

## Bacterial domination over Archaea in ammonia oxidation in a monsoon driven tropical estuary

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### Abstract

Autotrophic ammonia oxidizing microorganisms, which are responsible for the rate limiting step of nitrification in most aquatic systems, have not been studied in tropical estuaries. Cochin Estuary (CE) is one of the largest, productive and monsoon driven estuary in India opening into the southeast Arabian Sea. CE receives surplus quantities of ammonia through industrial and domestic discharges. The distribution of ammonia oxidizing -bacteria (AOB), - archaea (AOA) and anaerobic ammonia oxidizing bacteria (anammox) were studied using Fluorescence *in situ* hybridization (FISH) and their relative contribution to the process as well as the governing factors were examined and reported for the first time from CE. The order of occurrence of these assemblages was  $\beta$ -proteobacteria ( $0.79$  to  $2 \times 10^5$  cell ml<sup>-1</sup>) >  $\gamma$ -proteobacteria ( $0.9$  to  $4.6 \times 10^4$  cell ml<sup>-1</sup>) > anammox ( $0.49$  to  $1.9 \times 10^4$  cell ml<sup>-1</sup>) > AOA ( $0.56$  to  $6.3 \times 10^3$  cell ml<sup>-1</sup>). Phylogenetic analysis of DGGE bands showed major affiliation of AOB to  $\beta$ -proteobacteria, while AOA was affiliated to crenarchaeota. The abundance of AOB was mostly influenced by ammonia concentrations. The recovered ammonia oxidation rate of AOB was in the range of 45 - 65 % whereas for AOA it was 15-45 %, indicating that AOB were mostly responsible for the ammonia oxidation in CE during the study period. Overall, the present study provides an insight into the relevance and contribution of different groups of ammonia oxidizing bacteria in CE and emphasizes the need for further in depth studies across space and on season scale.

**Key words:** Ammonia oxidation, tropical, bacteria, archaea, anammox, Cochin estuary

## **Introduction**

Nitrogen (N) cycle involves the transformation of the element into various oxidation states that can affect key ecosystem processes such as primary production and decomposition. Ammonia oxidation is a critical process in linking biological N fixation, anaerobic N losses and decomposition of nitrogenous organic matter, thereby controlling the productivity of coastal and estuarine environments. Our understanding about the microorganisms involved in ammonia oxidation has evolved substantially in the last two decades with the identification of anaerobic ammonia oxidizing bacteria (anammox) [1] and ammonia oxidizing archaea (AOA) [2]. Most of the earlier studies from the temperate and subtropical estuaries were on the community structure and abundance of each rate limiting process, i.e., either AOB coupled or/with anammox or nitrite oxidizing bacteria (NOB) and the responses to environmental changes [3-6]. AOA were initially considered as an inhabitant of only the open ocean and extreme environments, but their presence in coastal oceans, estuaries and bays has been recently recognized [7-9]. Though a few reports on the relative abundance of AOB and AOA are available from marine and estuarine environments [10], information on their relative contribution to ammonia oxidation is very much limited, particularly from the tropical estuaries.

CE is the second largest wetland ecosystem opening into the south east Arabian Sea. The physio-chemical conditions of CE are influenced by anthropogenic and terrestrial inputs from six rivers, seawater influx from two bar mouths [Major opening at Fort Cochin (450 m wide) and minor opening at Munambam (250 m wide)] and the prolonged southwest monsoon. Ammonia in CE accounts for 50 – 65% of the dissolved inorganic nitrogen [11] and it experiences high nitrification rate of  $166 \text{ nmol N L}^{-1} \text{ h}^{-1}$  in moderate salinities [12]. Despite these reports, no information is available on the microbial community participating in ammonia oxidation in this estuary. In this study, we used a combination of Fluorescence *in situ* hybridization (FISH), DGGE and ammonia oxidation rate experiments to understand the distribution of different ammonia oxidizing microorganisms, the factors that are influencing their abundance and their relative contribution to ammonia oxidation.

## **Material and methods**

### **Study area and sampling**

CE is a monsoon driven tropical estuary, situated ( $9^{\circ} 30' - 10^{\circ} 12' \text{N}$  and  $76^{\circ} 10' - 76^{\circ} 29' \text{E}$ ) on the south west coast of the Indian peninsula. It covers an area of  $\sim 25600$  hectare. It receives fresh water from six rivers and opens in to the Arabian Sea through a major opening at Fort Cochin (450 m wide) and a minor one at Munambam (250 m wide). Surface and near-bottom samples were collected

using a Niskin water sampler (10 L capacity) from 5 stations (Fig. 1) across the estuary viz. Nedungadu (station 1), Varappuzha (station 2), Fort Cochin (station 3), Arookkuty (station 4) and Thaneermukkam (station 5). To study the spatial variability in the distribution of nitrifiers, sampling was carried out during the southwest monsoon when the estuarine condition was limnetic to reduce the influence of discrete salinity gradient.

### **Analysis of environmental variables**

Environmental variables were measured following standard protocols. Dissolved oxygen (DO) content in the ambient water was determined following Winkler's titration method [13]. Samples for nutrients (Ammonia, nitrite, nitrate, phosphate and silicate) were filtered and estimated spectrophotometrically within six hours of sampling [13].

### **Microbial abundance**

Abundance of total eubacteria, total archaea, AOB, anammox, AOA and nitrite oxidizing bacteria (AOB) were quantified using FISH following the protocol of Glockner et al [14] with oligonucleotide probes labeled with Cy<sub>3</sub> (Table 1). Briefly, paraformaldehyde (final concentration 1% v/v) preserved water samples were passed through 0.2 µm (Millipore GTTP2500) and 0.1 µm (Millipore VCTP02500) white polycarbonate membrane filters for bacteria and archaea, respectively. The filters were hybridized for 90- 120 min in a hybridization solution (0.9 M NaCl, 20mM Tris HCl (pH 7.4) and 0.01 % SDS), containing 50 ng nucleotide probes and 40 – 60 % formamide (depending on the probe sequence, Table 1). 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 Cy<sub>3</sub>. The interrelationships between the different groups of nitrifiers and environmental variables were assessed using Statistica version 6 (Stat Soft Inc, USA). The bacterial data were tested for its normal distribution and normalized before statistical analyses.

