Chapter 3.
Materials and Methods
3.1. Description of the study area

The Mandovi and the Chapora are two tropical estuaries lying in close geographic proximity on the west coast of India (Figure 2). The hydrological characteristics of these two estuarine systems are governed by the monsoon regime. The physical characteristics of the Mandovi and the Chapora estuaries have been described by Varma and Rao, 1975; Varma and Cherian, 1975; Murthy et al, 1976. Based on the environmental characteristics the Mandovi estuarine system is classified as a tide dominated coastal plain estuary and geomorphologically identified as drowned river valley estuaries [Murty et al, 1976]. The estuarine channel of the Mandovi is used to transport large quantities of ferromanganese ores from mines located upstream to the Murmagao harbor (Arabian Sea), while the Chapora is free from the movement of ferromanganese ore bearing barges. Lush mangrove vegetation fringes both the estuarine systems.

To study the influence of metal contamination on nitrification process, two sites were selected (Figure 2). The control site which is relatively free and less affected from metal pollution is located at Tuvem in the Chapora estuary at 15° 38.28' N and 73° 47.71' E. The experimental site which is exposed to enrichment of metal ores is located at Diwar in the Mandovi estuary at 15° 30.42' N and 73° 52.28' E. The Mandovi estuary is longer and the estuarine system is complex. The river has its origin from the Parwa Ghat of the Karnataka part of Sahyadri hills and joins the Arabian Sea through Aguada Bay, after traversing a stretch of about 70 km. In both the estuaries the pre- and post-monsoon flow is regulated.
Figure 2: Location of sampling sites in the Chapora and the Mandovi estuary.
by the semi-diurnal tides. The position of the stations was fixed using the
determined Global Positioning System (GPS) (Magellan GPS NAV 5000™, USA).

3.2. Sampling programme and sample processing

Field observations were carried out for a period of one year at both the
control and experimental sites from April 2005 to March 2006. Sampling was
carried out on monthly basis during the low tide period. The study period was
grouped according to three distinct seasons based on the south west monsoon,
namely Pre-monsoon (February to May), Monsoon (June to September) and
Post-monsoon (October to January).

Sediment cores were collected from fringing mangrove areas along the
Chapora (control site) and the Mandovi (experimental site) estuaries (Figure 2). A
PVC hand-held sediment corer was used to retrieve sediment cores of 12-15 cm
in length and 8 cm diameter. The cores were transported to the laboratory in cold
condition for analyzing physico-chemical and microbiological parameters, taking
necessary precautionary measures. In the laboratory, sub samples were taken at
2 cm intervals from surface to 10 cm, by carefully sectioning the core with a
sterile blade in a laminar flow.

Water samples from the adjoining streams were collected for measuring
salinity, temperature and dissolved oxygen. Water samples were also collected in
clean carboys for preparing bacteriological media.
3.3. Analytical techniques

3.3.1. Hydrological 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 was likewise measured using a hand refractometer (ATAGO 2442-W01) calibrated to zero with distilled water.

3.3.1.2. Dissolved oxygen

The dissolved oxygen (DO) concentration in the water samples was estimated using Winkler’s titrimetric method [Carpenter, 19651. Water samples were collected in 125 ml acid washed (10% HCl) glass-stoppered bottles and fixed immediately with 1 ml of manganous chloride (3M) and 1 ml of alkaline-iodide (8M-4M) solution (Winkler’s reagents). Samples were mixed well and the precipitate was allowed to settle. In the laboratory, 1 ml of sulphuric acid (10N) was added to dissolve the precipitate and the samples were titrated with 0.01N sodium thiosulphate using starch as indicator. The procedure was standardised by using potassium iodate. Results are expressed as ml l⁻¹.

3.3.2. Ambient nitrogen concentrations

3.3.2.1. Pore water extraction

Extraction of interstitial waters is usually done with pressure—operated squeezes or centrifugation. In the first method, the sediment core is crushed mechanically under high pressure to expel the water [Manheim, 1966; Kriukov and Manheim, 1982; Zimmermann et al, 1978; Bender et al, 1987; Nath et al,
1988]. Alternatively the pressure is generated by passing an inert gas through the core that displaces the pore water [Reeburg, 1967]. In the centrifugation method, the sediment core is placed in a tube and centrifuged at high speed to expel the pore water, which then is siphoned off. High vacuum suction has also been used to recover pore waters [Manheim, 1966]. An advantage of these methods is that since the sections of the core are well defined, it would be possible to obtain profiles of distribution of elements.

Pore water in this study was collected by centrifugation method. After sectioning the cores at 2 cm interval, each fraction was made into slurry with a known volume of saline and then loaded separately into centrifuge tubes. The tubes were spun at low R.P.M (5000) at 4 °C for 10 minutes (REMI Cooling Centrifuge). The water was then carefully siphoned out into a pre-cleaned 100 ml polyethylene bottle and allowed to stand for 15 minutes in cold conditions in order to sediment out the coarse particles. Further, the diluted pore water was filtered on GF/F and then subsequently filtered on 0.22 μ membrane filter. The filtrate was stored in cold (~4°C) until further analysis.

