNA polymerase (Sigma Aldrich) and 200  $\mu$ M each dNTPs (Sigma Aldrich). The PCR products were run through an agarose gel (1.5 %) electrophoresis to confirm the amplification of the targeted gene, purified using PCR Clean-Up System (Promega, Madison, USA) and ligated into the plasmid of pGEM®-T Easy Vectors system (Promega, USA). The recombinant plasmids were transformed into chemically competent *Escherichia coli* DH5 $\alpha$  (TaKaRa, India) by heat shock method to construct a clone library. The recombinant colonies with inserts of *amoA*/ 16S rRNA genes were picked and grown overnight in LB broth at 37 °C. A total of 350 clones that incorporated *amoA* gene fragments were selected from the 4 stations for plasmid extraction. Recombinant plasmids were purified using Nucleo-pore Genetix Brand SureSpin plasmid mini prep kit (Genetix Biotech, India). RFLP pattern of each clone was then produced (only for *amoA* clones). For RFLP, the DNA subsamples were digested with restriction enzymes, Alu I and Hae III for 1 hr and run through 1% agarose gel. Based on the unique RFLP patterns, representative clones from each sample (total 90 clones) were selected for sequencing PCR reactions. In combination with vector-specific primers, T7 and SP6, on an ABI sequencer. Following the same procedure, single 16S rRNA

gene clone library analysis was also performed by pooling all the PCR products to further confirm the diversity pattern of AOA in the CE. A total of 400 clones were randomly selected for sequencing of 16sRNA gene.

### **Phylogenetic analysis**

The partial *amoA* gene and 16S rRNA gene sequences were checked for the presence of any vector sequences contamination using the Vec Screen program of NCBI and BIOEDIT v. 7.0.0 was used for manual editing of the sequences ([http:// www.mbio.ncsu.edu/ Bioedit/ bioedit.html](http://www.mbio.ncsu.edu/Bioedit/bioedit.html)). The chimeric sequences detected using DECIPHER ([http:// decipher.cee.wisc.edu/FindChimeras.html](http://decipher.cee.wisc.edu/FindChimeras.html)) were also removed from the dataset. Further, the remaining sequences were clustered and classified into OTUs. Unique *amoA* sequences after removing the singletons were selected as OTUs for this functional gene whereas 16S rRNA sequences were clustered into OTUs with a 3% distance cut-off. Sequences from the representative OTUs were compared with the GenBank database using the algorithm BLAST-N, to identify the most similar *amoA* and 16S rRNA gene sequences. From the total archaeal 16S rRNA gene sequences, only those sequences that showed similarity with ammonia oxidizing archaea were selected for further analysis. The representative sequences then aligned with sequences obtained from the same database using the software package CLUSTAL X (Thompson et al. 1997). These sequences were multiply aligned using clustalW and the phylogenetic trees were constructed with the neighbor-joining and maximum parsimony methods using MEGA 5.0 (Tamura et al. 2013). Bootstrap re-sampling analysis for 1,000 replicates was performed to estimate the confidence of the tree topologies (Tamura et al. 2007).

**Nucleotide Sequence and Accession Numbers:-** The sequences obtained in this study were submitted in GenBank under the accession numbers MF693461-MF693527, KX 648315-KX648356, KX581347-KX581356.

### **PCR and denaturing gradient gel electrophoresis (DGGE)**

Spatio-temporal variations in the community structure of AOA were studied by analyzing the band pattern of *amoA* gene of AOA in a PCR-DGGE (Wuchter et al. 2006). The details of primer sequence and thermal program for amplification are given in Table 1. In the PCR, 50 ng of DNA sample was used as template for 50 µl reaction mixture containing 2 µl each of primers (10 picomoles µl<sup>-1</sup>), 5 µl 10X Taq polymerase buffer (NEB, Canada), 1 U Taq DNA polymerase (NEB, Canada) and 200 µM each dNTPs (NEB, Canada). Triplicate PCR reactions were performed for each sample and PCR products were pooled during DGGE.

The DGGE of amplified PCR products was 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 before using Nano-drop (Thermo-Fisher, USA) and an equal concentration of DNA per sample (1250 ng) was loaded into the DGGE gel. The PCR products were run on a 10 % polyacrylamide gel with 30 – 55 % of denaturing gradient of urea and formamide for 18 hr at a constant voltage of 75 V in 1×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).

### **Analysis of DGGE band profiles**

DGGE gel images were analyzed with Bionumerics software version 4.6 (Applied Maths USA). The software carries out a density profile analysis, detects the bands from each lane and calculates the relative contribution of each band to the total lane intensity. The number of DGGE bands in each sample were counted as the major phylotypes of that station (operational taxonomic units -OTUs). Gels were cross-checked visually as well as for the number of bands per lane. Cluster analysis of DGGE bands based on square root transformed community data matrix through Bray-Curtis similarity was performed with PRIMER v.6 software package (Plymouth Marine Laboratory). Unique bands were excised, re-amplified and cloned for confirming the amplification of *amoA* gene.

### **Statistical analyses**

One-way ANOVA was performed for finding out the significance of spatio-temporal 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. Shannon-Weiner diversity index of *amoA* gene was calculated using PRIMER software (version 6.1.5) (Clarke and Warwick 1994).

## **Results**

### **Environmental characteristics of the CE**

The water column temperature of the CE ranged from 24 to 32° C during the study period with higher temperature during pre-monsoon (Table 2). Salinity showed a marked gradation in the CE, ranging from 0 to 34.5 PSU. Average salinity in the low saline station (Stn.1) was  $1.9 \pm 2.1$  PSU, while it was  $15.6 \pm 11.3$  PSU in the intermediate saline stations (Stns.2 & 3) and  $23.3 \pm 9.3$  PSU in the high saline station (Stn.4). Salinity showed a significant seasonality and during the summer monsoon it was near to zero at all stations except Stn.4. The DO concentration ranged from 2.5 to 7.3 mg L<sup>-1</sup> and was maximum during the monsoon and minimum during the post-monsoon. The pH ranged from

7.1 to 8.5, with higher values at Stn. 4 and lower values at Stn. 2. The SPM varied from 3.2 to 138 mg L<sup>-1</sup>, with the maximum during the monsoon and minimum during the pre-monsoon. Seasonal and spatial variations in DIN were also evident during the study period. Ammonium was the major component of DIN and its concentration ranged from 0.2 to 30.8 μM. The comparatively higher concentration of ammonium was observed at the intermediate saline stations. Ammonium was maximum during the post-monsoon and minimum during the monsoon period. Nitrate ranged from 1.1 and 28.4 μM, while nitrite varied from 0.1 to 0.8 μM. The highest nitrate concentration was observed at the low saline station during monsoon and the lowest at the high saline station during pre-monsoon. Phosphate concentration ranged from 0.1 to 2.8 with a maximum during the monsoon. Silicate concentration ranged from 0.01 to 48.1 μM with higher and lower concentration during the monsoon and pre-monsoon periods, respectively. Most of the environmental variables exhibited statistically significant spatio-temporal variations, however, the surface bottom variations were not statistically significant (Supplementary Table 1).