### **Community structure of ammonia oxidizing bacteria and archaea**

#### **Extraction of DNA from water samples**

Genomic DNA from water samples were extracted following Boström et al [15] with slight modification. Briefly, 1 L of water sample was passed through 0.2 µm polycarbonate membrane filter (Millipore; GTTP2500), followed by incubation at 37 °C for 1h in lysis buffer (NaCl 400 mM, Sucrose 750 mM, EDTA 20 mM and Tris HCl 50 mM) containing 1 mg ml<sup>-1</sup> lysozyme. Subsequently, SDS (1 %) and proteinase K (100 µg ml<sup>-1</sup>) were added to the solution and continued incubation for 5 h at 55 °C. Further 0.6 volume of isopropanol was added and DNA was precipitated

by keeping at  $-20^{\circ}\text{C}$  for 60 min. DNA pellet was washed copiously with 70 % ethanol, dissolved in TE buffer and stored at  $-20^{\circ}\text{C}$  until used.

### **Denaturing gradient gel electrophoresis (DGGE)**

Community structure of AOB and AOA was studied using DGGE technique. Briefly, 5  $\mu\text{l}$  DNA sample was used as template for 50  $\mu\text{l}$  PCR reaction mixture containing 2  $\mu\text{l}$  each of primers (10 picomoles  $\mu\text{l}^{-1}$ ), 5  $\mu\text{l}$  10X Taq polymerase buffer (NEB, Canada), 1 U Taq DNA polymerase (NEB, Canada) and 200  $\mu\text{M}$  each dNTPs (NEB, Canada). 16S rRNA gene of ammonia oxidizing  $\beta$ -proteobacteria were amplified with an equimolar concentration of three forward primers (CTO189fA -GC and CTO189fB -GC and CTO189fC-GC), each with a GC clamp at 5' end and a reverse primer (CTO654r) containing a single ambiguous base [16]. The forward primers CTO189fA and CTO189fB (GGAGRAAAGCAGGGGATCG) and CTO189fC (GAGGAAAGTAGGGGATCG) were synthesized separately and collectively referred to as CTO189f-GC. The sequence CTO654r (CTAGCYTTGTAGTTTCAAACGC) was used as reverse primer. The cycling conditions were as follows: initial denaturation at  $95^{\circ}\text{C}$  for 5 min, followed by 30 cycles of denaturation at  $95^{\circ}\text{C}$  for 60 sec, annealing at  $55^{\circ}\text{C}$  for 60 sec, extension at  $72^{\circ}\text{C}$  for 60 sec and a final extension for 10 min at  $72^{\circ}\text{C}$ . For amplification of archaeal ammonia monooxygenase gene (*amoA*) of AOA, we used a combination of GC clamp attached forward primer *amoAf* (CTGAYTGGGTCYTGGACATC) and reverse primer *amoAr* (TTCTTCTTTGTTGCCAGTA). The GC clamp was attached to the 5' end of forward primer. The cycling conditions were as follows: initial denaturation at  $95^{\circ}\text{C}$  for 5 min, followed by 30 cycles of denaturation at  $95^{\circ}\text{C}$  for 30 sec, annealing at  $58^{\circ}\text{C}$  for 30 sec, extension at  $72^{\circ}\text{C}$  for 45 sec and a final extension for 10 min at  $72^{\circ}\text{C}$ .

The PCR products of AOB (480 bp) and AOA (256 bp) were run on an 8 % polyacrylamide denaturing gradient gel prepared with 35 – 50 % (for AOB) and 30 – 55 % (for AOA) formamide for 17.5 h at 75 V. The bands separated were stained with SYBR green and observed in a gel documentation system (BioRAD, USA). The bands were picked, incubated overnight in a 50  $\mu\text{l}$  TE and re-amplified with respective primers without GC clamps. The PCR products were purified using Nucleo-pore Genetix brand Sure Extract PCR clean up/ Gel extraction kit (Genetix Biotech, India) and cloned into TOPO vector (Invitrogen, USA), following supplier's manual and the plasmids were transformed into chemically competent *E.coli* DH5 $\alpha$  by heat shock method. The recombinant colonies were picked and grown at  $37^{\circ}\text{C}$  for overnight in LB broth, an aliquote of which was preserved in glycerol at  $-80^{\circ}\text{C}$  and the remaining was used for plasmid preparation. Recombinant plasmids were purified using Nucleo-pore Genetix brand SureSpin plasmid mini prep kit (Genetix Biotech, India) and used as a template for sequencing PCR reactions in combination with vector

specific primers, T7 and SP6 on an ABI sequencer. Sequence data obtained were analyzed and edited using Sequencher V4.10.1 (GeneCodes). Subsequently, the sequences were compared with those in the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm to determine approximate phylogenetic affiliations. The nucleic acid sequences showing the closest similarities were used as Reference sequences while constructing neighbor -joining tree using the software MEGA (version 5.0). DGGE approach was followed to get some insight into the overall distribution of AOB and AOA in CE. The sequences were submitted to NCBI Genbank (accession numbers KM386955 to KM386977 and KM404171 to KM404173).

### **Contribution of AOB and AOA in nitrification rate**

Contribution of AOB and AOA in ammonia oxidation rate were measured based on recovered ammonia oxidation [17]. All samples were pre-treated with acetylene to irreversibly inactivate ammonia monooxygenase, and upon the removal of acetylene, the recovery of ammonia oxidation rate was monitored in the presence and absence of bacterial or archaeal protein synthesis inhibitors [17]. The schematic flow chart is given in Scheme 1. Water samples were amended with sodium chlorate (20 ppm) to inhibit the conversion of nitrite to nitrate. Experiments were carried out (in triplicate) in dark at room temperature. Briefly, samples were purged with acetylene gas to inactivate residual ammonia monooxygenase. After six hours of inactivation, the residual acetylene gas was removed completely by degassing under vacuum for 10 min. In one set of experimental bottles, a cocktail of protein synthesis inhibiting class of antibiotics, kanamycin ( $750\mu\text{g ml}^{-1}$ ) and gentamycin ( $750\mu\text{g ml}^{-1}$ ), were added to prevent the contribution of AOB. Another set of bottles was incubated in the presence of cyclohexamide ( $650\mu\text{g ml}^{-1}$ ) to prevent AOA. A third set of bottles without any antibiotics was kept as control. Nitrite accumulation was monitored at six hour interval for 24 h following standard spectrophotometric technique. The suitability of this method was also tested by measuring ammonia oxidation rate of the water sample without acetylene treatment.