By spinning at low temperature and RPM it was made sure that minimal disturbance was caused to the benthic organisms, which on lysis could change the pore water chemistry. The advantage of using this technique was that it enhanced the possibility of profiling without compromising much on changes arising during the handling. Thus the measurements of nitrogen concentrations in interstitial waters or the rates of nitrogen transformation were least affected by external factors associated with handling.
3.3.2.2. Ammonium

In the method employed here [Koroleff, 1969] dissolved ammonia reacts with hypochlorite at basic pH to form a monochloramine and in the presence of phenol, forms indophenol blue colour. The reaction is catalysed by sodium nitroprusside and requires six hours for colour development at room temperature. Freshly prepared distilled water was taken as blank. Samples were analyzed every time with freshly prepared reagents. Ammonium chloride was used as a standard. The optical density was measured at 630 nm (precision: ±0.05 μM N-NH₄⁺ l⁻¹).

3.3.2.3. Nitrite

Nitrite was measured by the method described by Bendschneider and Robinson [1952]. In this method, nitrite reacts with sulphanilamide in an acid solution (pH <2) and the resulting diazo-compound reacts with N-(1-naphthyl)-1-ethylenediamine to form a highly coloured azo-dye. The optical density was measured at 543 nm (precision: ±0.01 μM N-NO₂⁻ l⁻¹).

3.3.2.4. Nitrate

The method of Wood et al [1967] was employed for measuring nitrate. The nitrate in the sample was reduced almost quantitatively to nitrite in a cadmium-copper column and the nitrite was measured by the method described earlier for nitrite (precision: ±0.1 μM N-NO₃⁻ l⁻¹).

An earlier method for nitrate measurement was that described by Morris and Riley [1963], where copper was used as the cathode instead of mercury. More recently, Jones [1984] has described an alternative method for nitrate...
reduction, in that reduction of nitrate is achieved by shaking of samples with spongy cadmium.

3.3.3. Bulk sediment parameters

3.3.3.1. Sediment temperature, pH and Eh

As soon as the sediment cores were brought to the laboratory the sediment temperature was measured at an interval of 2 cm by inserting a stainless steel temperature probe of a hand held traceable mini digital thermometer with a precision of 0.1 °C. At 2 cm interval pH and Eh were also measured using a digital pH/Eh meter (Thermo-Orion)) after calibrating it with the standard buffers of pH 4, 7 and 9.2. The calibration standard used for Eh was equimolar (M/300) solutions of potassium ferricyanide and potassium ferrocyanide in 0.1 M potassium chloride. The system has an Eh of 0.430 mV at 25°C [Zobell, 1946].

3.3.3.2. Total Organic Carbon

Total organic carbon (TOC) in the 2 cm sub-sample of core was measured by wet oxidation with chromic acid followed by titration with ammonium ferrous sulfate [El Wakeel and Riley, 1957] and expressed as percentage. This method has a precision of 0.01%.

3.3.3.3. Iron and manganese

Sediment cores sectioned at 2 cm intervals were prepared for metal analysis according to Balaram et al [1995]. Metal concentrations (Fe and Mn) were measured using a flame atomic absorption spectrophotometer (AAS, PerkineElmer Model 5000). The accuracy of the analytical procedures was
assessed using the certified reference material MAG-1 (USGS) that yielded results within the reference value range [Flanagan, 1967, 1976]. MAG-1 is a fine grained gray-brown clayey mud with low carbonate content, from the Wilkinson Basin of the Gulf of Maine. The collection site was approximately 125 km east of Boston, Massachusetts. The age of the sediment is Holocene, but probably includes reworked Pleistocene sediment from surrounding areas. Element concentrations were determined by cooperating laboratories using a variety of analytical methods. Certificate values are based primarily on international data compilations [Abbey, 1983; Gladney and Roelandts, 1987; Govindaraju, 1994]. USGS reports [Flanagan, 1967, 1976] provide background information on this material.

### 3.3.4. Tracer technique

Research on nitrogen dynamics in sediments using tracer techniques has been rather scanty till recently owing to the difficulties of handling of samples and analytical techniques. A majority of the $^{15}$N tracer studies conducted on sediments have only focused on ammonium regeneration rather than on the concurrent assimilation of nitrogen [Blackburn et al, 1988]. $^{15}$N isotopes dilution techniques employed for these studies were also extended to measurements of nitrification rates in marine sediments by [Koike and Hattori, 1978]. Several works on nitrification have since been carried out using these techniques [e.g. Henriksen et al, 1981]

Research on nitrogen cycling in mangrove sediments is rare, even using simple techniques such as colorimetry. In a study carried out in a Southeast
Asian mangrove forest, Kristensen et al [1988] examined the transformation and transport of inorganic nitrogen in the sediments. Nitrification was measured in aerobic sediment slurries as the accumulation of NO$_2^-$ after addition of chlorate. Chlorate inhibits NO$_2^-$ oxidation and results in its accumulation which can then be measured [Belser and Mays, 1980]. After the incubation period, the increase in NO$_2$ was measured spectrophotometrically [Strickland and Parsons, 1972].