### **Abundance of bacteria and archaea**

Total microbial abundance observed in the CE ranged from 0.77 to  $1.57 \times 10^9$  cells L<sup>-1</sup> (Table 3). At the low saline station, the total microbial abundance ranged from 1.12 to  $1.41 \times 10^9$  cells L<sup>-1</sup>. At intermediate saline stations it ranged from 1.01 to  $1.57 \times 10^9$  cells L<sup>-1</sup> and at the high saline station the counts ranged from 0.77 to  $0.93 \times 10^9$  cells L<sup>-1</sup>. However, during the pre-monsoon season, the abundance was higher at all stations compared to other two seasons. Bacterial abundance in the CE ranged from  $3.66$  to  $6.70 \times 10^8$  cells L<sup>-1</sup>. At the low saline station, it ranged from  $5.58$  to  $6.05 \times 10^8$  cells L<sup>-1</sup>, at the intermediate saline stations it ranged from  $5.79$  to  $6.70 \times 10^8$  cells L<sup>-1</sup> and at the high saline station it ranged from  $3.66$  to  $4.29 \times 10^8$  cells L<sup>-1</sup>. However, higher abundance of bacteria was observed at all the stations during pre-monsoon. Archaeal abundance in the CE ranged from  $1.93$  to  $4.46 \times 10^8$  cells L<sup>-1</sup> and was always lesser than the bacterial abundance. The abundance of archaea ranged from  $3.27$  to  $3.83 \times 10^8$  cells L<sup>-1</sup> at the low saline station,  $2.79$  to  $4.46 \times 10^8$  cells L<sup>-1</sup> at the intermediate saline stations and  $1.93$  to  $2.53 \times 10^8$  cells L<sup>-1</sup> at the high saline station. Like bacteria, higher abundance of archaea was also observed during pre-monsoon. Though the bacterial and archaeal abundance was high during pre-monsoon, the spatio-temporal variations were not statistically significant. Both bacteria and archaea showed significant positive correlation with DIN ( $r = 0.53$ ,  $p < 0.01$ ,  $n = 24$ ;  $r = 0.51$ ,  $p < 0.01$ ,  $n = 24$ , respectively) especially with ammonium ( $r = 0.41$ ,  $p < 0.05$ ,  $n = 24$ ;  $r = 0.46$ ,  $p < 0.05$ ,  $n = 24$ , respectively) (Supplementary Table 2). Other environmental variables did not show any significant influence on the bacterial and archaeal distribution in the CE.



## Diversity and seasonal distribution of AOA

The sequence analysis of clone libraries showed the presence of a diverse group of AOA in the CE, with 21 OTUs from *amoA* gene sequences (Fig. 2). Relatively high number of OTUs of *amoA* gene were found in the clone library of intermediate stations (13 to 16 nos), followed by the low saline station (13 nos) and the high saline station (12 nos). Figure 3 shows the phylogenetic affiliation of the *amoA* sequences obtained from the clone library analysis. About 72 % of the archaeal *amoA* sequences obtained in this study were phylogenetically grouped into the uncultured group1 marine Thaumarchaeota lineage. A 25 % of the remaining sequences were split into two lineages: *Nitrosopelagicus* and *Nitrosopumilus* lineages and the sequences were related to the sequences reported from the coastal Arabian Sea, the East China Sea, the Gulf of Mexico, the Changjiang Estuary of China and the Yellow River Estuary of China. Up to 15% of the total sequences were affiliated to OTU 5 and it formed the most abundant OTU in the CE. This OTU was closely related to the sequence obtained from the coastal waters of the Arabian Sea. Up to 12% of the sequences were affiliated to OTU 1 and OTU 9 and these OTUs were closely related to the sequences obtained from the East China Sea and the Changjiang Estuary of China respectively.

More clarity on the diversity pattern of AOA was obtained via sequencing the clone library of 16S rRNA gene. The AOA sequences were selectively picked from the 400 sequences of 16S rRNA gene obtained from the study area and these sequences were used for the phylogenetic analysis. About 33% of 16S rRNA genes sequence showed similarity with AOA and they were grouped into 37 OTUs (Fig. 4). A similar pattern of diversity was observed in the phylogenetic analysis of both *amoA* and 16S rRNA gene sequences in the CE. Up to 80 % of total AOA sequences were broadly grouped into uncultured marine group I Thaumarchaeota lineage. From this group 25 % of sequences were further sub-clustered in to *Nitrosopumilus* lineage. Majority of the remaining sequences (18%) were aligned with *Nitrosopelagicus* lineage. Similar to *amoA* gene sequence, most of the 16S rRNA gene sequences of AOA were also aligned with the sequences reported from the coastal Arabian Sea, the China Sea, and estuaries from China. Upto 17 % of the total AOA 16S rRNA gene sequence were affiliated to OTU 34 and 13 % of sequences were affiliated to OTU 29 and these two OTUs come under the *Nitrosopumilus* lineage. Remaining OTUs were less than 10% of the total sequences. Similar to *amoA* gene sequence, most of the 16S rRNA gene sequences were also aligned with the sequences reported from the coastal Arabian Sea, the China Sea, and estuaries from China.

