2.1 Description of Study Area

Cochin estuary, the largest estuarine system in the southwest coast of India, is a part of the Vembanad-Kol wetlands (09°40' 12" - 10°10'46"N and 76° 09' 52" - 76°23'57"E), included in the Ramsar Site (No.1214). This estuary is topographically divisible into two arms; a southern one extending from barmouth to Thanneermukkam and a northern one extending from Cochin barmouth to Azhikode. This tropical aquatic system is under the profound influence of the monsoon, which contributes to nearly 71% of the annual rainfall (Jayaprakash 2002) and constantly prevailing three seasonal conditions viz. monsoon (June-September), post-monsoon (October-January) and pre-monsoon (February-May). Tides at Cochin are a mixed semi-diurnal type, with the maximum spring tide range about 1m (Srinivas 1999), resulting in incomplete flushing. The existence of the abundant mangrove vegetation of these regions has been shrinking in area due to land reclamation and developmental activities. The Cochin estuary has been shrinking in its area from 315 km² in 1912 to 256 km² in 1980 (Gopalan et al. 1983).

The estuary is experiencing increased anthropogenic interventions since the last five decades. Indiscriminate reclamation, urban development,
discharge of untreated and partially treated sewage, industrial effluents, increased shipping activities and siltation are the major threats to this sensitive ecosystem. As per the Environmental Protection Act in 1986, the Cochin estuary has been classified under the ‘Ecologically Sensitive Zone’. The brief description of the sampling stations are given below,

**Station-1 (ST-1): Barmouth**

ST-1 is located near the Barmouth, Cochin estuary (Figure 2.1) and it is the gateway of the Arabian Sea. Intense traffic due to boating and shipping activities is taking place in this region with the regular dredging for the uninterrupted movement of ships. Water flow rate is high in this region. Average depth in this region is 14-16 m.

**Station-2 (ST-2): Bolgatty Island**

The sampling point is situated nearer to the Bolgatty Island, where river Periyar joins the estuary. Newly built International Container Terminal and marina for leisure craft are close to this station. As compared to the first sampling station, the depth of the sampling site is less (around 3-5 m).

**Station-3 (ST-3): Sulphur jetty**

This station is situated near to the Sulphur berth, which is exclusively used for the sulphur transport. Imports of sulphur via large ships are taking place and sulphur transportation to the destination by large barges taking place and making it an active zone.
Figure 2.1 Map of Cochin estuary showing sampling locations
Station-4 (ST-4): Shipyard

India’s one of the largest ship building and repairing facility is located near to this sampling station. On the opposite side of shipyard the ship repairing facility of Indian Navy is also located.

Station-5 (ST-5): Thevara Bridge

Station 5, where fresh water from Vembanad Lake split by Willington Island, is taking place at this point.

Station-6 (ST-6): Mattancherry Fishing harbour

This station is known for its largest fleet of fishing boats along the west coast of India and has 8-10 m average depth.

Station-7 (ST-7): Indian Oil Corporation -Oil storage facility

Station 7 is located near the storage facility of petroleum products. Also it contributes the berths for passenger ships and is placed in the northern tip of Willington Island.

Station-8 (ST-8): Kumbalam

This station is near to Kumbalam island and is situated to the southern part of the Cochin estuary where the river Chithrapuzha joins the estuary.

Station-9 (ST-9): Poothotta

Poothotta is the middle part of Vemband Lake and is a coastal village in Ernakulam, Kerala. It is the home town for the Poothotta Coir Vyavasaya (Industrial) Co-operative Society, a government owned firm involved in the manufacture of coir products. The place is located below
the sea level. It features an impressive system of dykes and bunds to facilitate paddy cultivation. Adjacent to this station there is a boat terminal, which connects the districts Ernakulam to Kottayam and Alappuzha.

**Station-10 (ST-10): Murinjapuzha Outlet**

Ittipuzha is another river which falls in the Cochin estuary. The samples were taken at the discharge point of the river.

**Station-11 (ST-11): Ittipuzha Outlet**

This river also flows into the Cochin estuary and samples were taken from the discharge point.