3.3.4.1. Measurement principle for nitrification rates

One of the first direct measurements of nitrification rates in marine sediments employed $^{15}$N isotope dilution technique with sediment slurries [Koike and Hattori, 1978]. This approach involved addition of $^{15}$N-NO$_3^-$ to a mixture of sea water and sediment. This was incubated for 48 hours open to atmosphere but without shaking. The observed changes in concentration and atom% enrichment of NO$_3^-$ over time were then used to calculate nitrification rate. Other reports of nitrification in sediments using $^{15}$N include those of Chaterpaul et al [1980]; Jenkins and Kemp [1984] and Nishio et al [1983]. In the present study, nitrification rates were measured by the method of Schell [1978]. In this method, the nitrite in the sample is extracted as a dye (1-benzene-azo-2-napthol) by using an organic solvent like CCl$_4$.

3.3.4.2. Experimental protocol of nitrite extraction

Each 2 cm intact section of the sediment core was transferred to a beaker (500 ml capacity) and 250 ml of filtered estuarine water was added. Samples were then incubated with $^{15}$N-NH$_4^+$Cl$^-$ in the dark for 24 hours. At the end of the incubation period, samples were gently mixed and pre-screened through a 200
μm mesh net. Samples were then filtered onto Whatman GF/F filter pads (pre-ignited at 400°C for 4 hours) and 200 ml of the filtrate was recovered for the extraction of nitrite.

A part of the filtrate recovered (200 ml) was transferred to a separating funnel of 250 ml capacity and unlabeled $^{14}$N-NaNO$_2^-\$ (1 cc = 1.25 N-Na$^+$NO$_2^-$ and 1 cc = 12 N-Na$^+$NO$_2^-$) were added to duplicate samples. Two concentrations of the vector were added to the filtrate as it was difficult to predict the nitrite concentration in the sample after incubation. This ensured that there was sufficient N for detection by emission spectrometry. The initial step involves the formation of a diazonium compound with 3 ml of aniline sulfate solution (5 ml/l of aniline sulfate in 1N HCl). After 5 minutes, 3 ml of β-napthol (5 g/l of β-napthol in 3N NaOH) was added to the separating funnel and the contents were well mixed. This resulted in the formation of a complex coloured compound - azo dye: 1-benzene-azo-2-napthol. The dye was then acidified with 1 ml of concentrated HCl to protonate the dye and allowing its efficient extraction. It was further extracted thrice using the organic solvent carbon tetrachloride (CCl$_4$).

In the first extraction, 5 ml of CCl$_4$ was added and the separating funnel was shaken vigorously for about 10 seconds. The phases were allowed to separate and the organic phase was drained into a clean, dry separating funnel taking care so as to avoid the passage of the organic film or to retain any traces of dye. The organic film present between the two phases is a potential source of organic nitrogen contamination and therefore was avoided. The subsequent extractions were carried out in the same way using 3 ml of CCl$_4$ each time. The
organic phases recovered at the end of each extraction were carefully pooled into a 10 ml beaker.

3.3.4.2.1. Isotopic analysis by emission spectrometry

There are two techniques for isotopic analyses of samples involving nitrogen tracers viz. emission spectrometry and mass spectrometry. Emission spectrometry was preferred since it is less complicated and requires no high vacuum for sample preparation; it requires nitrogen gas in the order of 0.2-10 μg in comparison to 30 μg – 3 mg for mass spectrometry and is less expensive. The precision of emission spectrometry is in the order of 0.01 atom % 15N compared to that of mass spectrometry, which is 0.001 atom% 15N.

3.3.4.2.2. Principle of detection

The 14N and 15N atoms in nitrogen gas are paired to form the nitrogen molecules 14N14N (28N), 14N15N (29N), 15N15N (30N). When an external energy source is supplied, the nitrogen molecules in the tube containing the sample get excited and on returning to the ground state, emit electromagnetic radiations of specific energy. These radiations are emitted in the ultra-violet region at different wavelengths (297.7 nm, 298.3 nm and 298.9 nm for 28N, 29N and 30N respectively). When the emitted light is resolved by a monochromator, the light intensities corresponding to the three wavelengths are detected by a photomultiplier-amplifier system and recorded as peaks. The measurement of the peak heights allows the 15N% abundance in the sample to be calculated as

\[
\%^{15}N \text{ abundance} = \frac{100}{2R+1}
\]

Where, \(R = \text{peak height of } ^{28}N / \text{peak height of } ^{29}N\)
3.3.4.2.3. Preparation of samples for emission spectrometry

Samples (particulate matter retained on the filters) were first processed by Kjeldahl digestion. The Kjeldahl method encompasses three steps: digestion, distillation, and titration. Digestion is accomplished by boiling the sample in concentrated sulfuric acid. The end result is an ammonium sulfate solution. This is further distilled by adding excess base (e.g., sodium hydroxide) to the digestion product to convert \( \text{NH}_4^+ \) to \( \text{NH}_3 \). Further, \( \text{NH}_3 \) is recovered by distilling the reaction product. Back titration with sulfuric acid quantifies the amount of ammonia in the receiving solution. The amount of nitrogen in the sample is then calculated from the quantified amount of ammonia ion in the receiving solution. In the present study, at the end of the titration step, a few drops of 0.01N standard HCl solution were added to the flask containing the distillate to make it acidic. The distillate was then evaporated to dryness by placing the flask at low temperature in the oven. The residue in the flask was re-dissolved in 10 ml deionized water and transferred to a stoppered tube (5 ml capacity). A second washing was necessary to remove all the traces of the residue. The tubes were dried at low temperature until the contents reached dryness, after which the PON was recovered in a known quantity of deionized water in such a way that 1 \( \mu l \) contained 1\( \mu g \) PON. Finally, the sample in each tube was withdrawn by capillary action into capillary tubes (5 cm in length) and these were placed on a rack and dried in the oven at low temperature.
3.3.4.2.4. Conversion to nitrogen gas

The determination of $^{14}$N/$^{15}$N isotope ratios is carried on nitrogen gas generated from a sample. Hence for the analyses, it is necessary to convert the nitrogen atoms in the sample to molecular nitrogen gas. There are three methods by which this conversion can be achieved: a) Kjeldahl-Rittenberg method; b) modified Dumas method or c) Kjeldahl-Dumas method.

