3.1 Description of the Study Area

3.1.1 Cochin estuary

The Cochin estuary (CE) is the second largest wetland ecosystem along the southwest coast of India, covering an area of ~25600 ha (Menon et al. 2000) extending from 9° 30’ - 10° 12’ N to 76° 10’ - 76° 20’ E. This estuary is running parallel to the Arabian Sea covering a distance of 80 kms, extending from Munambam at north to Alleppey at south and two permanent openings connect the CE to the Arabian Sea. Constant mixing with seawater through tidal exchanges has given it the characteristics of a tropical estuary. Since it is geographically located in the tropical region, the mean temperature of surface water is ~ 28°C during monsoon and 30°C during pre-monsoon seasons (Madhupratap et al. 1992). The depth of the estuary varies from 2 to 7 m, but the ship channels at the Cochin harbor region are dredged and maintained at 10 to 13 m. The annual rainfall in Cochin is around 3200 mm of which, nearly 75% occurs during the South-West (SW) monsoon (Qasim 2003). SW monsoon is the main cause for the seasonal variation in the CE. This complex micro tidal estuary (Joseph and Kurup 1989) undergoes a characteristic transformation from a river-dominated system during the monsoon
season (June–September) to a tide-dominated system during the pre-monsoon season (February–May). The most important hydrological variable encountered in the CE is salinity, similar to the conditions encountered in estuaries with a gradual declining of salinity from 30 at the entrance i.e. mouth of the estuary to nearly fresh water at the head of the estuary i.e. point of entry of the rivers.

The nutrient composition of the estuary is greatly influenced by terrestrial anthropogenic inputs, fresh water influx from six rivers, Periyar, Pampa, Manimala, Minachil and Achankovil mainly during the prolonged monsoon, and seawater influx from two bar mouths (Menon et al. 2000, Madhu et al. 2010). Previous studies have estimated that the CE receives high concentrations of industrial effluents of about $10^4 \times 10^3$ m$^3$ day$^{-1}$ and untreated domestic wastes of ca 260 m$^3$ day$^{-1}$ (Balachandran et al. 2005). These studies revealed that the system bears excess of inorganic nitrogen irrespective of seasons and the nutrient recycling suggests that this system is very sensitive to increased nutrient loadings.

### 3.1.2 Description of sampling sites

For this study, 4 stations were selected across a salinity gradient. Station positions were fixed using the Global Positioning System (Magellan NAV DLX 10, USA) and the station positions are given in Figure 2.1 Geographic position of stations are given below.

<table>
<thead>
<tr>
<th>Stations</th>
<th>Latitude</th>
<th>Longitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>Station 1</td>
<td>10° 01, 850’ N</td>
<td>76°15.918’ E</td>
</tr>
<tr>
<td>Station 2</td>
<td>9°59.147’ N</td>
<td>76°15.7’ E</td>
</tr>
<tr>
<td>Station 3</td>
<td>9°58.252’ N</td>
<td>76°14.278’ E</td>
</tr>
<tr>
<td>Station 4</td>
<td>10°00’ N</td>
<td>76° 05’ E</td>
</tr>
</tbody>
</table>

Station 1 is close to river entry and in this study designated as “low saline” with an average salinity of below 10. Stations 2 and 3 are considered as “intermediate saline” stations with salinity range of 10 to 25. These stations are estuarine in nature located close to the entry point of river and receive
Studies on nitrifying microorganisms in Cochin estuary and adjacent coastal waters

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anthropogenic inputs from domestic sewage and industrial effluents. Station 4 is a coastal station, about 20 kms away from station 3 with minimum of fresh water input except from precipitation during SW monsoon. This station is designated as “high saline” station with salinity range between 25 and 35. Average depth of the estuarine stations (Stns. 1, 2 & 3) is > 10 m and coastal station (Stn. 4) is around 25 m.

![Figure 3.1 Location of sampling stations in the Cochin Estuary](image)

**3.2 Sampling Strategy and Transportation**

Bimonthly sampling was carried out from all stations for a period of one year (2011) covering three different seasons: – pre-monsoon (January - April), monsoon (June - August) and post-monsoon (October - December). Two sets of water samples were collected from each station using a 5 L Niskin sampler, surface sample (~1m depth) and bottom sample (close to the bottom). Water samples for estimating physicochemical parameters were sub-sampled in polypropylene bottles and preserved at 4 °C during transportation and for microbiological analysis, the samples were transferred to sterile glass bottles and stored at 4-5 °C till analysis. All samples were processed immediately after transportation.
3.3 Analytical Studies

3.3.1 Environmental parameters

3.3.1.1 Temperature and salinity

Water temperature was measured by dipping the stainless steel temperature probe of a hand held traceable mini digital thermometer with a precision of 0.1°C into ambient water. Salinity of the samples was determined using a Digi Auto Salinometer (Model TSK, accuracy ±0.001) immediately after reaching the laboratory.