**Station-12 (ST-12): Thanneermukkam Bund**

This station is situated at the southern part of Vembanad Lake. A salinity barrier (bund) is built near to this station to prevent the intrusion of saline water during post-monsoon season, and thereby protecting the paddy field from saline water flow. Samples were taken from near the bund. Thanneermukkom Bund was constructed in 1974 and was functional since 1976. It is the largest mud regulator in India. This barrier essentially divides the lake into two parts - one with brackish water perennially and the other half with fresh water fed by the rivers draining into the lake. This barrier has helped the farmers in Kuttanad, where farming is done below sea level. However it has created ecological problems, primarily, the rampant propagation of the Water Hyacinth in fresh water. The backwaters were abundant with fish as a part of the staple food for the people residing in this region. The salt water barrier has caused deterioration of the catch of fish in their region and the fishermen are opposed to the bund from 2005 onwards.
Chapter -2

Station-13 (ST-13): Panavally

Panavally is an island in the middle part of Vembanad Lake. Samples were taken from the Boat jetty situated near the island. As there is no bridge, connecting this island from main land, boats are the means of transportation for people residing in this area.

Station-14 (ST-14): Perumbalam

Perumbalam is another island which is bigger than Panavally which is located in the middle part of Vembanad Lake. The samples were taken from the shores of boat jetty. Mussels collection from the estuary and Lake are the major activity along this place. Machines are deployed in this place to collect mussels from bottom.

2.2 Methodology

Sediment samples were taken from the same 14 stations described above which spread across the Cochin estuary on May 2007 to May 2008. Van Veen grab (0.042m²) sampler was used to collect the surface sediments from these estuarine stations. Samples were transported to the laboratory in ice box and stored in a deep freezer (-20⁰C) until analysis. All the analyses were carried out in triplicates and the average results were reported.

2.2.1 General Sedimentary Parameters

The sediment textural characteristics (sand, silt, and clay) were determined by pipette analysis (Krumbein and Pettijohn 1938) after removing the inorganic carbonates using 10% HCl and organic matter by 30% H₂O₂. This analysis is based on Stoke’s law. Sediment was dispersed
Materials and Methods

for overnight with sodium hexametaphosphate. It was then wet sieved through a 63 µm sieve to collect the sand fraction. The mud fraction was divided into silt and clay fractions by the timed gravimetric extraction of dispersed sediments (Folk 1974). Sediment samples were freeze-dried and finely powdered using agate mortar for further analyses. Powder X-Ray diffraction analysis was carried out to find the mineralogy of the sediments (Moore and Reynolds 1997). Total Carbon, Nitrogen and Sulphur were determined using Vario EL III CHNS Analyser. Sediment organic carbon was estimated by the method of El Wakeel and Riley (1956) and later modified by Gaudette and Flight (1974). The amount of Total Organic Matter (TOM) was obtained by multiplying the Organic Carbon (OM) values with 1.724 (Nelson and Sommers 1996).

The chloroplastic pigments (chlorophyll-a and pheoapigments) were analysed spectrometrically according to Lorenzen (1967). Pigments were extracted with 90% acetone (24 hr in the dark at 4°C). After centrifugation, the supernatant was used to determine the functional chlorophyll-a and acidified with 0.1N HCl to estimate the amount of pheoapigments. Protein (PRT) were determined after extraction with NaOH (0.5M, 4 h) as suggested by Hartee (1972). The absorbance was evaluated at 650nm using UV-VIS Spectrophotometer. Bovine albumin solutions were used as standard. Carbohydrates (CHO) were analysed according to Gerchav and Hacher (1972). This method is based on the same principle as the method of Dubios et al. (1956), and it specifically adopted for carbohydrate determination in sediments. The absorbance was measured at 490nm using Glucose as standard. Lipids (LIP) were extracted by direct elution with chloroform and methanol according to Bright and Dyer (1959). Absorbance
was measured at 367nm. Tripalnitine solutions were used as standard. Protein, carbohydrates and lipid concentrations were converted to carbon equivalents by using the following conversion factors; 0.49, 0.40, and 0.75 µg of C µg respectively (Pusceddu et al. 2000). The sum of total protein, carbohydrate and lipid carbon equivalents was reported as BioPolymeric Carbon (BPC). The protein to carbohydrate ratio (PRT: CHO) and lipid to carbohydrate (LIP: CHO) ratio were also calculated.