In the present study, the modified Dumas method was used for conversion of bound nitrogen to nitrogen gas. Samples containing the extracted dissolved nitrogen (nitrite from nitrification experiments) were directly processed by the modified Dumas combustion method. This involves the dry combustion of organic and/or inorganic nitrogen with an oxidant, copper oxide resulting in the complete reduction of ammonia to molecular nitrogen. The reaction takes place in vacuum in a sealed tube. The vacuum is necessary so that there is no dilution of the $^{15}$N in the sample with atmospheric nitrogen. Vacuum is also necessary for discharge of the tubes (4 torr). The capillaries were transferred to discharge tubes (volume = 4 cc) with an upper constricted end, and approximately 15 mg of copper oxide was added. Since the nitrogen content of the sample in the discharge tube should yield a pressure of around 4 torr, it is necessary to know the approximate nitrogen content of the sample to at least ± 20%. The total N content required depend on the volume of the discharge tube and was estimated to be approximately 26 µg. The discharge tubes were connected to a vacuum line in a vertical position and evacuated until the vacuum exceeds $10^{-3}$ (atmospheric pressure). At a time, eight tubes could be evacuated on the vacuum system. The
adsorbed gases on the inner walls of the tubes were removed by heating with a hand torch. After degassing the tubes while monitoring with a vacuum gauge, the tubes were carefully sealed at the constricted end with a hand torch. The conversion of sample nitrogen to nitrogen gas was then carried out by combusting the evacuated discharge tubes in the muffle furnace at 500°C for 6 hours followed by cooling to room temperature. The other gases produced during the combustion i.e. CO₂, H₂O and oxides of nitrogen were frozen out with liquid nitrogen, allowing the nitrogen gas in the tubes to be analyzed for its ¹⁵N content by emission spectrometry. This was done in a Jaco NIA-1 N-15 analyzer. Although the light intensity of the discharge is the highest at a pressure of 1.5 torr [Sommer and Kick, 1965], the discharge lasts longer when more nitrogen is present, and a more stable discharge is obtained at 4 torr. Enrichments were determined using a calibration curve (Figure 3) made using standards provided by the manufacturer. A detailed account on analyses of ¹⁵N:¹⁴N ratios have been documented by Fiedler and Proksch [1975].

The discharge tubes, before being connected to the vacuum line were washed with chromic acid, rinsed well with deionised water and heated in a muffle furnace at 500°C overnight to remove all traces of nitrogen. These were then wrapped in aluminium foil and stored in a desiccator. Copper oxide was also pre-heated at 500°C and stored in a desiccator.
Figure 3: Calibration curve for measurement of isotope ratios

\[ y = 1.0041x - 0.1016 \]

\[ R^2 = 0.9998 \]
3.3.4.2.5. Calculations

Nitrification rates were calculated using equations developed by Schell [1978].

Initial atom% excess of $^{15}$N in NO$_2^-$ in the dissolved fraction,

$$15^\text{NDI} = [(0.000365 \times N_c) + (0.95 \times T_c) + (0.00365 \times A_c) \times 100] - 15^\text{Na}$$

$$\text{TN}$$

Final atom% excess of $^{15}$N in NO$_2^-$ in the dissolved fraction (NDF),

$$15^\text{NDF} = 2 [(15^\text{Nex} \times (N_c + V_c) - 15^\text{Na} \times V_c) - 15^\text{Na}]$$

$$N_c \quad N_c$$

Where,

- $N_c$ = Ambient nitrite concentration (µg at N l$^{-1}$)
- $V_c$ = Vector concentration (µg at N l$^{-1}$)
- $T_c$ = Tracer concentration (µg at N l$^{-1}$)
- $A_c$ = Ambient ammonium concentration (µg at N l$^{-1}$)
- $\text{TN}$ = Total nitrogen concentration ($N_c + T_c + A_c$)
- $15^\text{Na}$ = Natural abundance of $^{15}$N (0.0365%)
- $15^\text{Nex}$ = Atom% excess of $^{15}$N in the azo dye fraction

Since it is measuring uptake of $^{15}$NH$_4^+$ into $^{15}$NO$_2^-$,

$$V \, (h^{-1}) = \frac{15^\text{NDF}}{15^\text{NDI}} \times \frac{1}{T}$$

Where, $T$ = incubation duration (24 hours)

Nitrification rate, $R$ (µg at N l$^{-1}$ h$^{-1}$) = $(V \times N_c) \times 1000$
3.4. Bacteriological techniques