3.3.1.2 pH

pH was estimated based on a pH measurement at constant temperature method (Gieskes 1969, 1970). A digital pH meter (Thermoelectron Corporation, USA) was used for determining pH of water samples after calibrating it with the standard buffers of pH 4, 7, and 9.2 (Fluka), respectively.

3.3.1.3 Dissolved oxygen (DO)

DO concentration was estimated using Winkler’s titrimetric method (Carpenter 1965). Water samples were carefully siphoned into 125 ml acid washed (10% HCl) glass stopper bottles without formation of air bubbles. Winkler A (1 ml of 3 M manganous chloride) and Winkler B (1 ml of 8 M alkaline iodide) were added to the samples and mixed properly. The precipitate formed was dissolved using 1 ml of 10 N H₂SO₄ and titrated with 0.01 N sodium thiosulphate using starch as indicator. Concentration of oxygen is expressed as mg L⁻¹.

3.3.1.4 Suspended particulate matter (SPM)

For determination of SPM, 250 ml of water samples were filtered through previously weighed millipore filter paper (0.45 µm pore size) subsequently, the filter was dried at 80° C to 90° C to constant weight to eliminate the water content and re-weighed. The differences in weight indicate the amount of suspended solids and the value expressed in mg L⁻¹.
3.3.2 Nutrient concentrations

3.3.2.1 Ammonia-N (NH\textsubscript{4})

Ammonia-N was determined according to the indophenol blue method of Koroleff, (1983). The measurement of ammonia included both free dissolved ammonia gas and the ammonium ions. This method estimates the sum of NH\textsubscript{4}\textsuperscript{+} and NH\textsubscript{3} and is denoted here as NH\textsubscript{4}-N. In a moderately alkaline medium, ammonia reacts with hypochlorite to form monochloramine, which in presence of phenol, catalytic amount of nitroprusside ions and excess of hypochlorite forms indophenol blue. The formation of monochloramine requires a pH between 8 and 11.5. At higher pH, ammonia is incompletely oxidized to nitrite. Calcium and magnesium ions precipitate in seawater as hydroxide and carbonate respectively above pH 9.6. However, their precipitation can be prevented by complexing them with citrate buffer. Adequate care was taken to ensure that samples, blanks and standards were not contaminated during the course of analysis. The samples were 'fixed' by the addition of reagents immediately after collection and the absorbance, after the colour development (after 6 hours) was measured at 630 nm using UV - Vis spectrophotometer (Shimadzu, 1650 PC Japan). The concentration was calculated based on the standard ammonium chloride (NH\textsubscript{4} Cl) solution (precision: ±0.05). Concentration is expressed in \textmu M N-NH\textsubscript{4}.

3.3.2.2 Nitrite-N (NO\textsubscript{2})

Nitrite was measured by the method described by Bendschneider and Robinson (1952). In this method, nitrite in the sample was allowed to react with sulphanilamide in an acid solution. The resulting diazo compound reacted with N-(1-naphthyl)-ethylene diamine to form a highly coloured azo dye. The absorbance was measured at 543 nm using a UV - Vis spectrophotometer. The concentration was calculated based on the standard Sodium nitrite (NaNO\textsubscript{2}) solution (precision: ±0.05 \textmu M). Concentration is expressed in \textmu M N-NO\textsubscript{2}.

3.3.2.3 Nitrate-N (NO\textsubscript{3})

The method described by Grasshoff (1970) was used for the estimation of nitrate. In this method the nitrate present in the sample was reduced to nitrite
using a reductor filled with copper coated-cadmium granules. The condition of reduction was adjusted so that nitrate is almost quantitatively converted to nitrite and not reduced further. Nitrite thus formed was estimated as the method mentioned in section 3.3.2.2 above. Potassium nitrate (KNO₃) was used for standardization. Concentration is expressed in μM N-NO₃.

3.3.2.4 Phosphate

The method of Murphy and Riley (1962) was adopted for estimating inorganic phosphate. Phosphate and ammonium molybdate were allowed to react in acid solution to give phosphomolybdic acid, which was reduced by ascorbic acid. Optical density was measured using a spectrophotometer (Shimadzu, Japan) after 10 min at 882 nm. Potassium dihydrogen phosphate (KH₂PO₄) was used as standard and the concentration is expressed in μM.