2.2.2 Trace Metals

1g of the dried finely powered sediment sample was repeatedly digested using 1:5 mixture of conc. HClO₄ and conc.HNO₃, and then evaporated to dryness (Loring and Rantala, 1992). The dry residue was dissolved in 0.1 HNO₃ and made up to 25ml. Further analysis was conducted by Atomic Absorption Spectrophotometer (Perkin Elmer 3110). The accuracy of the metal analyses was checked using triplicate analysis of a certified reference material (BCSS-1, National Research Council of Canada). The triplicate analysis of BCSS-1 showed a good accuracy and recovery rate.

2.2.3 Rare Earth Elements

The following sample preparation procedure was adopted for the dissolution of samples.

Open acid digestion method by Parijat Roy et al. (2007) was adopted. A test portion (0.5g) of the sample was added to each PTFE Teflon beakers. Each of the samples was moistened with a few drops of ultra-pure water. 10ml of an acid mixture (7:3:1 HF-HNO₃-HClO₄) was added to each sample. Samples were swirled until completely get moist. The beakers were
then covered with lids and kept overnight for digestion after adding 1ml of 5µg/ml Rhodium (Rh) solution which act as an internal standard and the beakers were heated on a hot plate at 200°C for about 1 hour, the lids were removed and the contents were evaporated to incipient dryness until a crystalline paste was obtained. The remaining residues were then dissolved using 10 ml of 1:1 HNO₃ –milli-Q water and kept on a hot plate for 10 minutes with gentle heat (70°C) to dissolve all suspended particles. Finally, the volume was made up to 250 ml and stored in polyethylene bottles. The samples were then analysed using the Perkin Elmer Sciex ELAN DRCII ICP-MS at National Geophysical Research Laboratory Hyderabad, India. MAG-1 is used as reference materials for REEs analysis. Triplicate analyses were performed and the average value was reported.

2.2.4 Antifouling Biocides

The analytical method for the antifouling biocide extraction was carried out by the below appended means:

For the organotin analysis the methods of Morabito et al. (1995), modified by Sangeeta et al. (2009) were followed. 2-5g wet/dry sediment is taken in a dry boiling tube. To this 15ml of 0.03% tropolone in methanol and 1ml conc.HCl were added. Tripropyltin chloride (TPrTCl) equivalent to 100 ng Sn were added as an internal standard. The samples were then vertexed for 1 hr to obtain organotins (OTs). It is then centrifuged at 2000 rpm for 10 mins and the supernatant (upper layer) were collected in a separating funnel. Repeated the vertexed for another 1hr with the same sample, centrifuged and collected in a previously used separating funnel. To this 20 ml dichloromethane and 100ml 5% NaCl were added and shaken for 15 min. The processes were repeated with another volume of dichloromethane and
NaCl for 15 min. Dichloromethane layer were collected after passing through anhydrous sodium sulphate (Na₂SO₄) to remove moisture. Immediately, 1ml iso-octane was added to dichloromethane layer to avoid evaporative loss. Then the volume was reduced to 0.2ml by passing N₂ or using rotary evaporator. Next 1ml pentyl magnesium bromide (Grignard reagent) were added carefully. Milli-Q water was added repeatedly until effervescence ceases. An extra volume of 1ml water was added, before the addition of Hexane (2ml) to this reaction mixture and repeated (2ml×2) twice. Hexane layer was pipetted out and passed it through the florisil or silica column (4g). 1:1 hexane-toluene mixture was used as the mobile phase. Sample was collected in vials and concentrated by using N₂ gas to 1ml. Finally injected into GC/MS.

For calibration, a standard mixture was prepared by adding 100µl of standard solution containing TBT (107ng Sn), DBT (103ng Sn), MBT (101 ng Sn), MPT (106 ng Sn), DPT (99.4ng Sn), TPT (102 ng Sn) and TPrT (105 ng Sn). Rest of the processes were similar to that has been done for sediment samples.