Spatial and temporal changes in the community structure of AOA were obtained from the Bray-Curtis similarity cluster analysis of the DGGE band pattern of *amoA* gene (Fig. 5). The number

of unique bands at each station varied between 15 and 24 (corresponding to the major phylotypes of each station). The DGGE banding pattern of AOA at low saline station (Stn.1) showed 7 to 16 bands from the individual sample and a total of 24 unique bands altogether (corresponding to 24 phylotypes). A maximum number of bands were observed in the DGGE gel during pre-monsoon and minimum during monsoon. The banding pattern of *amoA* gene at this station showed significant seasonal variations with two separate clusters with 59% Bray-Curtis similarity index. DGGE banding pattern at intermediate saline station, Stn. 2 showed 9 to 14 bands from individual sample and total of 21 unique bands altogether. The community structure of AOA showed seasonal fluctuations at this station also with two clusters, while the Bray-Curtis similarity index reached up to 77 %. The monsoon and non-monsoon samples formed separate clusters at Stn. 1 and Stn. 2, however, there was overlapping of a few samples. A higher number of bands were observed in the bottom water throughout the study period at both of these stations. The number of banding pattern at intermediate saline station, Stn. 3 showed 6 to 10 bands from individual sample and total of 15 bands from the station. The high saline station (Stn. 4) exhibited 9 to 12 bands from individual sample and total of 19 OTUs from the station altogether. Seasonal variations in the community structure were not significant at Stn. 3 and 4 and Bray-Curtis similarity index reached more than 80%. Karl Pearson's Correlation analysis showed that number of phylotypes of AOA in the DGGE gel have a significant negative correlation with DIN, whereas other environmental parameters did not show any significant relationship with the distribution of OTUs. Shannon-Weaver index H' (an indicator of 'diversity') fluctuated between 1.83 and 2.64 and were significantly higher at the freshwater stations than at all the other stations.

## **Discussion**

“Estuaries are one of those edges between two separate habitats that turn out to be hard to define specifically, though everyone believes they know what they are” (Blaber 2008). Environmental settings and biogeochemical processes of the estuaries in the tropical and temperate regions are distinctly different along with unique microbial composition (Eyre and Balls 1999). Estuaries are persistently exposed to anthropogenic activities that cause negative impacts on their biodiversity and habitat suitability.

## **Estuarine Characteristics**

The CE exhibited the characteristics of a typical micro-tidal tropical estuary, with salinity ranging from limnetic in the river mouth to 34 PSU in the bar mouth and this is similar to the situation encountered in any other Indian estuaries (Qasim 2003). Although the signatures of tidal intrusion

have been recorded even up to 60 km inside from the bar mouth during the pre-monsoon season (Balachandran et al. 2008), a significant portion of the CE remains limnetic during the monsoon season due to heavy freshwater discharge from seven rivers. The effect of freshwater runoff input during the summer monsoon reached up to the coastal station where the salinity of the surface water dropped from 34.5 to 10.57 PSU. Thus in the CE, the freshwater runoff mostly regulates the salinity gradient than the seawater intrusion.

The water column temperature ranged from 24 to 32° C in the CE, showing the nature of a tropical system. The water column was relatively cool during the monsoon period and intense solar radiation during pre-monsoon and post-monsoon seasons increased the water column temperature. The generally high DO concentration (av.  $4.9 \pm 1.3$  mg L<sup>-1</sup>) observed in the water column indicates the healthy oxygenated condition of the CE. This is mainly due to mixing of the water column by the tides and freshwater input. Higher concentrations of dissolved inorganic nitrogen recorded in this study, were similar to the previous studies, and this has been attributed to the contribution of industrial effluents and domestic sewage (Madhu et al. 2007; Qasim 2003), discharged through river water or terrestrial runoff. The present study corroborates the earlier observation on nutrient overloading in the CE, leading to eutrophication (Martin et al. 2011).

### **Distribution of bacteria and archaea**

The total microbial count, that ranged from 0.77 to  $1.99 \times 10^9$  cell L<sup>-1</sup> was in agreement with earlier studies in the CE (Parvathi et al. 2014; Thottathil et al. 2008) and other Indian estuaries (Ram et al. 2007; Sarma et al. 2011). Bacteria dominated the estuarine microbial community and accounted for 37.5 to 58.2% (av. 46%) of the total microbial counts, while archaea accounted for 24.9 to 32.7% (av. 27%). The archaea in the CE, though comparatively less in number, formed a significant percentage of the total microbial population. The abundance of bacteria and archaea detected in the CE using FISH was comparable with other world estuaries and coastal system (DeLong et al. 1999; Eilers et al. 2000; Glöckner et al. 1999; Quan et al. 2010). In the present study it was observed that depth did not have a significant effect on the microbial abundances and this may be due to the mixing of water column and also shallowness of the study region. Inorganic and organic nutrient availability may profoundly influence not only the microbial metabolism but its community composition also (Rappé and Giovannoni 2003). Both bacteria and archaea were found to be affected by DIN especially ammonium concentration. Salinity and other environmental factors did not show any significant correlation with microbial abundance. Moreover, the distribution of bacteria and archaea did not show any significant seasonal variation throughout the study period. This response

indicates the nutrient-rich and well-oxygenated condition of the study area which supports higher microbial abundance throughout the year irrespective of seasons.

### **AOA diversity and distribution**

The ubiquitous distribution of putative archaeal *amoA* genes and their numerical dominance over the bacterial counterparts in most of the marine ecosystems suggest that AOA play an important role in the global nitrogen cycling. However, their evolutionary history and phylogenetic arrangements are still in its infancy as very few cultivable represents and full genome sequences are available currently and when coming to the tropical estuary, the studies on AOA is further limited. The major fraction of the putative archaeal *amoA* sequences obtained from this study fell within uncultured group 1 marine Thaumarchaeota lineage (Brochier-Armanet et al. 2008). The sequences retrieved from the CE showed similarity with sequences from other estuarine and coastal waters, especially from the subtropical region (Zhang et al. 2015; Zheng et al. 2014). A total of 20-30 % of the sequences of both *amoA* and 16S rRNA genes of AOA from the CE were grouped into the *Nitrosopumilus* and *Nitrosopelagicus* lineages. These two lineages are the most widely reported ones from the estuarine and coastal system (Bollmann et al. 2014; Francis et al. 2005; Liu et al. 2014). After translation, the resultant protein sequences showed sequence similarity among each other and had relatively high identities (97–100%) to the nearest matched GenBank sequences. Interestingly, *Nitrososphaera* lineage, which has been recently reported from various soil and freshwater regions of estuaries (Bollmann et al. 2014), was not observed in the present study. Archaeal *amoA* gene sequences also showed variations among the sampling sites and indicated that intermediate salinity region was the preferred environment for the higher diversity of AOA in the CE. This result was consistent with the previous study in the Plum Island Sound estuary and Yangtze Estuary, China (Bernhard et al. 2010; Zheng et al. 2014). The diversity observed using *amoA* gene sequence in the current study is comparatively lesser than that observed from other world estuaries, (Bernhard et al. 2010; Zheng et al. 2014). However, Singh et al. (2010) have obtained similar results in the sediment samples of the Mandovi and Zuari estuaries (India), and have reported 9 to 27 OTUs for *amoA* gene of AOA.