3.3.2.5 Silicate

Silicate was estimated using protocol of Grasshoff (1964). Sample was allowed to react with ammonium molybdate resulting in the formation of silicomolybdate, phosphomolybdate and arsenomolybdate complexes and oxalic acid was added to reduce to silicomolybdous acid and the absorbance of blue colour was measured at 810 nm. Sodium fluorosilicate (Na₂SiF₆) solution was used as standard and the concentration is expressed in μM.

3.4 Microbiological Enumeration

3.4.1 Direct total counts (DTC)

Total prokaryotic abundance was determined by DAPI (4,6-Diamidino-2-Phenylindole) (Porter and Feig 1980). Samples for total bacterial counts were fixed with buffered formalin 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-μm polycarbonate nuclepore filter (Millipore, USA). Samples were enumerated at 100X magnification under a Nikon epifluorescence microscope, and at least 10 fields of ≥30 cells per field were counted. Total prokaryotic abundance was expressed as cells ml⁻¹.
3.4.2 Fluorescent In Situ Hybridization (FISH)

FISH is a molecular tool used for rapid independent monitoring of phylogenetically defined bacterial populations in environmental samples using nucleic acid probes. In the present study, abundance of total eubacteria, archaea, ammonia oxidizing bacteria and nitrite oxidizing bacteria in the water were estimated using FISH.

3.4.2.1 Sample fixation and preservation

About 200 ml of water sample from each station was fixed with formaldehyde to a final concentration of 2-4% and kept for 1 hour at room temperature (27± 2°C). The samples were sonicated for 20 to 40 seconds in order to detach the particle associated cells. Then 10-25 ml of sample was filtered onto a white 0.2-μm polycarbonate membrane filter (Millipore GTTP04700) that was placed over cellulose nitrate pre-filter (Millipore AP1504700). Low pressure was applied during filtration to avoid cell damage. Filters were washed with 30 ml of sterile phosphate-buffered saline (pH 8) followed by 30 ml of sterile distilled water and air-dried over absorbent paper in individual sterile petri dishes. The filters were immediately hybridized or stored at -20˚C until hybridization.

3.4.2.2 Hybridization and DAPI staining

Modified protocol of Glöckner et al. (1999) was used for hybridization. Replicate filters were used for hybridization with suitable probes labeled with fluorescent dye CY3. Each filter was cut into four sections with a razor blade, and each section was placed onto a microscope slide. A slit was made in the right edge of each filter section to ensure that the side containing the bacteria was facing upwards. The filter sections were hybridized with 20 μL of hybridization buffer, containing 0.9 M NaCl, 20 mM Tris- HCl (pH 8), 35 to 60% formamide (formamide % varies depending on probe), 0.01% sodium dodecyl sulphate (SDS), and 50 ng of oligonucleotide probe. Each slide with filter sections was placed into a polyethylene tube (in a horizontal position). A piece of blotting paper was put into the polyethylene tube and soaked it with 1ml hybridization buffer to create a humid atmosphere. These caped polyethylene tubes incubated in the dark for 90 to 120
minutes at 46˚ C hybridization chambers. Following incubation, filter sections were quickly transferred into pre-warmed (48˚C) washing buffer prepared in individual falcon tubes. The prewarmed wash buffer contained 20 mM Tris-HCl, 5 mM EDTA, 0.01% SDS and 20 to 60 mM NaCl (5M NaCl depending on % formamide in hybridization buffer), and incubated for 15 min at 48˚C in a stirred water bath. The wash buffer with filter section was poured into a Petri dish and the filter section was picked and rinsed by placing them into a Petri dish with distilled H2O for several seconds. Each filter section was dried at room temperature over absorbent paper, placed on a glass slide. Dried filters were counter-stained with 50 μl of DAPI (1 μg ml⁻¹) for 3-8 minutes. After staining, each filter section was washed with 1 ml of filtered Milli Q water, dried over absorbent paper and mounted on a glass slide in glycerol medium (Citifluor #1; Citifluor). Hybridization and counting were completed within one month after preparation. The details of FISH probes used, its sequences and reference are given in the Table 3.1. Target sites of the probes, formamide concentration in hybridization buffer and NaCl concentration in the wash buffer are given in the Table 3.2. (Preparation of all buffers and solutions are given in Appendix section.)