### **Results and Discussion**

Low salinity (0 to 2.29) was recorded at all stations in CE except in the bottom waters of station 3 (Table 2), where the stratification of water body during the high tide was observed with fresh water at the surface (0-5 m depth) and seawater at the bottom (5 m depth) (Fig. 2). High nutrient loading was observed during the study period. The concentrations of ammonia ( $2.3 - 9.9 \mu\text{M}$ ), nitrite ( $0.3 - 1.0 \mu\text{M}$ ) and nitrate ( $15.1 - 36.1 \mu\text{M}$ ) were high due to increased discharge of nutrient rich industrial effluents [18] and formation of perennially undulating water bodies or null zones due to its geomorphology (ox-bow shaped) and meandering flow [19-21]. High nutrient loading during

monsoon season has been earlier documented not only in CE [11] but also in other tropical estuaries such as Mandovi-Zuari [22], Godavari [23] and Hoogly [24] around the Indian peninsula.

Abundance of eubacteria, archaea, AOB, anammox and AOA in CE was enumerated using FISH technique and representative images are given in supplementary Figure 1. In CE we observed eubacterial dominance ( $1.8$  to  $3.5 \times 10^5$  cell  $\text{ml}^{-1}$ ) by an order of magnitude higher than archaea ( $1.7$  to  $2.7 \times 10^4$  cell  $\text{ml}^{-1}$ ) at all the stations (Fig. 3). The abundance of eubacteria was consistent with the earlier reports from other Indian estuaries [25]. No significant variation in eubacterial abundance was noticed with stations. Abundance of archaea could not be compared with that of other Indian estuaries as the same is being reported for the first time. Nevertheless, our results corroborate with the earlier findings on the dominance of eubacteria over archaea in Chesapeake Bay [26] and Columbia River estuary [27].

Among the two classes of AOB,  $\beta$ -proteobacteria ( $0.79$  to  $2.0 \times 10^5$  cell  $\text{ml}^{-1}$ ) showed significant dominance, which was approximately one order of magnitude higher than  $\gamma$ -proteobacteria [nitrosococcus ( $0.9$  to  $4.6 \times 10^4$  cell  $\text{ml}^{-1}$ )] (Fig. 4). Their abundance was higher in the surface waters compared to the bottom. Phylogenetic analysis of the DGGE bands showed major affiliation of AOB to  $\beta$ -proteobacteria, while one band showed similarity with  $\gamma$ -proteobacteria *Nitrosococcus* sp (Fig. 5 and Supplementary Fig. 2a). The nutritional gradient influences the community structure and nitrification efficiencies of ammonia oxidizing microorganisms [28]. It was observed that ammonia concentrations in the water column had positive influence on the abundance of  $\beta$ -proteobacteria ( $r=0.639$ ,  $p<0.05$ ) and nitrosococcus ( $r=0.814$ ,  $p<0.01$ ) (Supplementary Table 1). Our result is in agreement with the earlier findings that the available ammonium limits the abundance of AOB [5, 29]. Other critical environmental variables which are known to be influencing the ammonia oxidation in the estuaries, such as salinity, temperature and dissolved oxygen [30, 31] had little effect on the distribution of AOB in CE. Since CE was limnetic during the study period, the effect of salinity on abundance of ammonia oxidizing microorganisms was not evident. Moreover, AOB is also known to exhibit a broad range of salinity tolerance [6].

Anammox and AOA were the other ammonia oxidizing microorganisms recorded in CE (Figs. 6 & 7). The ubiquitous presence of anammox has been reported from sediments of estuaries and freshwater and also from hypoxic region of ocean [7, 32, 33]. In CE anammox was observed in the water column and its abundance ranged from  $0.49$  to  $1.9 \times 10^4$  cells  $\text{ml}^{-1}$ , with minimum in the surface waters at station 5 and maximum in the bottom waters at station 3 (Fig. 6). The anammox present in the water column may be resuspended from sediments, due to excessive mixing of water

column and continuous dredging operations in CE. Anammox mediated oxidation of ammonia to nitrogen gets inhibited at higher concentration of DO (more than 2  $\mu\text{M}$ ) [34]. Therefore, we presume that the contribution of anammox may be negligible compared to the other groups of microorganisms in CE as the level of oxygen was ca 3.9 to 7.9  $\text{mg L}^{-1}$  in the water column during this period.

We observed the presence of AOA in the surface (0.56 to  $6.3 \times 10^3$  cells  $\text{ml}^{-1}$ ) and bottom (0.32 to  $2.9 \times 10^3$  cells  $\text{ml}^{-1}$ ) waters of CE, which was less than AOB (Fig.7). The diversity of AOA was monitored using PCR DGGE analysis of ammonia monooxygenase gene (*amoA*) (Supplementary Fig. 2b). Phylogenetic analysis of the DGGE bands showed major affiliation of AOA to Crenarchaeota. Similar results have been reported from Elkhorn Slough estuary in California where the organic load due to agricultural impact was higher [35]. Ammonia monooxygenase of archaea is tuned to work more efficiently in oligotrophic conditions [36], and hence AOA outnumber AOB in the open ocean. Recently, AOA dominance has been reported in sediments from Plum Island Sound estuary, Massachusetts [37] and Pearl River estuary China [38].

Although both AOB and AOA are present in CE, it is important to understand the differential contribution of these two groups of organisms to ammonia oxidation. The ammonia oxidation recovery rate assays showed that AOB mediated ammonia oxidation was higher than that of AOA in CE (Fig. 8). Ammonia oxidation activity of about 50 – 75 % could be recovered in control bottles after removing acetylene gas, which confirms the active recovery of ammonia oxidation. The protein synthesis of bacteria was inhibited by a combination of kanamycin and gentamycin while archaeal protein synthesis was inhibited by cyclohexamide. It was observed that 40 -65 % of ammonia oxidization activity could be recovered in the bottles supplemented with archaeal protein inhibitors, which may be contributed by AOB. On the other hand, recovery of ammonia oxidation rate was reduced to 15 – 45 % in the samples treated with bacterial protein synthesis inhibitors, which indicates that the contribution of AOA in CE was considerably low during the period of sampling. While no significant difference was noticed in the relative contribution of AOA between surface and bottom waters, spatial differences were observed. Maximum recovery of AOA mediated ammonia oxidation was observed in the surface and bottom waters at station 2 (45 %) while it was < 19 % at station 3. AOB mediated recovery of ammonia oxidation rate was > 50 % in the surface and bottom waters at stations 1 and 2, while it was 40 – 45 % at station 3. Many reports are available on the abundance of AOB and AOA in marine and estuarine environments, but their relative contribution to ammonia oxidation is hitherto not studied [4, 17, 39, 40]. Although both AOB and AOA harbor ammonia monooxygenase gene, the structure and mode of action of the respective enzymes are

different [36]. Archaeal ammonia monooxygenase gets triggered at lower concentrations of ammonia and switches off at higher concentrations [36], while that of bacterial gets triggered at higher concentration of ammonia. CE contains high concentrations of ammonia, i.e. 50 -65 % of the dissolved inorganic nitrogen [11], and therefore the AOB's contribution in ammonia oxidation could be higher.