Estuarine water column is subjected to wide fluctuations in various physical (tides, salinity, temperature, and turbidity) (Cunha et al. 2000; Shish and Ducklow 1994; Wikner et al. 1999) and chemical properties (DO, Nutrients, pH) (Amon and Benner 1996; Covert and Moran 2001; Thingstad and Billen 1994). To understand the influence of these environmental variables on the distribution of AOA in the CE, the spatio-temporal variations in the community structure of AOA in the water column were determined using the PCR-DGGE method. The DGGE technique is considered as an ideal method for comparing a large number of samples with short processing time

and at low cost (Green et al. 2010). Dominant microbial groups that function in an ecosystem can be evidenced by DGGE fingerprinting using appropriate target genes, even though the method is qualitative or semi-quantitative. It has been successfully used to understand the responses of AOA to dynamic environmental variables in many studies (Sahan and Muyzer 2008; Wankel et al. 2011; Zhang et al. 2015). AOA showed 15 to 24 unique bands during this study, which may be representing the predominant phylotypes of AOA in the CE. A high number of bands observed in the bottom waters compared to the surface waters was owing to the re-suspension of ammonia oxidizers from the sediments. Similar banding pattern of AOA has been reported previously from various estuarine environment worldwide. DGGE analysis from agriculturally impacted Elkhorn Slough Estuary (USA), observed 10 to 24 bands for AOA (Wankel et al. 2011) and the Westerschelde estuary (Netherlands) showed 7 to 15 bands for AOA (Sahan and Muyzer 2008). Up to 21 bands of AOA-*amoA* gene were observed from Yong River estuary, China (Zhang et al. 2015).

The low saline and intermediate saline stations (Stns.1 and 2, respectively) showed relatively higher number of bands in the DGGE gel of AOA. A large input of allochthonous nutrients along with microbial load including nitrifiers enter into the estuary throughout the year from various sources such as domestic sewage and industrial waste and also nutrient-rich fresh water from rivers (Sheeba et al. 2017; Vipindas et al. 2018). This nutrient input supports the AOA community to maintain its relatively higher diversity throughout the year especially at the low and intermediate saline stations, where sufficient nutrient concentration is available along with optimum salinity environment.

Apparent seasonality among predominant ammonia oxidizers was also observed in the DGGE banding patterns of low and intermediate saline stations, while such seasonality was not evident in the Stn.3 and 4. At Stns.1 and 2 monsoon samples showed a relatively lower number of AOA bands and also exhibited separate clustering in the Bray-Curtis similarity clusters. These results indicate that monsoon runoff is the major factor regulating the seasonal distribution of AOA in the CE. During the peak monsoon, a large amount of fresh water through rain and river water influx enters the estuary and it flushes the estuary many times (Vinita et al. 2015). This flushing reduces the residential time of water column and thus minimizes the growth and activity of the estuarine microorganisms before reaching the marine ecosystem. Whereas during pre-monsoon the flushing rate of water column is very less and this allows maximum residential time for AOA to grow and proliferate with higher nitrification activity. Relatively high nitrification rate has been reported from the CE during pre-monsoon season (Miranda et al. 2008; Vipindas et al. 2018). Since the water column of the estuary is nutrient rich throughout the year, the seasonal variation in nutrient

concentration is not a significant factor in determining the distribution of AOA phylotypes. Our statistical analysis also showed similar results where most of the nutrient variables did not show any significant correlation with AOA distribution, whereas dissolved total N concentration showed negative correlation. This negative correlation indicates that, further addition of N after attaining the optimum level, may negatively influence the AOA distribution. At the same time, the seasonality of AOA was not evident in the Stn. 3 and Stn. 4 where the salinity was higher than other stations and the influence of sea water was comparatively higher. Thus it maintains same major phylotypes, which withstand the high salinity throughout the year. The temporal variation of AOA in the low and intermediate saline stations may also be due to their less adaptability towards the variations in salinity and nutrient levels in the estuary (Liu et al. 2013; Wang et al. 2014). The seasonal distribution behavior of AOA in the CE is just opposite to the behavior of AOB observed in the CE. Our previous study reported that AOB community in the CE is relatively stable throughout the year, while abundance and activity of organisms showed significant seasonal variations (Vipindas et al. 2018).

## **Conclusion**

The present study reports the diversity and seasonal distribution pattern of AOA in the water column of a tropical estuary along the southeast Arabian Sea for the first time. Though the percentage contribution of AOA to the nitrification process in the Cochin estuary is less than that of AOB, a broad diversity of AOA was observed in the system. Clone library analysis of both *amoA* gene and 16S rRNA gene sequences showed that the major AOA sequences are well distributed in the CE as documented from subtropical and temperate regions. Summer monsoon plays a critical role in regulating the seasonality of AOA distribution along the salinity gradient in a dynamic tropical estuary like the Cochin estuary.

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## Conflicts of Interest

The authors declare no conflict of interest.

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**Table 1** Primers and PCR conditions used for the PCR amplification.

	Target gene	Primer (5'-3')		Amplicon length	Thermal profile	References
DGGE	AOA <i>amoA</i>	amoAf	CTGAYTGGGCTGGACATC	256 bp	95 °C for 5 min, followed by 30 cycles of 30s at 95 °C, 30s at 58 °C, 45s at 72 °C final extension for 10 min at 72 °C.	Wuchter et al. 2006
		amoAr	TTCTTCTTTGTTGCCAGTA			
Clone Library	AOA <i>amoA</i>	Arch- <i>amoAF</i>	STAATGGTCTGGCTTAGACG	635 bp	95 °C for 5 min, followed by 30 cycles of 60s at 95 °C, 45s at 53 °C, 60s at 72 °C final extension for 10 min at 72 °C.	Francis et al. 2005
		Arch- <i>amoAR</i>	GCGGCCATCCATCTGTATGT			
	Archaeal 16sRNA	Arch21F	TTCCGGTTGATCCYGCCGGA	850 bp	95 °C for 5 min, followed by 30 cycles of 60s at 95 °C, 60s at 55 °C, 60s at 72 °C final extension for 10 min at 72 °C.	Bano et al. 2004 Stahl 1991
		Arch-915R	GTGCTCCCCGCAATTCCT			