The GC-MS system (Shimadzu QO 2010) with electron impact ionization mode (70ev) was used for the analysis. The organotins were separated and detected using RESTEK Rtx-5MS capillary column (30m, 0.32mm i.d. 0.25 µm) and selected ion monitoring (SIM) mode. Two microlitres of sample or blank or standard mixture was injected using a programmable column injector. The injector was kept at 60°C for 1 min and then temperature was raised at 100°C min⁻¹ to 240°C, and maintained at the same temperature for the run time. Initial column temperature was 80°C. After 2 min, the oven temperature was programmed to reach 280°C at
10⁰C min⁻¹. The interference temperature of the MS was maintained at 280⁰C. Helium was used as the carrier gas (3 ml min⁻¹). The total run-time was 24 min. Data analyse was done by data processing software Shimadzu GC-MS solution Version 2.21.

Quantification of organotins was performed by using TPrT as internal standard. Fresh standards were prepared to obtain calibration curves along with procedural blanks. The minimum detection concentration for butyltins and phenyltins is 0.2 ng Sn/g.

For biocides like Irgarol 1051, chlorothalonil and dichlofluanid, the method of Voulvoulis et al. (1999) were adopted. 50 g of dry sediment sample were taken in 250 ml Teflon bottles and spiked with 1ml working standard solution. 60 ml of acetone and 50ml of dichloromethane (6:5 ratio) were added. The bottles were shaken on a rotary shaker overnight and centrifuged at 2000 rpm for 10 min. The supernatant was filtered through a Buchner funnel and collected in a round bottom flask. Anhydrous sodium sulphate (Na₂SO₄) was added and the solution was reduced by volume using rotary evaporator. Dried with a gentle stream of nitrogen and the extract was re-dissolved in 60 ml acetone and transferred into a separating funnel. 120 ml of 2% sodium sulphate were added and the compounds were subsequently extracted with portion of petroleum ether (3×30ml). The ether fractions were combined and again reduced to incipient dryness. 1ml of acetone was then added to re-dissolve the residue which was transferred to a vial for further analysis by GC-MS (Agilent 6890). Separation was achieved on a fused silica capillary column (DB5). The sample was injected splitless with the injector temperature maintained at 260⁰C. The chromatographic temperature conditions were as follows; 70⁰C held for 3 mins, increased at 30⁰C /min to 120⁰C held for 1min,
then 15°C/min to 180°C, held for 3 mins and 25°C/min to 300°C final temperature, held for 15 min. Helium was used as carrier gas with a flow rate of 0.5 ml/min. The mass spectrometer was in the selected ion monitoring (SIM) mode at 70 eV ionization energy and scanned from 50 to 500 Dalton. The individual compounds were identified by comparison with authentic standards and interpreted by mass spectrometric fragmentation patterns.

For biocide Chlorothalonil, method of Voulvoulis et al. (1999) and Kazos et al. (2008) were adopted. 100 g of dried sediment sample were extracted with 60 ml of acetone and 50 ml of dichloromethane (6:5 ratio) for 17 hrs and centrifuged at 2000 rpm for 10 min. The supernatant was filtered through a Buchner funnel and collected in a round bottom flask. Sodium sulphate (Na₂SO₄) was added and the solution was reduced in volume by rotary evaporation. It was then taken down just to dryness with a gentle stream of dry nitrogen and then the extract was re-dissolved in 60 ml of acetone and transferred to a separating funnel. 120 ml of 2% sodium sulphate were added and the compounds were subsequently extracted with portion a petroleum ether (3×30 ml). The ether fractions were combined and again reduced to incipient dryness. 1 ml of acetone was then added to redissolve the residue which was transferred to a vial for further HPLC analysis.