NOB, such as nitrospira and nitrobacter, were observed in the range of  $1.8$  to  $6.9 \times 10^4$  and  $2.5$  to  $7.4 \times 10^4$  cells  $\text{ml}^{-1}$ , respectively (Supplementary Fig. S3), which were less than the ammonia oxidizing  $\beta$ -proteobacteria of respective station. Interestingly, we observed a positive correlation of anammox with  $\beta$ -proteobacteria ( $r=0.747$ ,  $p< 0.05$ ) and Nitrobacter ( $r = 0.732$ ,  $p< 0.05$ ). Similarly, positive correlations of AOA with  $\beta$ -proteobacteria ( $r=0.695$ ,  $p< 0.05$ ) and Nitrobacter ( $r = 0.860$ ,  $p< 0.05$ ) were also observed. The  $\gamma$ -proteobacteria (nitrosococcus) showed strong positive correlation with nitrospira ( $r = 0.894$ ,  $p <0.01$ ), whereas  $\beta$ -proteobacteria showed correlation with nitrobacter ( $r = 0.766$ ,  $p < 0.01$ ). Our results show the possibility of a cross feeding between ammonia oxidizing microorganisms (AOB, anammox and AOA) and NOB (Fig. 9). The cross feeding between AOB and NOB has been reported earlier in biofilms, using confocal microscopy and microautoradiography-fluorescent *in situ* hybridization (MAR-FISH) techniques [41-44]. Microscopic observations often showed the presence of NOB in the close proximity of AOB, which not only indicates the metabolic association between these two groups, but also confirms that their association is not mutually toxic [41-44]. It has been established that in controlled environments the interactions between these microorganisms vary with the level of ammonia and dissolved oxygen present [29, 45], which is equally applicable in CE also. During the study period, DO was maintained at higher levels in CE except in the bottom waters of Fort Cochin, which incidentally accounted for higher numbers of AOB and NOB.

Our results showed the dominant role of AOB in modulating the ammonia oxidation in a monsoon driven nutrient rich tropical estuary. The process is fuelled by cross feeding among the organisms in the proximity, which in turn is dictated mainly by ammonia and dissolved oxygen.

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## Legends to Figure

**Figure 1.** Map showing the sampling stations. Station numbers in parenthesis

**Scheme 1.** Schematic representation of experimental approach used for measuring differential contribution of AOB and AOA in ammonia oxidation rate

**Figure 2.** Salinity variation at station 3 showing stratification

**Figure 3.** Abundance of Eubacteria (a) and Archaea (b) in the surface (blue bars) and bottom (red bars) waters from five sampling stations in Cochin estuary.

**Figure 4.** Abundance of ammonia oxidizing  $\beta$ -proteobacteria (a) and Nitrosococcus (b) in the surface (blue bars) and bottom (red bars) waters from five sampling stations in Cochin estuary.

**Figure 5.** Rooted neighbor-joining phylogenetic tree based on 16S rRNA gene of ammonia oxidizing  $\beta$ -proteobacteria sequences retrieved from DGGE bands.

**Figure 6.** Abundance of anammox in the surface (blue bars) and bottom (red bars) waters from five sampling stations in Cochin estuary.

**Figure 7.** Abundance of Archaeal ammonia oxidization gene (amoA) in the surface (blue bars) and bottom (red bars) waters from five sampling stations in Cochin estuary.

**Figure 8.** Comparison of ammonia oxidation recovery rate of control (A), AOA (B) and AOB (C) in surface and bottom waters of sampling stations 1, 2 and 3

**Figure 9.** Schematic representation of interactions among nitrifiers in Cochin estuary (\*  $p < 0.05$ , \*\*  $p < 0.01$ )

**Supplementary Figure 1.** Representative images of samples hybridized with FISH probes for eubacteria (a), archaea (b), ammonia oxidizing  $\beta$ -proteobacteria (c) and Nitrosococcus (d)

**Supplementary Figure 2.** Representative images of DGGE pattern of ammonia oxidizing  $\beta$ -proteobacteria (A) and archaeal ammonia monooxygenase (amoA) (B) in surface (s) and bottom (b) samples of sampling stations

**Supplementary Figure 3.** Abundance of Nitrospira (a) and Nitrobacter (b) in the surface (blue bars) and bottom (red bars) waters from five sampling stations in the Cochin estuary.

Table 1: FISH probes used in the study

<b>Group</b>	<b>Probe</b>	<b>Sequence</b>	<b>Target site</b>	<b>Target organism(s)</b>	<b>Formamide (%)</b>	<b>Reference</b>
	<i>EUB 338</i>	<i>GCTGCCTCCCGTAGGAGT</i>	<i>16S</i> (338–355)	<i>Domain Bacteria</i>	55	[46]
<b>AOB</b>	<i>Nso190</i>	<i>CGATCCCCTGCTTTTCTCC</i>	<i>16S</i> (190–208)	<i>Ammonia-oxidizing β-proteobacteria</i>	55	[47]
	<i>NmV</i>	<i>TCCTCAGAGACTACGCGG</i>	<i>16S</i> (174–191)	<i>Nitrosococcus mobilis lineage</i>	40	[48]
<b>NOB</b>	<i>NIT</i>	<i>CCTGTGCTCCATGCTCCG</i>	<i>16S</i> (1035–1048)	<i>Nitrobacter spp.</i>	45	[49]
	<i>Ntspa 712</i>	<i>CGCCTTCGCCACCGGCCTTCC</i>	<i>16S</i> (712–732)	<i>Phylum Nitrospira</i>	40	[50]
<b>Archaea</b>	<i>ARCH 915</i>	<i>GTGCTCCCCCGCCAATTCT</i>	<i>16S</i> (934–915)	<i>Archaea</i>	40	[51]
	<i>Arch-amo AFA</i>	<i>ACACCAGTTTGGYTACCWTC DGC</i>	<i>amoA gene</i>	<i>Archaeal ammonia oxidizing gene</i>	40	[52]
<b>Anammox</b>	<i>BS820</i>	<i>TAATTCCTCTACTTAGTGCCC</i>	<i>16S</i> (820–840)	<i>Annamox16S</i>	35	[53]

Table 2: Environmental parameters of surface (S) and bottom (B) waters in Cochin estuary stations