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Probe Name</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>EUB 338</td>
<td>GCTGCCTCCCCTAGGAGT</td>
<td>(Amann et al. 1990)</td>
</tr>
<tr>
<td>Archaea</td>
<td>ARCH 915</td>
<td>GTGCTCCCCCGCAAATTCTCT</td>
<td>(Stahl and Amann 1991)</td>
</tr>
<tr>
<td></td>
<td>β-AOB</td>
<td>Nso 190</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N. mobilis</td>
<td>NmV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NOB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrobacter sp.</td>
<td>NIT</td>
<td>CCTGTGCTCCATGCTCCG</td>
<td>(wagner et al. 1996)</td>
</tr>
<tr>
<td>Nitrospira sp.</td>
<td>Ntspa 712</td>
<td>CGCCTTCGCCACCGCCTCC</td>
<td>(Daims et al. 2000)</td>
</tr>
</tbody>
</table>
Table 3.2 Target sites of the probes, formamide concentration in the hybridization buffer and NaCl concentration in the wash buffer.

<table>
<thead>
<tr>
<th>Probe Name</th>
<th>Target Sites</th>
<th>Concentration</th>
<th>Formamide (%) in hybridization buffer</th>
<th>NaCl (mM) in the wash buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB 338</td>
<td>16S (338–355)</td>
<td>55</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>ARCH 915</td>
<td>16S (934–915)</td>
<td>40</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Nso190</td>
<td>16S (190–208)</td>
<td>55</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>NmV</td>
<td>16S (174–191)</td>
<td>40</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>NIT</td>
<td>16S (1035–1048)</td>
<td>45</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Ntspa 712</td>
<td>16S (712–732)</td>
<td>40</td>
<td>56</td>
<td></td>
</tr>
</tbody>
</table>

3.4.2.3 Counting of FISH cells

The slides were either examined immediately using an Olympus BH-2 epifluorescent microscope, equipped with a 100X oil objective, Cy3 filter (41007-HQ) and DAPI filter (UG-1), or were stored at -20°C for a maximum of 2 days before microscopic analysis. As Cy3 fluorescence fades much more rapidly than DAPI fluorescence (Pernthaler et al. 2001) direct counts of hybridized cells were completed first, followed by DAPI counts (under UV light), for total bacteria, in the same field of view.

3.5 Community Structure of AOB and AOA using DGGE

3.5.1 Sample preparation

3.5.1.1 DNA extraction

DNA extraction was performed following the protocol of Boström et al. (2004) with slight modification. Different steps involved in DNA extractions are briefly given below.

1. One to two liters of water samples were filtered through 0.2 µm cellulose nitrate filter and stored at -20°C until DNA extraction.
2. Frozen filters were cut into small pieces and put into 2 ml microcentrifuge tube containing 525 µl of lysis buffer.

3. Into the tube 11 µl of lysozyme (1mg/ml) was added and incubated at 37˚ C for one hour in water bath.

4. After incubation 60 µl of 10% SDS and 3 µl of proteinase (K 100 µg ml⁻¹) were added and incubated at 55˚C for 6 hours.

5. An approximately equal volume (0.7 to 0.8 ml) of chloroform/isoamyl alcohol (24:1) was added to the above, mixed thoroughly by inverting the tube, and spinnd for 4 to 5 minutes in a microcentrifuge. A white interface was visible after centrifugation.

6. The aqueous and viscous supernatant was removed to a fresh microcentrifuge tube, leaving the interface behind.

7. About 0.6 volume of isopropanol was added and mixed by inverting the tube to precipitating the nucleic acids.

8. The tube was centrifuged for 10 minutes at 10000 rpm. The supernatant was discarded. The pellet was washed with 1000 µl of 99% ethanol and centrifuged for 10 minutes at 1000 rpm to purify the DNA.

9. The supernatant was discarded and the purified DNA pellet was dried under vacuum.

10. The DNA pellet was then dissolved in 50 µl of 1X TE buffer for analysis. Preparation of all buffers, solutions and reagents are given in Appendix section.