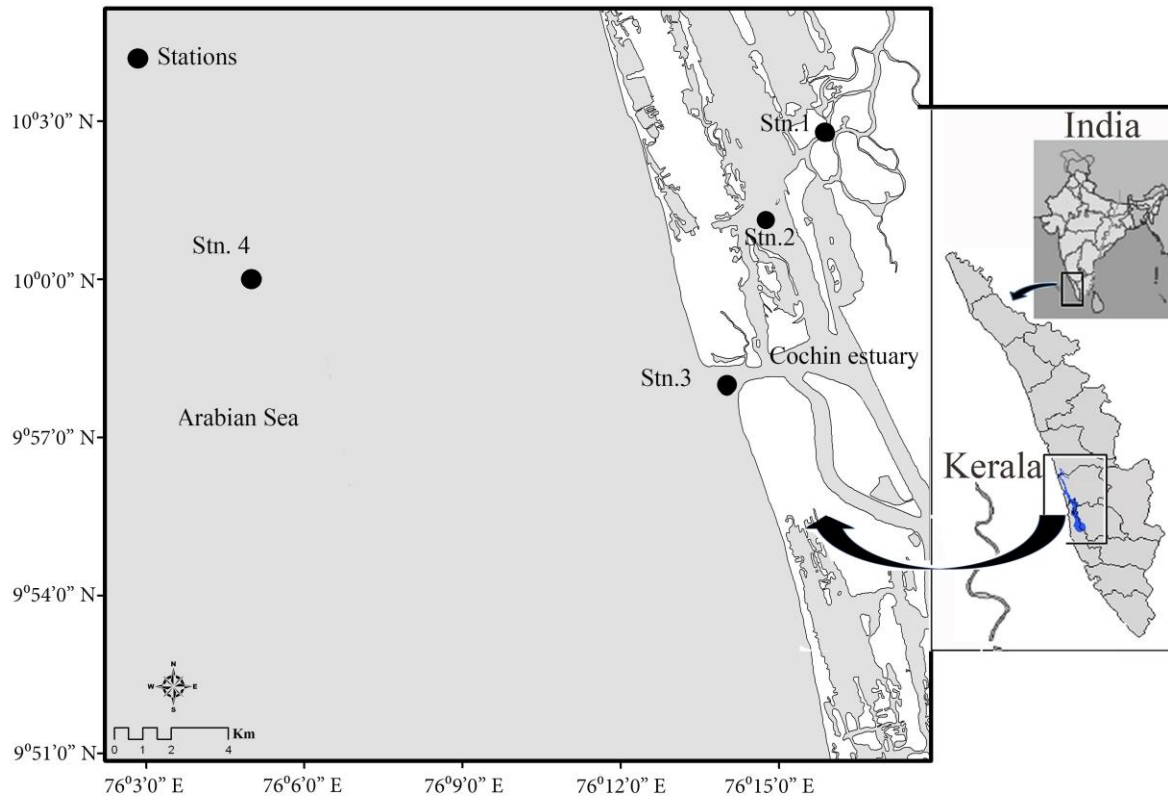
**Table 2** Spatio-temporal variations of environmental variables during three study season in the Cochin estuary (**PreM - pre- monsoon**, **Mn- monsoon, PstM- Post-monsoon.**

Parameters		Station 1 (Avg. depth 3m)			Station 2 (Avg. depth 5m)			Station 3 (Avg. depth 10m)			Station 4 (Avg. depth 25 m)		
		PreM	Mn	PstM	PreM	M	PstM	PreM	Mn	PstM	PreM	Mn	PstM
Surface Sample	Salinity (PSU)	0±5.4	0±2.3	3.4±1.6	17.3±0.6	1.3±5.1	12.1±4.5	20.3±3.6	1.4±11.7	25.8±4.7	24.7±7.2	10.6±2.1	34.5±4.1
	Temperature (°C)	31±0.8	29±0.5	28±0.3	32±0.8	30±0.3	30±0.1	30±0.5	29.5±0.5	29±0.5	32±0.3	29±0.2	30±1
	DO (mg L <sup>-1</sup> )	5.1±1.2	5.8±0.5	2.8±1.1	7.3±0.7	5.5±0.5	3.8±1.8	5.6±0.6	5.1±0.1	4.2±0.2	6.1±0.7	4.8±0.5	4.3±1.6
	Ammonium (µM)	5.3±12.9	4.7±8.9	11.6±0.5	2.6±2.3	5.5±14.8	12.7±3.4	28.4±9.5	10±19.6	18.1±3.5	0.2±9.12	7.1±10.6	12.2±21.7
	Nitrite (µM)	0±0.1	0.7±0.01	0.3±0.1	0.1±0.2	0.3±0.1	0.1±0.1	0.1±0.1	0.3±0.1	0.6±0.03	0.2±0.1	0.5±0.2	0±0.1
	Nitrate (µM)	7.7±0.6	28.5±3.6	13.3±4.1	2.7±0.8	5±10.7	9.8±2.4	7.4±1	6.3±2.6	4.2±0.6	3.3±1	2.6±1.1	0.3±2.1
	Phosphate (µM)	0.5±0.02	1.5±0.4	0.6±0.4	0.1±0.9	1±0.5	0.5±0.6	1.1±0.3	2.8±1.2	1.3±0.1	0.1±0.1	1.6±0.7	2.1±0.9
	N:P	28.5	22.3	43.7	45.2	10.4	47.9	32.4	5.9	17.6	36.4	6.5	5.9
SPM (mg L <sup>-1</sup> )	3.2±1.9	4.8±10.8	25.2±11	24±7	14.8±13	30±12.6	25.6±33.4	16.8±11.6	60.8±1.2	31.2±7.8	28±56	54±0.2	
Bottom Samples	Salinity (PSU)	4.9±4.0	0±2.4	3.6±1.7	22.7±3.2	2.3±1.2	8.1±1.8	33.9±1.7	13.2±3.5	33.2±6.1	27.5±2.7	18.5±1.9	34.3±3.6
	Temperature (°C)	31±0.8	27±1.5	27.8±0.2	30±0.3	29±0.5	29.5±0.3	30±0.8	25±1.5	29±1	30±0.5	24±1.02	29.5±0.8
	DO (mg L <sup>-1</sup> )	4.8±1.2	5.7±0.5	2.5±1.2	6.3±0.2	4.9±0.7	3.5±1.2	4.3±1.1	2.7±0.5	3.4±0.6	7.3±0.5	4.2±0.03	4.3±0.3
	Ammonium (µM)	5.7±21.1	3.7±14.8	11.6±1.5	1.3±2.6	6.4±16.3	18.6±5.7	30.8±13.3	7±11.9	15.4±4.4	1.9±4.7	4±8.7	6.4±2.1
	Nitrite (µM)	0.2±0.2	0.8±0.2	0.2±0.1	0.1±0.2	0.3±0.1	0.1±0.2	0.1±0.2	0.6±0.2	0.3±0.4	0.1±0.01	0.2±0.04	0.1±0.3
	Nitrate (µM)	7.2±1	23.6±1.9	15.2±3.5	1.3±1	3.6±7.5	6.6±0.7	4.2±0.9	4.2±1.3	1.2±0.1	3.4±1	1.9±0.8	1.3±0.9
	Phosphate (µM)	0.9±0.1	2.5±1.1	0.5±0.5	0.6±0.6	1.4±0.5	0.8±0.6	0.8±0.4	2.4±1.1	1.1±0.3	0.2±0.01	2.8±1.3	0.3±0.4
	N:P	15	11.1	54.1	5	7.6	33.2	45	4.8	15.8	27	2.2	24.3
SPM (mg L <sup>-1</sup> )	16.8±7.4	4.4±20.8	30±12	34.4±48.4	15.2±20.6	34.4±10.8	43.6±53.8	138.8±9	99.6±28	23.2±9.6	76.8±61.8	43.6±5.6	