Parameters	Stations									
	1		2		3		4		5	
	S	B	S	B	S	B	S	B	S	B
pH	7.2	7.3	6.7	6.7	6.8	7.7	6.6	6.6	7.0	7.0
Salinity	0.84	1.09	00	00	2.29	20.6	00	00	00	00
DO (mgL <sup>-1</sup> )	7.2	6.0	6.9	6.7	6.5	3.4	6.8	6.8	7.9	7.0
NH <sub>4</sub> -N (μM)	3.5	5.6	6.9	5.9	9.9	7.7	8.8	7.7	5.1	2.3
NO <sub>2</sub> -N (μM)	1.0	0.7	0.4	0.3	0.6	1.5	0.8	0.4	0.3	0.4
NO <sub>3</sub> -N (μM)	15.9	17.6	36.1	21.1	18.2	19.8	15.1	17.2	22.4	22.6
PO <sub>4</sub> -P (μM)	2.6	3.1	0.8	0.7	2.1	1.8	2.2	1.4	0.5	0.6

Scheme 1

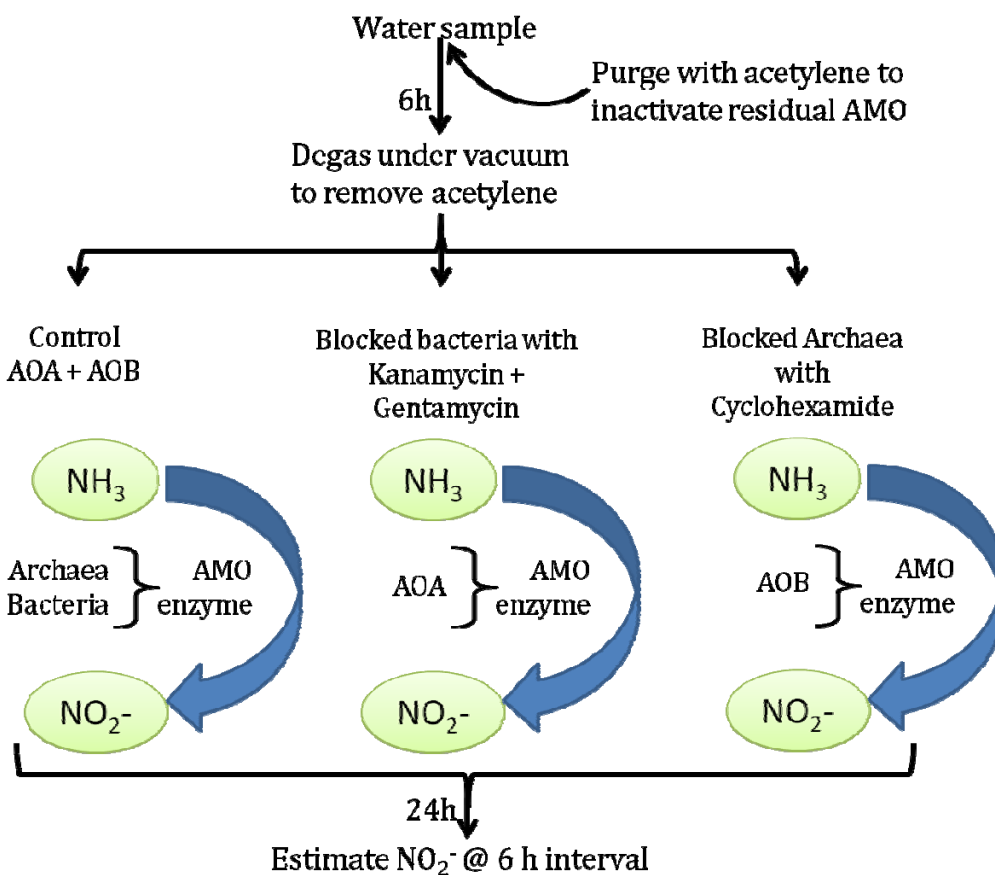


Figure 1

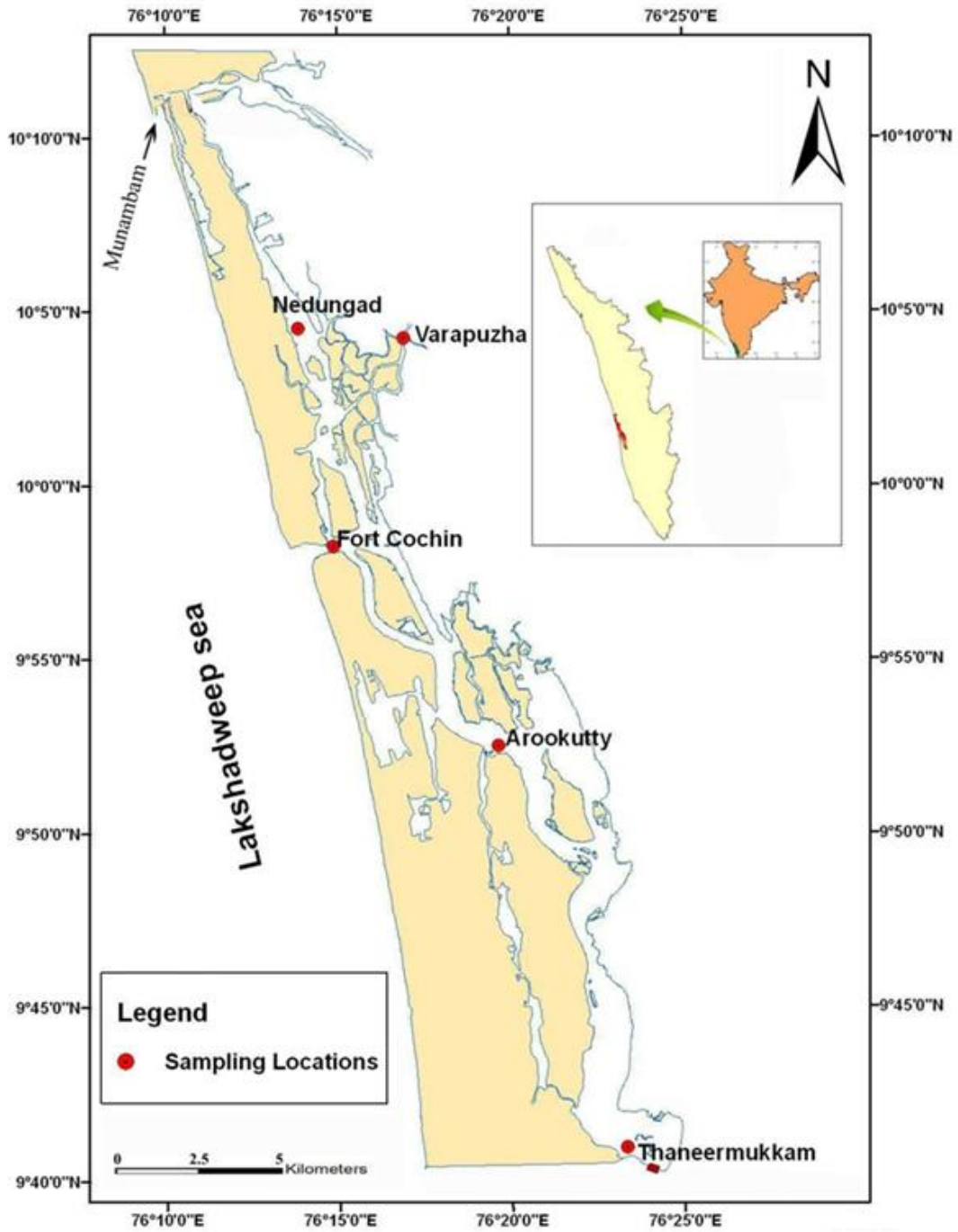