3.5.1.2 DNA detection by agarose gel electrophoresis

Accurately weighed 1.5 g of electrophoresis grade agarose was added to 100 ml of electrophoresis buffer (1X TAE). The agarose was melted in a microwave oven (IFB, USA) and was swirled in between to ensure even mixing. The molten agarose was allowed to cool down to 50-60˚C. Prior to the preparation of molten agarose the gel caster (BioRAD, USA) was leveled using the leveling feet and leveling bubble. The comb was placed on the appropriate slots of the tray. The molten agarose was poured into the gel tray. The gel was allowed to solidify for 20-30 minutes at room temperature. After the gel was hardened, the comb was
withdrawn and the gel tray removed carefully. The gel casting tray was placed in the electrophoresis tank and sufficient 1X TAE buffer was added to cover the gel to a depth of 1mm. The extracted DNA samples were mixed with 6X loading dye in the ratio 1:5 and loaded into the gel wells with a micropipette at the negative electrode. Three µl of 500 bp DNA molecular weight marker was loaded as reference. The leads were connected and voltage applied so that DNA could migrate toward the anode. The gel was electrophoresed at 100 V until the dye front had migrated to about three-fourth of the gel. The DNA fragments were visualized using the gel documentation system (BioRAD excel, USA) and the image was captured using image lab software (BioRAD, USA).

3.5.1.3 Estimation of DNA by spectrophotometry

DNA was quantified by measuring absorbance at 260 nm using Nanodrop Spectrophotometer (Nanodrop Technologies, USA). An absorbance value of 1 at 260 nm is equivalent to the concentration of 50 μg ml⁻¹ for double stranded DNA. Purity of DNA was checked by measuring the ratio of absorbance at 260 nm and 280 nm. If the ratio A260/A280 is approximately equal to 1.8, it indicates pure DNA. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants.

3.5.2 PCR amplification of DNA for AOB

The purified DNA was subjected to PCR in a Thermal cycler (Eppendorf, Germany) for the amplification. All PCRs for DGGE were carried out with an equimolar mixture of three forward primers (Kowalchuk et al. 1997) (CTO189fA-GC, CTO189f BGC, and CTO189 f C-GC), each with a GC clamp and a reverse primer containing a single ambiguous base without GC clamp. The forward primers CTO189fA and CTO189fB (CCGCCGCGCGCGGCGGGGGGGGGGCACGGInterrupted]) and CTO189fC-GC (CGCCCGCGCGCGGCGGGGGGGGGGGCAGCGCGCAGCGCGGGGATCG) were synthesized separately and collectively referred to as CTO189f-GC.
The reverse primer sequence was CTO 654r (CTAGCYTTGTAGTTTCAACGC). The primers amplify 465 bp partial rDNA sequences of β-subdivision ammonia oxidizer. Amplification was carried out in a 50 μl reaction mixture in duplicate. Each mixture containing 50 ng of purified DNA (DNA samples were diluted in milliQ water for required ng concentration), 3 mM concentration of MgCl₂, 2.5 mM of each dNTP, 10 pM of each primer and 3 U/μl of Taq polymerase in 1X of Taq buffer (NEB Canada). The PCR conditions used were: initial denaturation at 95°C for 5 minutes, 30 cycles consisting of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and elongation at 72°C for 45 seconds and final extension at 72°C for 10 minutes. Two reactions, a positive control with DNA template known to be successfully amplified and a negative control lacking template DNA was set up in each PCR run. Amplification was confirmed by gel electrophoresis. Duplicate PCR products of 465 bp length were mixed to minimize PCR bias and this was used for DGGE.

3.5.3 PCR amplification of DNA for AOA

Ammonia monoxygenase gene (amoA) of AOA was amplified for the community structure analysis of AOA. A combination of GC clamp attached forward primer amoAF (CTGAYTGGGCYTGGACATC) and reverse primer without GC clamp amoAR (TTCTTCTTTGTTGCCCAGTA) were used as primer sets (Wuchter et al. 2006). The GC clamp was attached to the 5’ end of the primer. Amplification was carried out in a 50 μl reaction mixture in duplicate. Each mixture containing 50 ng of purified DNA, 3 mM concentration of MgCl₂, 2.5 mM of each dNTP, 10 pM of each primer and 3U/μl of Taq polymerase in 1X of Taq buffer (NEB Canada). The PCR conditions used were: initial denaturation at 95°C for 5 minutes, 30 cycles consisting of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute and elongation at 72°C for 30 seconds and final extension at 72°C for 10 minutes. Amplification of 256 bp length of AOA gene was confirmed by gel electrophoresis.
3.5.4 Denaturing Gradient Gel Electrophoresis (DGGE) analysis

DGGE of PCR products generated were performed by the method described by (Muyzer and Smalla 1998) with the use of a D-Gene system (Bio-Rad Laboratories).