Liquid chromatograph (Perkin Elmer 200 series) with a UV spectrometric detector operated at 242 nm was used to analyse Chlorothalonil. Separation was achieved with a C8 column preceded by a guard column of similar packing at 30°C. A gradient elution was carried out with acetonitrile (solvent A) and phosphate buffer solution 10 nmol, pH 5.0 (solvent B). The mobile phase composition was programmed as follows: initially 30% A, then a linear gradient to 70% A in 16 min, kept constant upto 18 min, then returning
linearly to 30% A in 20 min and equilibrated for 10 min. The flow rate was at 0.6 ml/min and the injection volume was 20 µl. The solvents were degassed by sonication. Data acquisition and data analysis were performed with the Total Chrom software package supplied by Perkin Elmer.

All solvents and florisil were purchased from Merck (Darmstadt, Germany). Gas chromatographic and HPLC standards for biocides were purchased from Sigma-Aldrich (USA).

2.2.5 Acetylcholine esterase Enzyme Assay

The method used was a combination of the modified method of Ellman et al. (1961) later by Dellali et al. (2001) and Bonacci et al. (2004).

Total soft tissues were homogenised at 4°C in a 1:5 ratio (w/v) of 0.1M phosphate buffer optimised at 12000×g for a period of 30 min, after which supernatant were obtained and immediately used for assay of AChE activity.

AChE activity was measured using acetylthiocholine iodide (ASChI) as substrate. ASChI is hydrolysed by AChE, producing thiocholine and acetic acid. The thiocholine released from the hydrolysis was made to react with 5,5’-di-thi-bis-2-nitrobenzoic acid (DTNB) yielding the yellow compound 5-thio-z-nitro-benzoic acid (TNB) which absorbs at 412 nm. 50 µl of supernatant was placed into a reaction mixture consisting of 850 µl phosphate buffer (0.1M, pH 7.5), 1.875 mM DTNB and 50 µl of 2 mM ASChI to start the enzymatic reaction. The optical density at 412 nm was measured using a spectrometer at regular intervals continuously for 30 minutes. The reaction time was maintained at 25°C throughout the period. AChE activity was expressed in nmol/min mg protein. The quantity of protein present in the supernatant was determined using the Bradford
method (Breadford 1976) with bovine serum albumin as the standard. Absorbance for protein determination was measured at 595 nm.

2.2.6 Statistical Techniques

All data were subjected to statistical analysis wherever necessary. Pearson correlation was employed to find out the inter relationship between different parameters. Principal Component Analysis was done to find out the factors contributing to different biogeochemical processes occurring in estuarine system. Cluster analysis was done to identify the relationship between sampling stations.

A) Enrichment Factor (EF)

EF is a good tool to determine the source of metal between anthropogenic or naturally occurring sources (Morillo et al. 2002; Adamo et al. 2005; Valdes et al. 2005). EF was calculated to determine whether the levels of metals in sediments were of anthropogenic origin (i.e. Contamination) or not. The geochemical normalisation was performed using Fe as the reference element for the following reasons (1) Fe is associated with fine solid surfaces; (2) its geochemistry is similar to that of many trace metals; and (3) its natural sediment concentration tends to be uniform (Daskalakis and O’connor 1995).

The following equitation was used to estimate the EF of metals from each sediment station using Fe as a normaliser to correct the differences in sediment grain size and mineralogy:

\[ EF = \frac{(M_{c}/Fe)_{sample}}{(M_{c}/Fe)_{average \ shale \ value}} \]

Where \((M_{c}/Fe)_{sample}\) and \((M_{c}/Fe)_{average \ shale \ value}\) are respectively, the metal concentration (mg/kg dw) in relation to Fe levels (% dw) in sediment
samples and average shale values taken from Turekian and Wedepohl (1961) respectively. EF values were interpreted as suggested by Brich (2003) where $\text{EF}<1$ indicates no enrichment; $<3$ is minor; $3-5$ is moderate; $5-10$ is moderately severe; $10-25$ is severe; $25-50$ is very severe; and $>50$ is extremely severe enrichment.