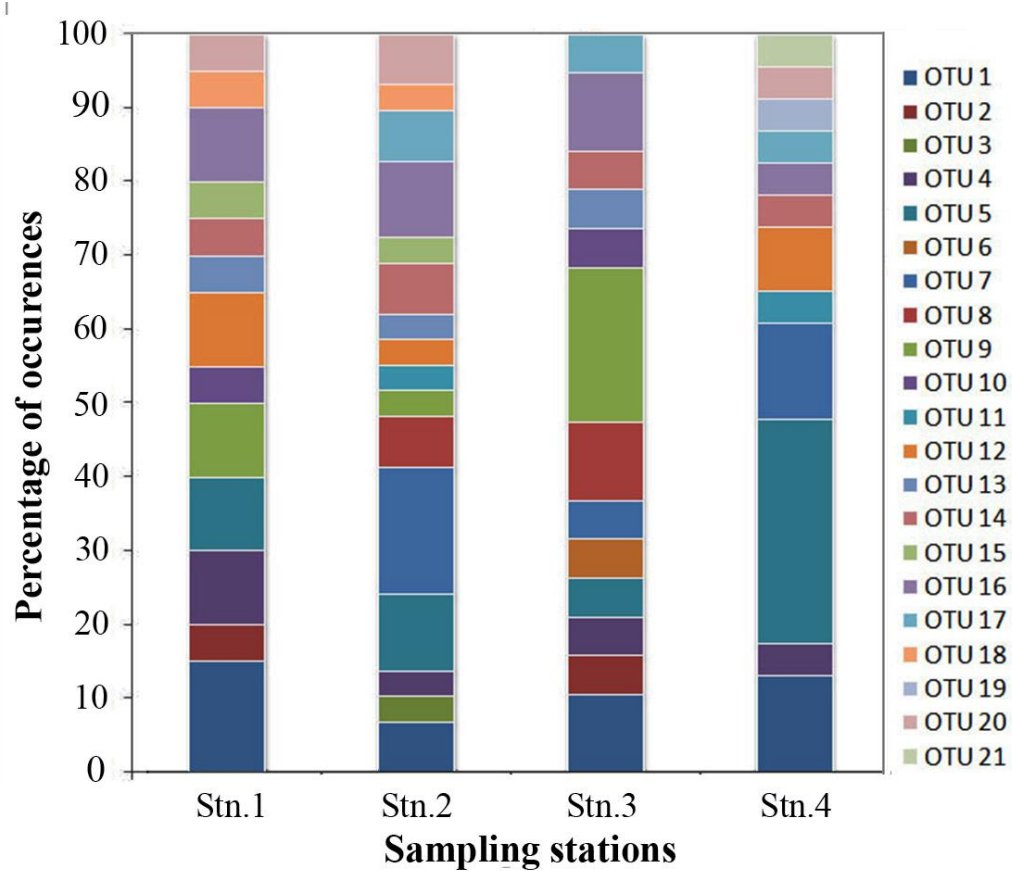
**Table 3** Seasonal distribution of total microbes and bacteria and archaea separately in the surface and bottom water of the Cochin estuary (**PreM - pre- monsoon, Mn - monsoon, PstM - post-monsoon**).

Cell counts (cells x 10 <sup>8</sup> L <sup>-1</sup> )		Station 1			Station 2			Station 3			Station 4		
		PreM	Mn	PstM	PreM	M	PstM	PreM	Mn	PstM	PreM	Mn	PstM
Surface	Total Microbes	13.7±1.6	11.23±0.1	11.8±0.7	15.12±2.1	10.14±0.9	13.6±0.1	13.16±1.9	11.14±0.2	12.12±0.5	7.84±3.1	8.95±2.8	8.01±0.5
	Bacteria	5.58±0.2	5.87±0.06	6.05±0.2	5.98±0.2	5.9±0.2	6.29±0.3	6.2±0.02	5.9±0.3	6.2±0.01	3.99±0.2	4.29±0.1	3.87±0.3
	Archaea	3.84±0.2	3.33±0.03	3.42±0.1	3.89±0.6	2.79±0.6	3.61±0.6	3.59±0.6	3.28±0.6	3.63±0.6	1.94±0.07	2.54±0.3	2.15±0.1
Bottom	Total Microbes	14.1±0.4	11.53±0.1	12.86±0.6	15.74±1.6	10.58±1.1	14.04±1.2	13.67±1.5	11.14±0.3	12.17±0.1	7.77±2.5	9.25±3.2	8.09±4.1
	Bacteria	5.94±0.4	5.64±0.09	5.72±0.06	5.9±0.1	5.71±0.4	6.7±0.4	5.79±0.01	6.04±0.8	6.61±0.7	3.66±0.04	4±0.08	3.77±0.1
	Archaea	3.84±0.3	3.28±0.03	3.48±0.1	3.93±0.3	3±0.1	3.5±0.4	4.46±0.2	3.15±0.05	3.5±0.01	2.24±0.01	2.45±0.03	2.15±0.2