3.4.1. Total and viable bacterial counts

As soon as the sediment cores were brought to the laboratory it was sectioned in sterile condition at 2 cm intervals up to a depth of 10 cm. The sub-samples were serially diluted to $10^{-2}$ with physiological saline and were fixed immediately with buffered formalin for total bacterial counts. Bacterial abundance was determined by acridine orange direct count method [Hobbie et al 1977]. Sediment dilutions (2 ml) preserved with 2% (final concentration) buffered formalin were stained with acridine orange (Hi-Media, Mumbai) (final concentration 0.01% w/v) for five minutes before filtering it through 0.22 μm black Nucleopore track etched membrane filter (Whatman). Samples were enumerated at 1250x magnification in an Olympus (BH) epifluorescence microscope, using a 515 nm barrier filter and at least 10 fields of >30 bacteria field$^{-1}$ were counted. Bacterial abundance was expressed as numbers per g wet weight of the sediment. Direct viable counts were made following the method of Kogure et al [1984]. Serially diluted sediment samples were incubated at 28± 2 °C for 6 h with nalidixic acid (0.02% w/v), piromidic acid (0.001% w/v), pipemidic acid (0.01%w/v) (Sigma, USA) and yeast extract (0.01%). Incubation was terminated by the addition of buffered formalin (final concentration 2%). Samples were stained with acridine orange (as mentioned for Total Counts) and observed under epifluorescence microscope. Only enlarged and elongated cells were enumerated as viable bacterial cells and their counts are expressed as numbers per g wet weight of the sediment.
3.4.2. Most Probable Number method

Nitrifiers were also enumerated by the most probable number (MPN) method of Alexander and Clark [1965] on both inorganic and organic media. Inorganic nitrifying media (seawater amended with ammonium chloride of 2 mM final concentration) was distributed in 5 ml quantities in 15 ml screw capped tubes. The organic nitrifying media was prepared by adding 0.01% final concentration of glucose to the inorganic nitrifying media. From each dilution, ranging from $10^{-1}$ to $10^{-6.7}$, 500 µl was inoculated in triplicate in both the nitrifying media (inorganic and organic) until the inoculation from the highest dilution into the culture tubes yielded negative results. The culture tubes were incubated in the dark for a period about 60 days at 28(±1) °C. After incubation, the tubes were tested for the presence of NO$_2^-$ and/or NO$_3^-$. The combinations of positive and negative tubes were scored and MPN was assessed from McCready's table [Rodina, 1972].

3.4.3. Plate counts

Sub samples of approximately 5 g wet weight sediment from each of 2 cm sediment core were sampled using sterile syringe cores. The sub samples were transferred to 45 ml of full strength sterile seawater ($10^{-1}$ dilution). Tween80 (50 µl) was added and the mixture was sonicated at 40 mHz for 10 seconds. Serial dilutions of the sediment samples were made in autoclaved seawater to yield dilutions from $10^{-1}$ to $10^{-6}$ to $10^{-7}$. Medium for the isolation of heterotrophic bacteria was prepared using nutrient strength of 25%, which correspond to 25% nutrient broth + 2% agar. A concentration of 100% corresponds to 8 g nutrient broth
(HiMedia Laboratories Pvt. Ltd., Bombay, India) per 1000 ml seawater. Medium for the enumeration and isolation of nitrifying bacteria was prepared with a mineral medium which is essentially a modified Winogradsky medium [Rodina, 1972] with pure agar (Difco) as gelling agent. The medium was substituted with ammonium chloride at 2 mM (final concentration). About 100 μl from $10^{-2}$ dilution was plated onto each medium. Inocula from positive MPN tubes, representing both the inorganic and organic enrichments from various sections were plated on respective solid medium. Bacterial counts in the form of colony forming units (CFU) formed on the medium were recorded after a 15-day incubation period at $28\pm1^\circ$C. Bacterial colonies were enumerated and is expressed as colony forming units (CFU) per g wet weight of sediment.

3.4.4. Isolation and purification techniques

Representative cultures of nitrifying bacteria representing different colony morphologies on the nitrifying plates were isolated. Each isolate represented a definite fraction of the plate count having similar colony and cell morphology. These were transferred to plates with nitrifying medium and upon growth they were checked for purity on the basis of microscopic examination. The isolates were stored in nitrifying medium slant tubes at 4 °C for taxonomic identification and characterization.

3.4.5. In vitro measurement of nitrification activity of the isolates

Nitrifier isolates were checked for their activity (ammonium oxidation and nitrite oxidation) in both inorganic and organic nitrifying media [Section 3.4.2 for media composition]. Aliquots of various media were dispensed in sterile,
disposable microplates. Further a 100 µl of the culture suspension in saline of 0.1 OD$_{600}$ was inoculated into the wells. The cultures were incubated in the dark for a period about 10 days at 28±1 ºC. After incubation, the wells were tested colorimetrically (qualitative) for the fall of NH$_4^+$ as well as the rise and fall of NO$_2^-$. Care was taken to have uninoculated controls wherever possible. Further, based on the fall of ammonium as well as the rise/fall of NO$_2^-$ with respect to the controls, the cultures were grouped into three viz. 1) ammonium oxidizers [AO], 2) nitrite oxidizers [NO] and 3) coupled ammonium-nitrite oxidizers [CAoNo]. Based on visual (colorimetric) observations on the changes in levels of ammonium and nitrite in the microplates with respect to controls, irrespective of whether the activity was noted in inorganic or organic nitrifying media, ten isolates each of AO, NO and CAoNo were selected for detailed kinetic studies.