3.5.4.1 Preparation of acrylamide gel

For DGGE of AOB Polyacrylamide gradient gels (8% polyacrylamide; 1.5 mm thick; 0.53 TAE; 37:1 acrylamide-bisacrylamide; 35 to 50% denaturant) using gel sandwich sizes of 16x16 cm were poured with the aid of a gradient maker (CBS, Del Mar, Calif.). Denaturing acrylamide (100%) was defined as 7 M urea with 40% formamide (24). A 10% poly acrylamide gel with 30 to 55% denaturant was used for AOA. Gels were poured from bottom to top. The gel was allowed to set for about one hour at room temperature. Ammonium Persulfate (APS) and Tetramethylethylenediamine (TEMED) were used for the polymerization of the gels as per the instructions in the equipment’s manual. Meanwhile the electrophoresis tank was filled with 7 L of 1X TAE buffer. The casted gel was inserted into the core after removing the comb. The other side of the core was also fitted with a gel sandwich so that the upper buffer chamber is formed. The wells of the gel were washed several times with same buffer to remove partially polymerized particles. The core along with the gels was carefully placed in the buffer tank. About 350 ml of running buffer was added to the buffer tank. The temperature control module was placed over the tank, switched on and the temperature was set at 60°C.

3.5.4.2 Sample loading

The heater and power were switched off and the sample was loaded (35 μl) after mixing with 5 μl of 6X loading dye. The power was switched on; the heater and the pump were also switched on. As soon as temperature 60°C was attained, the electrophoresis unit was switched on. Gels were run for 6.5 hours at 200 V in 0.53 TAE buffer at a constant temperature of 60°C or 16 hours at 75 V. After electrophoresis run was completed, the power was switched off. The core sandwich assembly was removed from the buffer tank. The gel sandwiches were carefully detached and the gel removed.
3.5.4.3 Viewing the Gel

The gels were stained in MilliQ (Millipore B. V., Etten-Leur, Netherlands) water containing 0.5 mg of ethidium bromide/liter and destained twice in 0.53 TAE buffer prior to UV transillumination. The bands separated were observed in a gel documentation system (BioRAD, USA) and the images were taken using image lab software.

3.5.4.4 Analysis of DGGE profiles

DGGE gel images were analyzed with Image lab software from BioRad (ver. 4.65). 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 numbers of operational taxonomic units (OTU) in each sample were counted as number of DGGE bands. Gels were cross-checked visually as well for number of bands per lane. An intensity matrix was constructed based on the relative contribution of the band to the total intensity of the lane. DGGE bands were detected and transformed into a presence/absence binary matrix. Cluster analysis of DGGE bands based on square root transformed community data matrix through Bray-curtis similarity were performed with PRIMER v.6 software package (Plymouth Marine Laboratory).

3.6 Phylogenetic Analysis of AOB

3.6.1 Excision of bands and re-amplification

The documentation of the DGGE gel was followed by printing of the gel picture. The bands were carefully marked and labeled. The gel was placed on a UV transparent acrylic plate. The UV lamp was switched on and unique bands were excised with a sterile surgical blade. The UV lamp was immediately switched off in order to minimize the damage to the DNA bands in the gel. The gel pieces were transferred to the labeled 1.5 ml micro-centrifuge tube with 25 μl of sterilized deionized water. The tubes were incubated at 4°C for overnight. The tubes were spunned for about 30 seconds. The supernatant was aspirated and stored. About 2 μl of the supernatant was used as a template for re-amplification using the same...
primers but without GC clamp. PCR was performed at similar condition mentioned in the DGGE PCR. The resulting PCR products were run on 1.5% agarose gel for assessment of quality and quantity (as mentioned above).

3.6.2 Cloning of PCR product

The PCR product was cloned using TOPO®- Vector System as per the manufacture’s instruction and contains following steps:

1. TOPO vector and salt solution (1 μl each) were added to 4 μl of fresh PCR product. The mixture was incubated for 30 minutes at 24°C after gently mixing, centrifuged for a few seconds after incubation to collect the contents at the bottom.

2. Frozen JM109 high efficiency competent cells were taken from storage and placed in an ice until just thawed (about 5-30 minutes). Mixed the cells by gently flicking the tube, as the competent cells were extremely fragile excessive pipetting was avoided.

3. Ligation reaction of 5 μl each was transferred to 50 μl One Shot® Chemically Competent E. coli cells taken in 1.5 ml microcentrifuge tube on ice and mixed gently. Another tube on ice with 0.1 ng uncut plasmid also set up for determination of the transformation efficiency of the competent cells was. The tubes were gently flicked and placed on ice for 30 minutes.