Figure 2

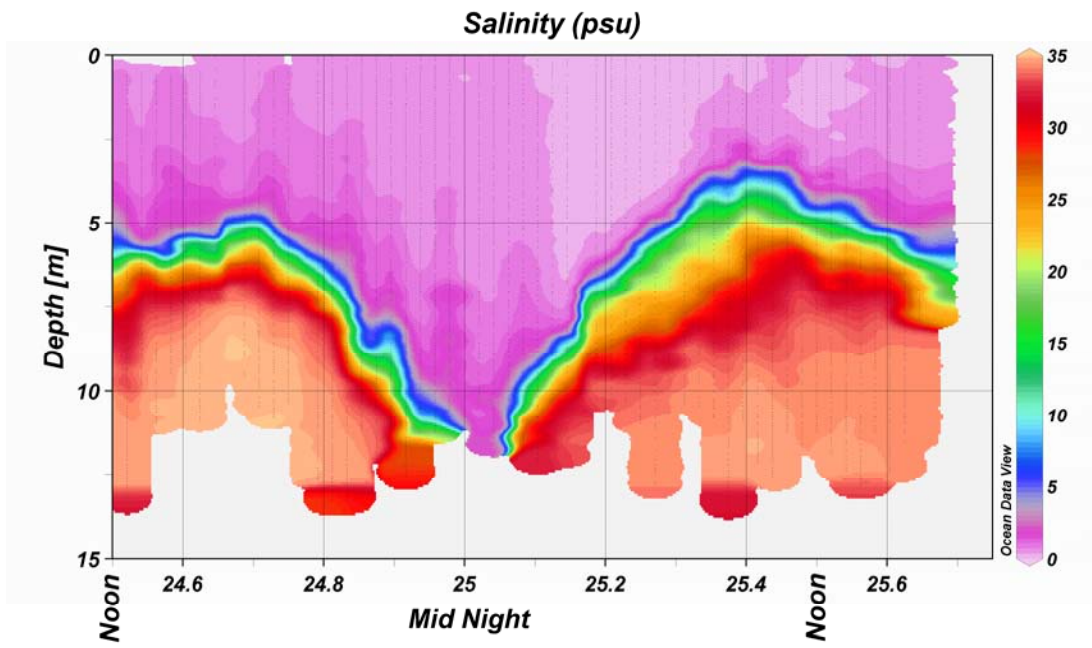


Figure 3

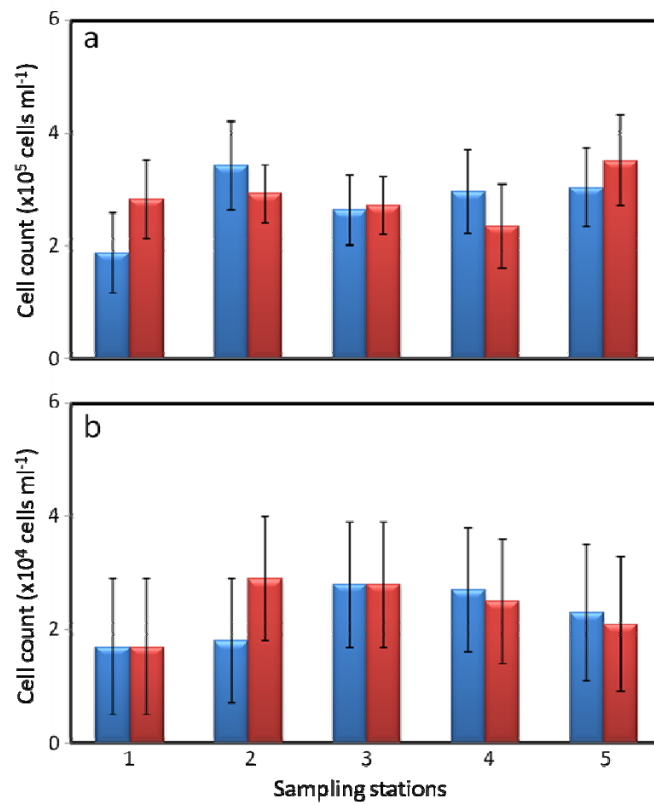


Figure 4

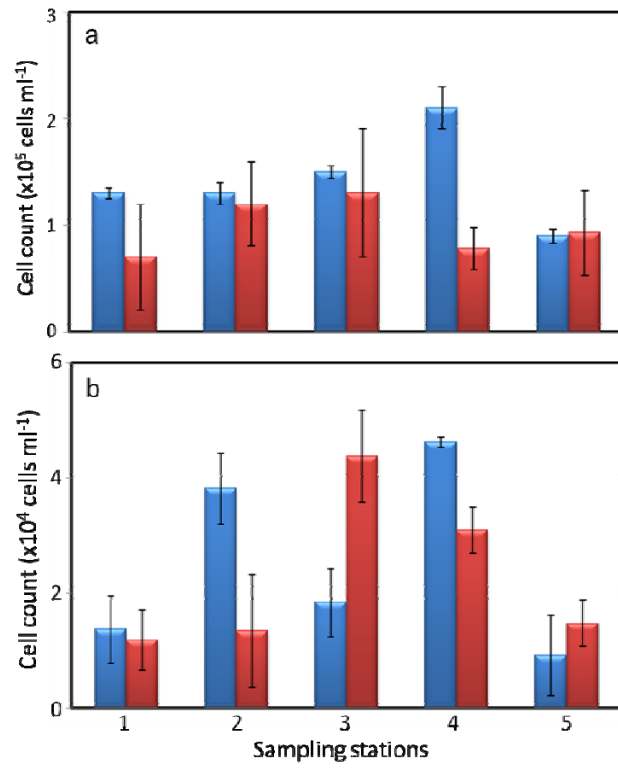


Figure 5

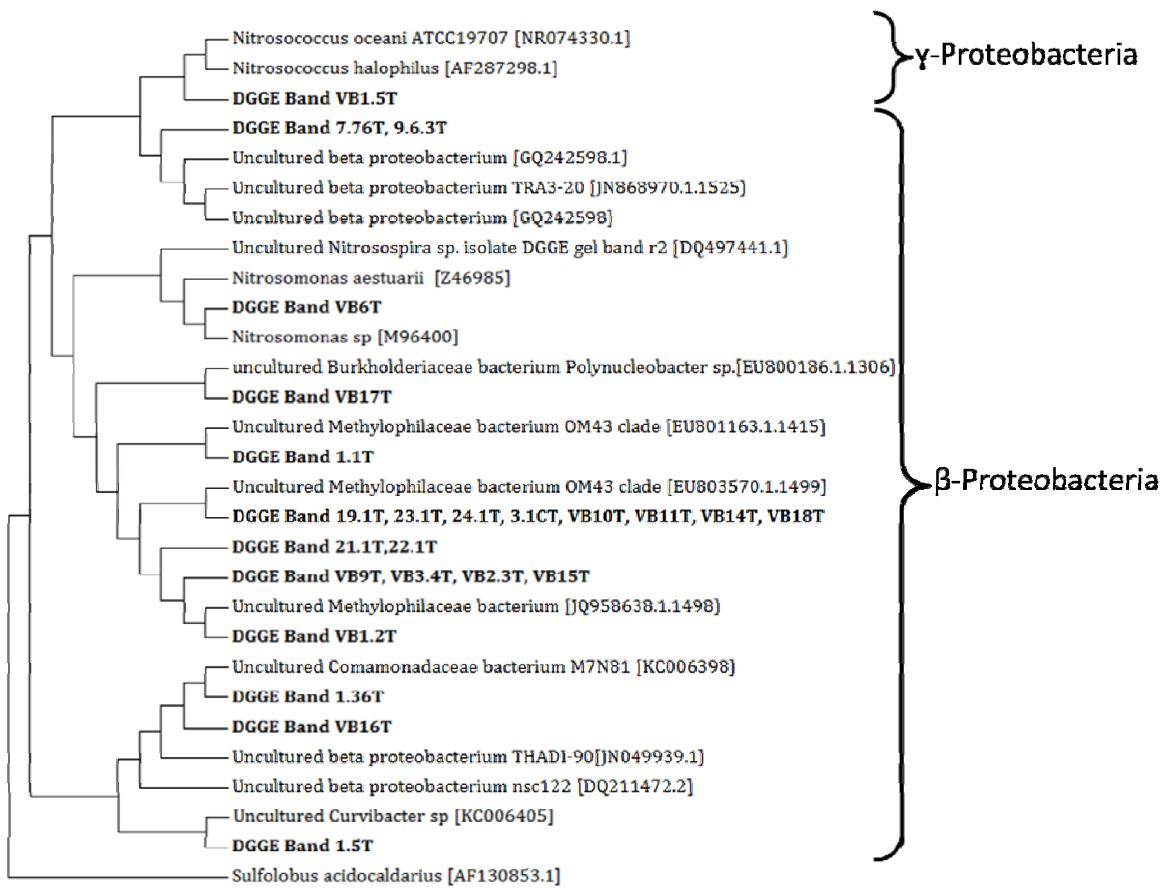


Figure 6

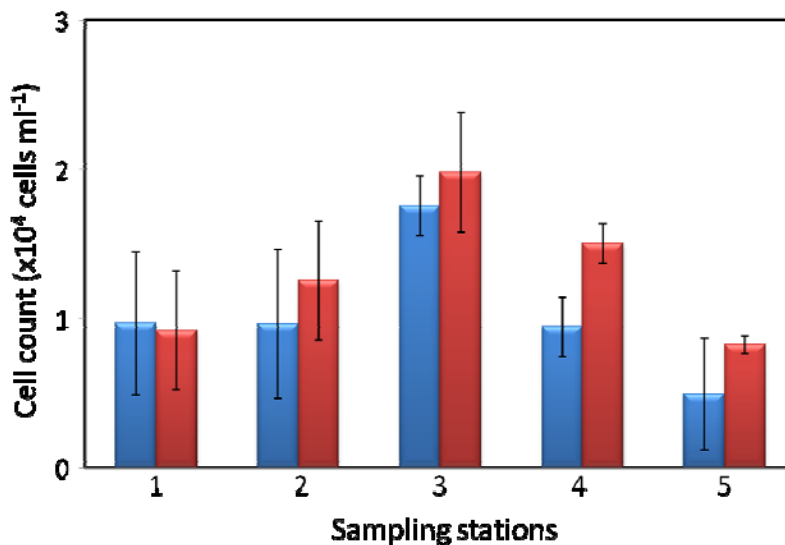


Figure 7