For preparing the inoculum, the isolates were grown in aged offshore seawater amended with 0.01% glucose for a period of 120 hours. The cells were then spun at 8000 RPM at 4 ºC and washed with physiological saline and suitable aliquots were used as inocula for subsequent experiments. Growth of all the isolates was monitored over a period of 120 hours at an interval of 24 hours under various experimental conditions. Direct cell counts for bacterial growth measurements were made using a cell counting chamber. Growth of AO, NO, CAoNo cultures were monitored during ammonium oxidation, nitrite oxidation and coupled ammonium-nitrite oxidation, respectively. The media used for each isolate was same one in which it was tested positive. [Section 3.4.2 for media composition].
Kinetics of ammonium, nitrite, and coupled ammonium-nitrite oxidation by batch cultures of various isolates in their respective medium [Section 3.4.2 for media composition] were monitored for a period of 120 hours under 28±1°C and at 1 atm pressure in triplicate. Ammonium concentration at the beginning of AO and CAoNo experiments was ~20 μM NH₄⁺-NI₁⁻¹. For nitrite oxidation experiments, an initial concentration of ~2 μM NO₂⁻-Ni¹ was maintained. Incubations were done in dark and changes in the level of ammonium, nitrite and nitrate were monitored as previously described (section 3.3.2.2 to 4) along with the increase in cell numbers at an interval of 24 hours for a period of 120 hours.

3.4.6. Phenotypic characterization of isolates in BIOLOG plates

Since, in the present study all the thirty isolates were Gram negative, the Biolog plate used was Biolog GN2 MicroPlate. The Biolog GN2 MicroPlate is designed for identification and characterization of a very wide range of aerobic gram-negative bacteria. Biolog's MicroPlates and databases were first introduced in 1989, employing a novel, patented redox chemistry. This chemistry, based on reduction of tetrazolium, responds to the process of metabolism (i.e. respiration) rather than to metabolic by-products (e.g. acid). Since the GN2 MicroPlate is not dependent upon growth to produce identifications, it provides superior capability for all types of gram negative organisms: fermenters, non-fermenters, and fastidious organisms all are identified in a single panel. The Biolog GN2 MicroPlate performs 95 discrete tests simultaneously and gives a characteristic reaction pattern called a "metabolic fingerprint".
Bacterial cultures (thirty isolates) showing enhanced nitrification activity (Section 3.4.5) were grown on the respective medium of isolation. However, use of Biolog Universal Growth w/5% Sheep Blood agar media enhanced the speed of growth of some strains. Further, the isolates were swabbed from the surface of the agar plate, and suspended in GN/GP Inoculating Fluid (Biolog); 150 µl of bacterial suspension was pipetted into each well of the GN2 MicroPlate. The MicroPlate was then incubated at 30°C for about 48 hours and then read using a microplate reader (BMG Optima).

3.4.7. Biochemical Characterization

The physiological and biochemical properties were examined according to standard methods of Gerhardt et al [1981]. General cell morphology was studied under an Olympus inverted microscope using young cultures of the strains grown on mineral media plates.

3.5. Molecular techniques

3.5.1. Fluorescence In Situ Hybridization (FISH)

Determining the structure and dynamics of bacterial communities is a core component of microbial ecology. Fluorescence In Situ Hybridization (FISH) with rRNA-targeted nucleic acid probes is a molecular tool for rapid and cultivation independent monitoring of phylogenetically defined bacterial populations in environmental samples. rRNA molecules are ideal target molecules for detection of prokaryotes because they are ubiquitously distributed, contain conserved and
variable sequence regions and are naturally amplified within microbial cells as integral parts of the ribosome.