4. After 30 minutes incubation, heat-shocked the cells for 45-50 seconds in water bath at exactly 42°C (without shaking) and immediately returned the tubes to ice for 2 minutes.

5. LB broth (950 μl) of room-temperature was added to the tubes containing cells transformed with ligation reactions and 900 μl to the tube containing cells transformed with uncut plasmid and incubated for 1.5 hours at 37°C with shaking (~150rpm).

6. Each transformation culture of 100 μl was plated on to duplicate kanamycin added LB plate. For the transformation control, a 1:10 dilution with SOC medium was plated. Incubated the plates overnight (16-24 hours) at 37°C.
7. The colonies were counted and picked for further analysis after 8-12 hours. The bacterial colonies were streaked for single colony on LB plates containing 100 μg ml⁻¹ kanamycin and incubated at 37°C.

8. A single colony was isolated and inoculated into 5 ml of LB containing 100 μg ml⁻¹ kanamycin and grown overnight with shaking at 37°C.

9. Mixed 0.85 ml of culture with 0.15 ml of sterile glycerol and transferred into a cryo-vial and stored at -80°C for long term storage. The remaining broth was pelletized and this pellet was used for plasmid extraction.

3.6.3 Plasmid extraction

Triplicate positive clones were selected at random and plasmids were extracted using plasmid extraction kit. Extracted plasmid was quantified by measuring absorbance at 280 nm using Nanodrop Spectrophotometer (Nanodrop Technologies, USA) and diluted to concentration of 200 ng μl⁻¹.

3.6.4 Sequencing PCR

Genes in the plasmids were amplified using the vector primers M13F-GTAAAACGACGGCCA and M13R-CAGGAAACAGCTATGAC. Amplification was carried out in a 10 μl reaction mixture. Each mixture containing 200 ng of template, 10 pM of primer and 1 μl of Big dye. The PCR conditions used were: initial denaturation at 95°C for 30 seconds, 25 cycles consisting of denaturation at 95°C for 15 seconds, annealing at 65°C for 15 seconds and elongation at 72°C for 30 seconds and final extension at 72°C for 5 minutes. The PCR are products were clean up using PCR clean up kit as per manufacturer’s instructions and the PCR products were then sequenced (Sci genome, Cochin).

3.6.5 Phylogenetic tree

Sequence data obtained were analyzed and edited using Sequencher V4.10.1 (Gene Codes). Subsequently, the sequences were compared with those in the Gen Bank 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 neighbour-joining tree using the software MEGA (version 5.0). Bootstrap analysis was carried out using 1000 iterations.

**Submission of sequences in GenBank**

The partial 16S rRNA gene environmental clone sequences obtained from this study were deposited in the Genbank database (Genbank accession numbers KM386955 to KM386977).

**3.7 Estimation of Activity**

**3.7.1 Nitrification rate**

Chemical inhibitor method was used for determining the nitrification rates in the water column of the CE followed by the protocol of Bianchi et al. (1994).

**Principle:** Nitrification rates were obtained by measuring the increase or decrease of nitrite concentrations in subsamples containing allylthiourea (ATU) or sodium chlorate (NaClO₃), the well-known inhibitors of the oxidation of ammonium and nitrite, respectively.

**Protocol:** Five hundred ml of sample was dispensed into nine one liter capacity bottles and the bottles were divided into three sets as shown in Figure 3.2. First set was kept as control without any inhibitor, the second set received ATU, at 100 mg L⁻¹ final concentration and the third set received NaClO₃ at final concentration of 10 mg L⁻¹. Preliminary experiments were carried out to prove that the inhibitor concentration was sufficient to inhibit the oxidation process before the actual analysis. Samples were incubated in dark for 36 hours at room temperature. Sub-samples (25 ml) were taken out at 6 hours intervals from 0 to 36 hours for analyzing the nitrite concentration. Ammonia oxidation activity was demonstrated by the increase of nitrite concentration in the triplicate receiving NaClO₃, as the increase in nitrite concentration in these bottles were only due to ammonia oxidation. The nitrite oxidation was demonstrated by the
decrease in nitrite concentration in the triplicate receiving ATU as the decrease these bottles were due to nitrite oxidation in sample of no further ammonia oxidation ay the action of ATU. Rates were estimated during the exponential phase of the nitrite decrease or increase and the results were expressed as μM N day⁻¹. All the analysis were performed in triplicate.