## Figures

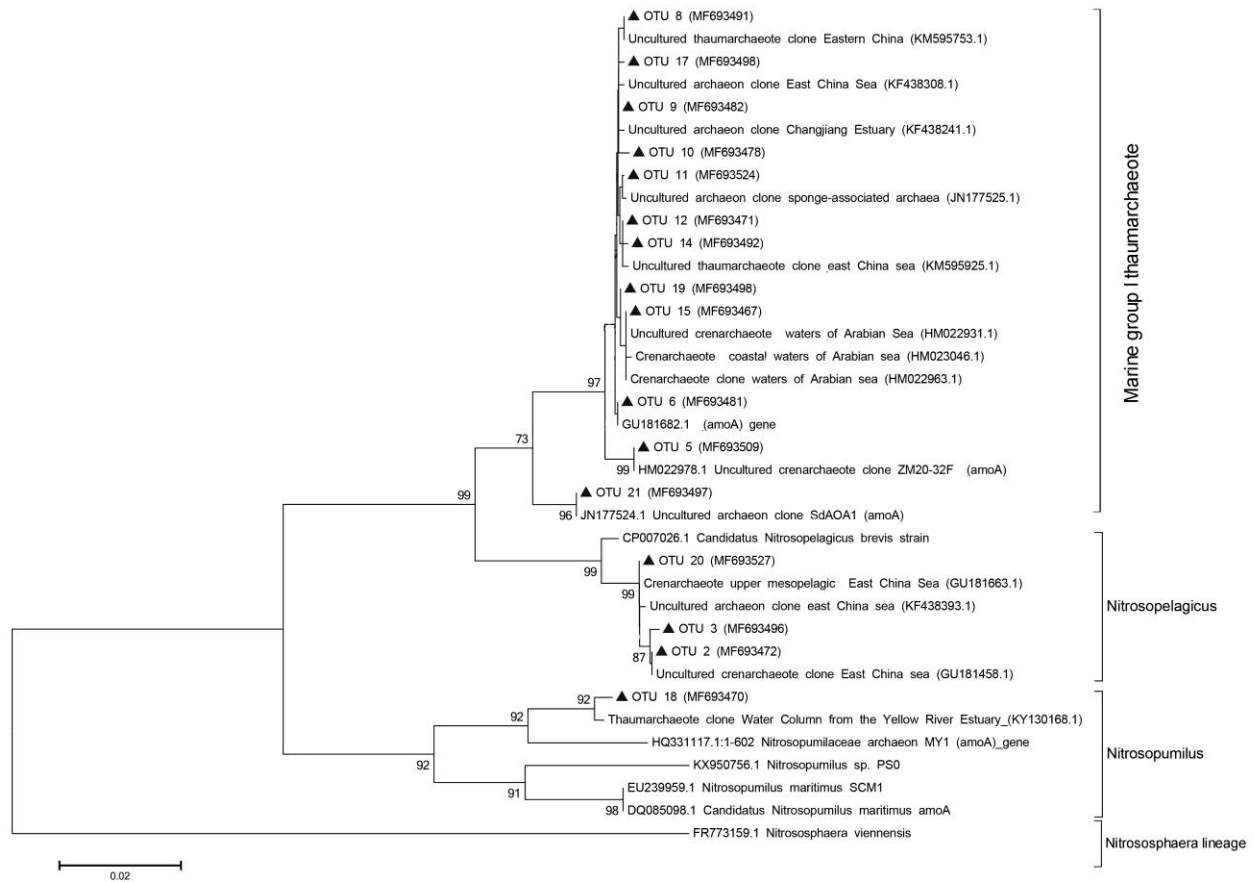


**Fig. 1** Map showing the sampling stations along salinity gradient in the Cochin estuary, (Station. 1- low saline, Station. 2 & 3 –intermediate saline and Station. 4- high saline).