In the present study, the sediment cores collected during the months of April (Pre-Monsoon), July (Monsoon) and October (Post-Monsoon) were sectioned at 2 cm intervals in sterile conditions to obtain representative samples up to 10 cm depth. Sub samples were re-suspended in phosphate-buffered saline (1x PBS), pH 7.4, consisting of 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄ per liter of distilled water. The samples were fixed with 4% paraformaldehyde (in 1x PBS) for one hour at room temperature. The samples were then washed twice with 1x PBS by centrifuging for 5 minutes at 4°C at 10000 RPM. This was done to remove residual paraformaldehyde. The fixed samples were suspended in a solution of 50% PBS and 50% ethanol and stored at -20°C. Before the commencement of FISH, aliquots of the samples were subjected to ultrasonic cleaning for dispersing cells adhered to sediment particles. For FISH, 10 µl of this sample was applied on a gelatin coated glass slide and dried for 1 h at 46°C, and subsequently dehydrated in solutions of 50%, 80% (vol/vol, in 10 mM Tris-HCl, pH 7.5), and 96% ethanol for 3 minute each. To start hybridization, 10 µl of hybridization buffer (with a composition dependent on the used probe) (Table 1) with 1 µl of fluorescently labeled probe (at a concentration of 50 ng/µl) were applied on the dehydrated sample and when necessary an unlabeled competitor (at 50 ng/µl) was added to the mixture (eg. when probed for Nitrobacter spp.). The samples were hybridized at 46°C for 1 h in a humidified chamber. Following hybridization, a stringent washing step was
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<th>Probe</th>
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<th>Flurochrome</th>
<th>Target organisms</th>
<th>Conc*</th>
<th>Formamide (%)</th>
<th>NaCl (M)</th>
<th>Tm (°C)</th>
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<tr>
<td>EUB338</td>
<td>GCTG CCTCCC GTAGG GT</td>
<td>Alexa fluor 350</td>
<td>Bacteria</td>
<td>0</td>
<td>0.9</td>
<td>55</td>
<td></td>
<td>Amann et al [1990]</td>
</tr>
<tr>
<td>Nso1225</td>
<td>CGC CAT TGT ATT ACG TGT GA</td>
<td>Fluorescein - isothiocyanate (FITC)</td>
<td>Betaproteobacterial ammonia-oxidizing bacteria</td>
<td>35</td>
<td>0.08</td>
<td>50</td>
<td></td>
<td>Mobarry et al [1996]</td>
</tr>
<tr>
<td>NIT3</td>
<td>CCT GTG CTC CAT GCT CCG</td>
<td>Fluorescein - isothiocyanate (FITC)</td>
<td>Nitrobacter spp.</td>
<td>40</td>
<td>0.056</td>
<td>55</td>
<td></td>
<td>Wagner et al [1996]</td>
</tr>
<tr>
<td>CNIT3</td>
<td>CCTGTGCTCCAGGCTCCG</td>
<td>Competitor to NIT3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Wagner et al [1996]</td>
</tr>
</tbody>
</table>

*Concentrations presented as percentage of formamide in hybridization buffer or molar concentration of NaCl in wash buffer.

Table 1: Probes used for FISH and the corresponding hybridization and washing conditions
performed for 10 min at 48°C in a buffer with the appropriate NaCl concentration (Table 1). Care was taken to include positive control for all the probes. All oligonucleotide probes were obtained from Molecular probes (Invitrogen). Microscopic observations were made with an Olympus BX51 microscope, equipped with mirror units U-MWU2, U-MWB2 and U-MWB2. Digital images were taken with an Olympus DP-70 12.5 million-pixel cooled digital color camera. Details on the fluorochrome used for each probe is given in the Table 1. A detailed version of this method was reported by Egli et al [2003]. The type cultures, *Escherichia coli* (ATCC:9637), *Nitrosomonas europaea* (ATCC:19718) and *Nitrobacter winogradkyi* (ATCC:25391) were used as positive control for the probes EUB338, Nso1225 and NIT3 respectively.

3.5.2. Bacterial taxonomy – 16SrDNA based identification

Five cultures each showing enhanced ammonium oxidation, nitrite oxidation and coupled ammonium-nitrite oxidation capability were checked for purity and further identified. A brief protocol for the same is given below:

16S rDNA sequencing based bacterial identification can be divided into three phases: i) extraction of bacterial DNA, ii) amplification of 16S rRNA gene by polymerase chain reaction (PCR) and iii) DNA sequencing of the amplified product and phylogenetic analyses of the sequence.

Bacterial DNA was extracted by repeated freezing (-80°C) and heating (at 95°C) of the bacterial cells for 30 min each in Tris buffer at pH 8. The cells were centrifuged at 10,000 x g for 10 min at 4°C to separate the cells. To the
supernatant, few microlitres of proteinase K, DNAase free RNAase and SDS were added and incubated at 37°C for half an hour. Further extraction and precipitation was done following Sambrook et al [1989]. An approximately 1500 bp-segment of the 16S rRNA gene was amplified by PCR with a forward primer pA (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer pH' (5'-AAGGAGGTGATCCAGCGCA-3'). The PCR cycle involved denaturing of the strand at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 30 seconds, primer annealing (for 60 seconds at 55°C), extension (for 90 seconds at 72°C) and denaturing at 95°C (for 1 min). A final extension for 10 min at 72°C was carried out before the amplified sequences were loaded on 0.8% agarose gel and separated by gel electrophoresis. The gel was then immersed in ethidium bromide for 2 h and the products were detected using a transilluminator. The PCR products were then purified. DNA sequence and phylogenetic analyses were carried out using a DNA sequencing machine (Applied Biosystems). The sequences obtained were aligned and compared with those available in the Genebank of the National Centre for Biotechnology Information (NCBI), USA [Altschul et al 1990].

3.6. Experiments – factors influencing nitrification

Sediment cores from both the control and experimental sites were collected during the post-monsoon season (October) as described earlier [Section 3.2]. Each sediment core was transferred to a separate sterile beaker (500 ml capacity) and 250 ml of autoclaved estuarine water was added to form
For dissolved oxygen amendment studies the beaker capacity was 1000 ml and the volume of autoclaved estuarine water used was 600 ml. In the present study ammonium [section 3.6.1.], nitrite [section 3.6.2.], nitrate [section 3.6.3.], organic loading [section 3.6.4.], dissolved oxygen [section 3.6.5.], liquid hydrocarbon - diesel [section 3.6.6.], pesticide - chloropyrifos [section 3.6.7.] and fertilizer - NPK 80:40:30 [section 3.6.8.] were used as amendments in the slurry described above to study their potential impacts on nitrification rates. All the amendments were made at three final concentrations (levels) as given in sections 3.6.1-3.6.5 with controls (unamended). After amendments adequate care was taken to gently mix the slurry to homogenize the contents.