**B) Contamination Factor (CF)**

Various calculation methods for quantifying the degree of metal enrichment in sediments have been put forward. One of them is the Contamination Factor (CF) (Perkey et al. 2004; Muthu and Jayaprakash 2007). The CF, calculated as the ratio between the sediment metal content at a given station and the normal concentration level reflects the metal enrichment in the sediment:

$$\text{CF} = \frac{C_s}{C_b}$$

CF was classified into four groups in Hokanson (1980) and Perkey et al. (2004). When $\text{CF}<1$, there is no metal enrichment by natural or anthropogenic inputs; $1 > \text{CF} < 3$ for a particular metal means that the sediment is moderately contaminated by the element; $3 > \text{CF} < 6$ means that there is considerable contamination; and if $\text{CF} > 6$, then there is very high contamination for that metal. Taylor’s (1972) crustal average contamination of the trace metals for the background concentrations were used in this study.

**C) Geoaccumulation Index (I$_{geo}$)**

Geoaccumulation index ($I_{geo}$) was introduced by Muller (1969) and allows the determination of the sediment analysis with organic and inorganic pollutants comparing the present concentration with background levels. Concentrations of geochemical background are multiplied each time
by a factor of 1.5 in order to allow content fluctuations of a given substance in the environment and very small anthropogenic influences. Values of geoaccumulation index can be defined as follows;

\[ I_{\text{geo}} = \log_2 \left( \frac{C_n}{(1.5 \times B_n)} \right) \]

Where ‘\( C_n \)’ is the measured concentration of the heavy metal (n) in the examined bottom sediment and ‘\( B_n \)’ is the geochemical background value in the average shale (Turekian and Wedepohl 1961) of element ‘n’; 1.5 is the background matrix correction factor due to lithogenic effects. The index of geoaccumulation includes even grades from 0 (non-contaminate) to 6 (very strong).

**D) Cluster Analysis (CA)**

Cluster analysis (CA) is a multivariate technique, whose primary purpose is to classify the object of the system into categories or clusters based on their similarities. The objective is to find an optimal grouping for which the observations or objects within each cluster are similar, but the clusters are dissimilar to each other. Hierarchical clustering is the most common approach in which clusters are formed sequentially. Hierarchical clustering provides intuitive similarity relationships between any one sample and the entire data set, and is typically illustrated by a dendrogram/tree diagram (Singh et al. 2004; Shrestha and Kazama 2007). The most similar objects are grouped first, and these initial groups are merged according to their similarities. Eventually as the similarity decreases all subgroups are fused into a single cluster. The dendrogram provides a visual summary of the clustering processes, presenting a picture of the groups and their proximity, with a dramatic reduction in dimension of the original data. The Euclidean distance usually gives the similarity between two samples, and a
distance can be represented by the difference between analytical values from the samples. CA was applied using a single linkage method, the distances or similarities between two clusters A and B is defined as the minimum distance between a point in A and a point in B.

\[ D(A,B) = \min \{ d(y_i, y_j), \text{for } y_i \text{ in A and } y_j \text{ in B} \} \]

Where \( d(y_i, y_j) \) is the Euclidean distance in Equation

### E) Factor Analysis (FA)

Factor analysis (FA) is designed to transform the original variables into new uncorrelated variables called factors, which are linear combinations of the original variables. The FA is a data reduction technique and suggests how many variances are present in the data. Principal component method (PCA) is used for extraction of different factors. The axis defined by PCA is rotated to reduce the contribution of less significant variables (Richard and Dean 2002; Alvin 2002). This treatment provides a small but approximately the same amount of information as the original set of observations. The FA can be expressed as:

\[ F_i = a_{1}x_{1j} + a_{2}x_{2j} + \ldots + a_mx_{mj} \]

Where ‘\( F_i \)’ is the factor, ‘\( a \)’ is the loading, ‘\( x \)’ is the measured value of variable, ‘\( I \)’ is the factor number, ‘\( j \)’ is the sample number and ‘\( m \)’ the total number of variables.

Factor scores can be expressed as:

\[ Z_{ij} = a_{1}f_{1j} + a_{2}f_{2j} + \ldots + a_{m}f_{mj} + e_{ij} \]

Where ‘\( z \)’ is the measured variable, ‘\( a \)’ is the factor loading, ‘\( f \)’ is the factor score, ‘\( e \)’ the residual term accounting for errors or other sources of variation.
Chapter -2

Reference


Materials and Methods


Ecotoxicological Assessment of Antifouling Biocides in the Sediments of Cochin Estuarine System