**Figure 3.2** Schematic representation of analytical approach used for measuring nitrification rate.

**3.7.2 Differential Contribution of AOA and AOB in Ammonia Oxidation**

Recovery of Nitrification Rate (RNR) analysis was used to understand the differential contribution of AOA and AOB in ammonia oxidation process in the CE using the protocol described by Taylor et al. (2010)

**Principle:** Chemical inhibitors and protein synthesis inhibiting antibiotics were used so that bacterial and archaeal ammonia oxidation were reversibly inhibited by acetylene which irreversibly inactivates the
ammonia monoxygenase enzyme. But upon the removal of acetylene, NH₃ oxidation could be resumed in ammonia oxidizers after a 1 to 2 hours delay by re-synthesis of the protein ammonia monoxygenase (AMO) as described by Hyman and Arp (1992) and the antibiotics that prevent protein synthesis could be used to discriminate between ammonia oxidation activities of AOA and AOB during the recovery of nitrification rate after acetylene exposure.

**Protocol:**

For assays of the recovered ammonia oxidation rate, 3 sets of 500 ml of water samples were dispensed in one L bottles with black phenolic caps fitted with gray butyl stoppers. A schematic diagram of experimental procedure is given in Figure 3.3. Another three sets of samples were also arranged at the same time for actual nitrification rate measurement as shown in the above section.

![Figure 3.3](image) Schematic representation of analytical approach used for measuring differential contribution of AOB and AOA in ammonia oxidation rate.

Acetylene was purged to the headspace for 6 hours (0.025%, vol/vol, or 0.025 kPa). Preliminary experiments were conducted to prove a 6 hours acetylene
exposure was sufficient to inactivate all NH\textsubscript{3} oxidation in the samples used in the study. Acetylene inhibition and recovery steps were carried out at 32°C. These conditions in the absence of any enzyme inhibitors were considered to be the standard. Samples incubated without any acetylene purging was considered as nitrification rate controls and water samples were amended with sodium chlorate (20 ppm) to inhibit the conversion of nitrite to nitrate. Acetylene removal after purging was achieved by placing the bottle under vacuum and degassing for 10 minutes. After degassing, all bottles were incubated with caps loosened to permit aeration. Aqueous solutions of antibiotics and nitrification inhibitors were added to the samples after acetylene was displaced. Preliminary experiments were carried out with a range of concentrations of the antibiotics to determine their minimum effective concentrations. The water-soluble bacterial protein synthesis inhibitors kanamycin and gentamicin (final concentration 750 µg ml\textsuperscript{-1} each) were added to inhibit bacterial protein synthesis. Water soluble eukaryotic protein synthesis cycloheximide (final concentration 650 µg ml\textsuperscript{-1}) was used for protein synthesis in AOA. Nitrite accumulation was monitored at 6 hours interval for 36 hours following standard spectrophotometric technique as given above. The values were expressed as µM N day\textsuperscript{-1}. All the analysis were performed in triplicate.

3.8 Statistical Analysis

Following statistical analysis was performed using suitable software package in order to better explanation of microbial and environmental data sets. The data were normalized by log transformation before analyses.

3.8.1 Karl Pearson’s correlation

Karl Pearson’s correlation was used for understanding the statistically significant relationship or associations between environmental variables, microbial abundance and nitrification activity.
3.8.2 Three-way ANOVA

The environmental and bacterial abundance data were subjected to statistical analysis for significant variation across the sampling period, over the study stations and between surface and bottom along with their first order interactions using three-way ANOVA. Thereafter with student’s t test for paired comparison between stations and between months to estimate the significance of the spatial and temporal variations of these parameters along with biological parameters with SPSS, V13 software (Jayalakshmy, 1998).

3.8.3 Canonical Discriminant Analysis (CDA)

CDA was performed for the discrimination between stations based on (1) environmental data and activity and (2) based on environmental parameters and abundance data for ecologically identifying the station locations in the CE. The classification success of the discriminant analysis was checked using jackknifed cross-validation (SPSS Inc., 1999) technique. To visualize the station differences, first and second factors discriminant scores were plotted.

3.8.4 Principal components analysis (PCA)

PCA was carried out for environmental variables and AOB and NOB abundance and also for environmental parameters and nitrification rate. All the variables were normalized and analysis was done based on the correlation matrix using the statistical program PAST version 2.02. The biplot was drawn according to the correlation biplot of (Legendre 1998).

3.8.5 Step up multiple regression model (SMRM)

The significance of the environmental and biological parameters in influencing the bacterial abundance were also determined with step up multiple regression model with interaction effects SPSS, V13, (Jayalakshmy, 1998) after normality testing and applying the appropriate transformation using Tuckey’s test of additivity (Federer 1968).

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