For all the three levels of amendments and the control, three sets of incubations were carried out viz., - 1) Nitrification was inhibited by adding Allyl thio urea to a final concentration of 86 µM [Ginestet et al, 1998], 2) Denitrification was inhibited by adding Chloramphenicol to a final concentration of 300 mg L$^{-1}$ [Brooks et al, 1992] and 3) Sub-samples of third set were monitored without adding any inhibitors. All incubations were closed (without exchange of water or other nutrients) and conducted in the dark at 28±2$^\circ$C. Flow chart for the experimental set up is shown in Figure 4.

The initial concentrations of ammonium, nitrite and nitrate in the pore water were analyzed. Further, all the experimental and control systems, except for dissolved oxygen amendment studies [section 3.6.5] were sampled at 2-day interval for 20 days. For dissolved oxygen amendment studies, samples were collected at 24-hour intervals for 8 days. A poly vinyl chloride (PVC) cylinder (2.5
Sediment core (Control/Experiment)

Preparation of slurry with core in a 500ml sterile beaker with 250ml sterile estuarine water

Amendment 1 (eg. 50 μM N-NH₄⁺I of slurry)
Amendment 2 (eg. 250 μM N-NH₄⁺I of slurry)
Amendment 3 (eg. 750 μM N-NH₄⁺I of slurry)
Control (Unamended)

Nitrification blocked system
Denitrification blocked system
Unblocked system

Figure 4: Flowchart of protocol for experiments designed to understand the influence of various abiotic parameters on nitrification rates
cm diameter and 10 cm height) was pushed into the sediment of the microcosms where it remained till the end of the experiment. Inside this PVC cylinder a second cylinder of polymethylmethacrylate (1 cm diameter and 10 cm height) was pushed and used to take a sediment core. In this way the sediment profile in the system was not disturbed. Pore water was extracted [as mentioned in section 3.3.2.1] and concentrations of ammonium [as mentioned in section 3.3.2.2], nitrite [as mentioned in section 3.3.2.2] and nitrate [as mentioned in section 3.3.2.2] were measured. Variations in the abundance of nitrifiers were monitored in the incubations where denitrification was blocked as previously describe in section 3.4.2. Only the rates are presented in the results section.

3.6.1. Ammonium

In addition to the ammonium present in the pore water at the beginning of incubation, ammonium was added to the system as ammonium chloride solution at three different concentrations viz, ~50, 100 and 150 μM N-NH₄⁺ l⁻¹ of slurry.

3.6.2. Nitrite

In addition to the nitrite present in the pore water at the beginning of incubation, nitrite was added to the system as sodium nitrite solution at three different concentrations viz, ~5, 15 and 20 μM N-NO₂⁻ l⁻¹ of slurry.

3.6.3. Nitrate

In addition to the nitrate present in the pore water at the beginning of incubation, nitrate was added to the system as sodium nitrate solution at three different concentrations viz, ~10, 25 and 50 μM N-NO₃⁻ l⁻¹ of slurry.
3.6.4. Dissolved organic carbon

Glucose is one of the most assimilable forms of organic carbon having immediate impact on growth and metabolism of microbes in the experiments defined in the previous section [3.6]. Hence glucose was added to the system at three different concentrations viz. 10, 25 and 50 mg glucose-C l\(^{-1}\) of the sediment slurry.

3.6.5. Dissolved oxygen

For altering the levels of dissolved oxygen in the overlying water of the sediment slurry, differential aeration process was employed. One set was left as it is without any aeration till the end of the experiment (8 days). The second set was aerated with a one-hour on/off cycle and the third set was aerated continuously till the end of the experiment. Aeration was done using commercially available aerators and care was taken to switch off the aerators approximately one minute prior to sediment sampling.

3.6.6. Liquid hydrocarbon

Commercial diesel (density of 0.820 kg/L at 15\(^{\circ}\)C) was used to study the effects of liquid hydrocarbon on nitrification rates. Diesel was preferred in the study as it is used as fuel in fishing trawlers, barges and other vehicles used for water transport. The different concentrations at which diesel was used were 10, 50 and 100 mg/g wet weight of the sediment.
3.6.7. Pesticide

Chloropyrifos is a toxic crystalline organophosphate insecticide that inhibits acetylcholinesterase and is being used to control insect pests. It is one of the most widely used organophosphate insecticides in India. Chloropyrifos used in the present study has a minimum purity level of 94% and exists as a semi solid mass. The different concentrations at which the pesticide was used were 10, 50 and 100 ppm of the sediment slurry.

3.6.8. Fertilizer

The fertilizer used in the present study is NPK 80:40:30, which contain ~53% nitrogen, 27% phosphates and 20% potassium. The different concentrations at which the fertilizer was used were 1, 5 and 10% w/w the sediment in the slurry.

3.7. Statistical tools

Measures of Central Tendency, Analysis of Variance (ANOVA), and Correlation coefficient were done using the statistical package of Microsoft Office Excel 2003 and Multiple Regression Analysis was done using SPSS for Windows (Release 7.5.1). Guidelines for interpretation of statistical data were obtained from Bailey [2004].