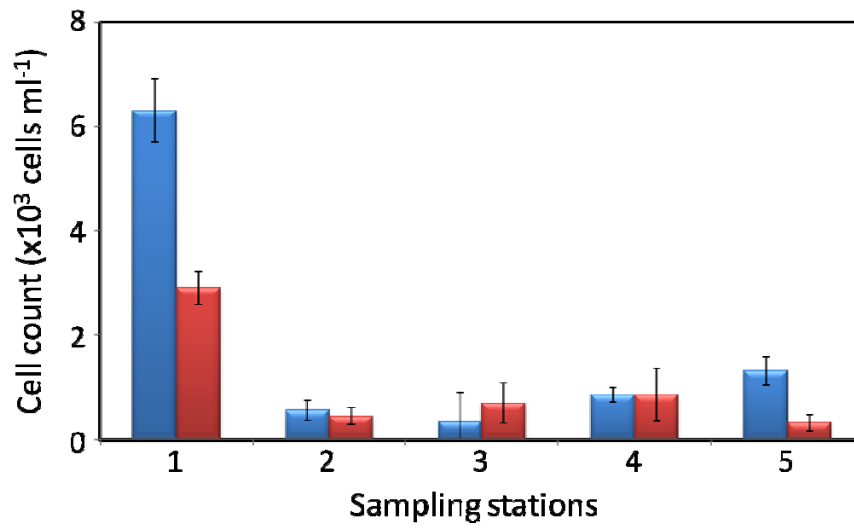


Figure 8

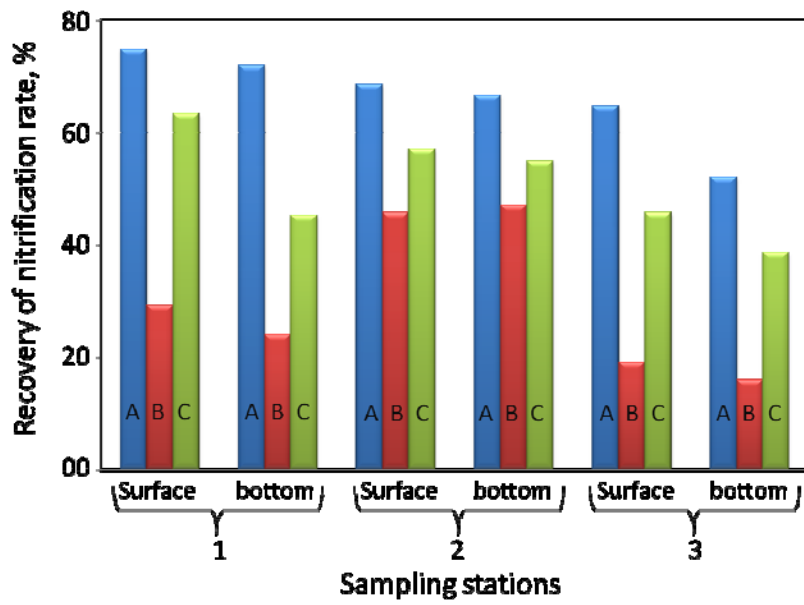
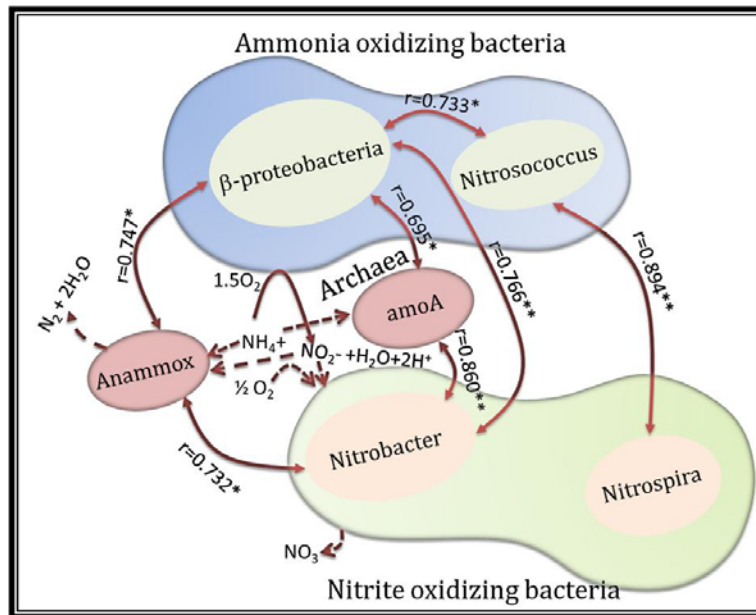
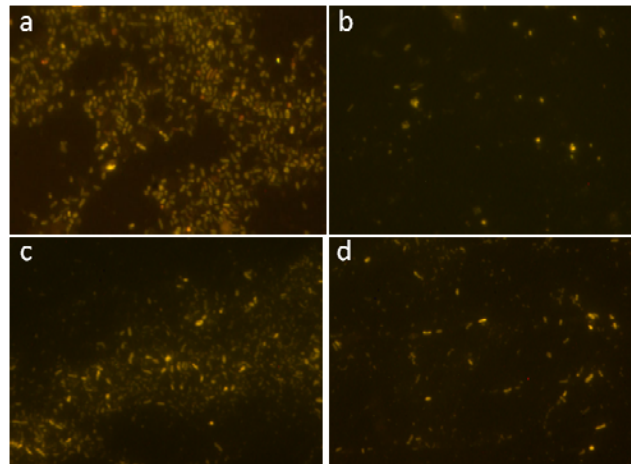


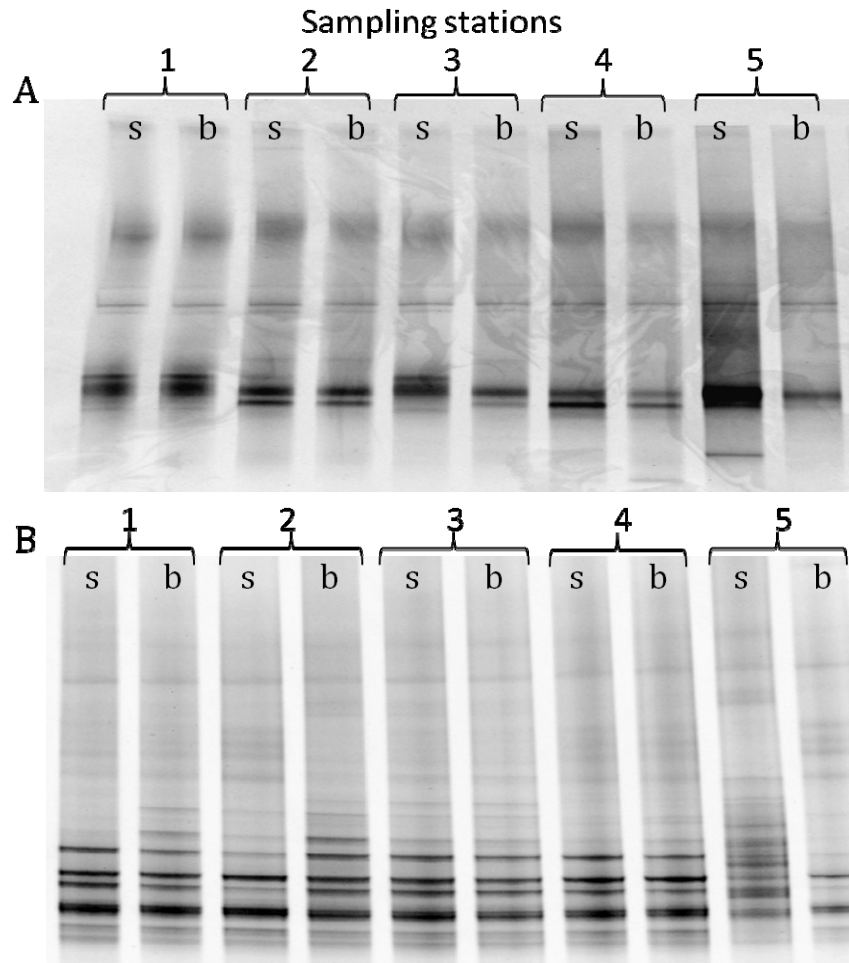
Figure 9



Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