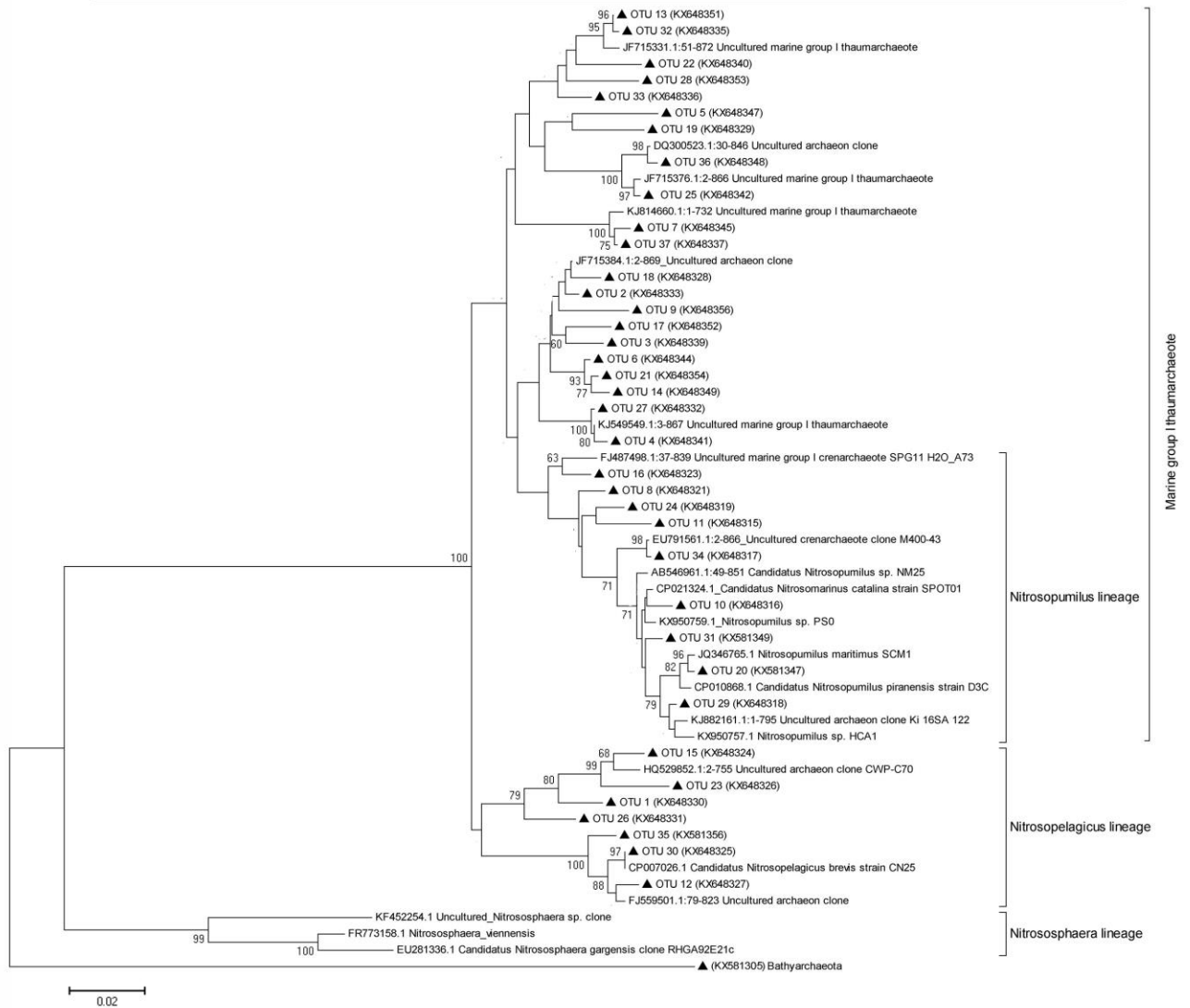


**Fig. 2** Distribution and relative abundance of AOA *amoA* gene OTUs in the CE. Unique *amoA* sequences after removing the singletons were selected as OTUs for this functional gene. 70 AOA *amoA* gene sequences selected from 350 clones (nearly 90 clones from each station).

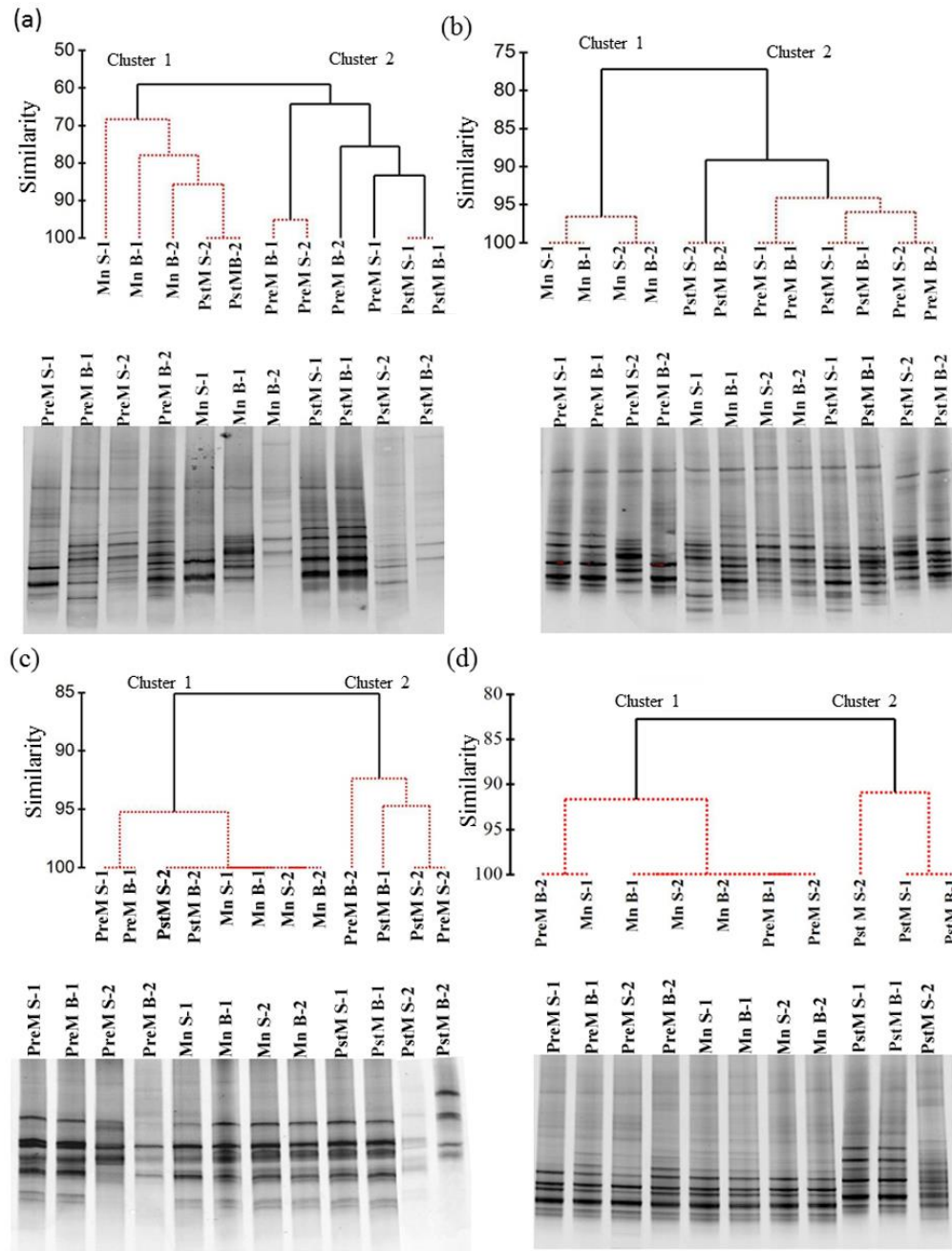




**Fig. 3** Phylogenetic tree based on the *amoA* gene sequences of ammonia-oxidizing archaea. Trees were constructed (using 590 base positions) with the neighbor-joining method using 1,000 bootstrap replicates. Unique *amoA* sequences after removing the singletons were selected as OTUs for this functional gene. The bootstrap values more than 70% are shown in the figure.



**Fig. 4** Phylogenetic tree based on the 16S rRNA gene sequences of ammonia-oxidizing archaea. Trees were constructed (using 710 base positions) with the neighbor-joining method using 1,000 bootstrap replicates. The OTUs were clustered based on 3 % distance cut-off from 400 total archaeal 16S rRNA gene sequences. The bootstrap values more than 70% are shown in the figure.



**Fig. 5** DGGE banding pattern showing the spatio- temporal variation in the community structure of ammonia oxidizing archaea in the study area. Bray-Curtis similarity based on hierarchical clustering of seasonal samples depicted through dendrogram (a) station 1; (b) station 2; (c) station 3; (d) station 4. For DGGE analysis two samples were collected from each season and mentioned as 1 and 2 to differentiate it. S and B is used to mark surface and bottom. PreM - pre-monsoon , Mn- monsoon, PstM - post-monsoon, S1- surface sample 1, S2 surface sample 2, B1- bottom sample1, B2- bottom sample 2.