CHAPTER 2

MATERIALS AND METHODS
2.1. STUDY AREA:

2.1.1 Geographical Features

The state of Goa, having a coastline of about 100 kilometers, lies on the mid-eastern coast of India, between Lat 14°54' N - 15° 48' and Long. 73° 41' E - 74° 21’ E [Fig. 2(a)]. Among the seven rivers flowing through the plains and hills of Goa, Mandovi and Zuari are of major importance and are called the lifeline of Goa.

River Mandovi originates from the Parva Ghat of the Karnataka part of Sahyadri Hills and after traversing a stretch of about 70 km joins the Arabian Sea through the Aguada Bay near Panaji. Its width at the estuary mouth is about 3.2 km, while upstream it narrows down to about 0.25 km. Large number of tributaries join this river along its course which is characterized by a number of deltic islands. It is fed by monsoon precipitation from the discharge of a catchment area of about 1150 km². The Mandovi basin constitutes about 42% of the land area and covers about 1530 km² of the entire state. The occurrence of sandbar near the entrance of the Mandovi in the Arabian Sea has been known for centuries. The mechanism of sand transport, wave action and circulation of Mandovi estuary has been studied by Murty et al., (1976).

River Zuari originates in the Dighi Ghat of the Karnataka part of the Sahyadri Hills and after flowing a zigzag stretch of about 67 km joins the Arabian Sea at Mormugao- Dona Paula point. Its width at the mouth of the estuary is about 5.9 km while upstream it narrows down, and at the upper reaches the
width is less than 1 km. Zuari basin covers an area of about 973 km$^2$ and receives discharge from a catchment area of about 550 km$^2$.

These two rivers, Mandovi and Zuari are joined by Cumbarjua canal giving rise to a major estuarine system. The area covered by these two river basins is about 69% of the total basin area of the riverine system in Goa. There are a number of iron and manganese mines located along the banks of these two rivers from where bulk of iron ore is transported to Mormugao harbour through these rivers. The banks of both these rivers are provided with thick vegetation of mangrove forest. Geologically speaking, the Mandovi and Zuari river estuaries could be classified as drowned river valley estuaries formed due to the Holocene rise in the Sea level (Anon, 1978). The two river estuaries are rich in resources and are used for fishing activity, practically throughout the year and particularly during the monsoon months when sea-fishing gets suspended.

2.1.2 Climate:

In Goa normally three seasons prevail in a calendar year. They are premonsoon (February – May), southwest monsoon (June-September) and postmonsoon (October – January). The premonsoon season is the warmest period of the year and experiences occasional showers towards the end of May. The average relative humidity is 80%. This season is followed by the southwest monsoon (hereafter referred to as the monsoon season), during which the state receives most of its rainfall, with an average of about 3000 mm. The postmonsoon season is a fair and stable season. Normally, atmospheric temperature shows two peaks, one in October when warm and humid conditions
exist and second in May, which is usually the hottest month of the year. The temperature of seawater varies between 26.5 to 31.0°C. Heavy rainfall, freshwater runoff, sandbar formation in the mouth region of estuary occur during monsoon and is followed by recovery during postmonsoon and stability during pre-monsoon (Qasim and Sen Gupta, 1981). Salinity decreases considerably (3psu) during monsoon due to freshwater runoff and rainfall. The average annual freshwater runoff is 7 km³ for Mandovi and 9 km³ for Zuari estuaries (Anon, 1979). The estuaries may be classified as stratified during the monsoon season which gradually evolves towards a well mixed one during the post monsoon period, according to the definition given by Pritchard (1952). It’s pre and postmonsoonal flows are regulated by the semi-diurnal tides having amplitude of 2-3 m (average of 2.3m) during spring tides. The currents are mainly influenced by tides during monsoon. Maximum distance of penetration of seawater (0.9 x 10⁻³) is about 67 km away from the mouth in May, which comes down to a minimum distance of about 10-11 km in July – August. The estuarine complex is fringed with extensive mangroves (Wafar et al., 1997), which are biologically productive nursery grounds for a variety of commercially important fin and shellfish (Parulekar et al., 1980; Qasim and Sen Gupta, 1981). Earlier work on benthos (Parulekar et al., 1973; Parulekar and Dwivedi, 1974; Parulekar et al., 1975, 1980; Harkantra and Parulekar 1981, 1985) revealed high benthic production consisting of clam beds, polychaetes, other molluscs etc. These estuaries are extensively used for fishing, aquaculture, ore transport, harbour development, water recreation, waste disposal and adjacent land for human
settlements (Parulekar et al., 1980; Qasim and Sen Gupta, 1981; Parulekar et al., 1986).

In Zuari estuary, tides of mixed semidiurnal type with a minimum range of about 2-3 m are encountered causing the exchange of appreciable amount of saltwater into the system from the adjacent sea, the rate of which varies considerably with season (Cherian et al., 1975). During the pre and post-monsoon period the flow is regulated by the tides of semi-diurnal type like that of Mandovi. The freshwater discharge into this estuary during the monsoon season is high. However, the amount of freshwater received by Zuari is less as compared to Mandovi estuary. During post and premonsoon period, the estuary, to a distance of about 14 km is primarily tide-dominated due to meagre or negligible freshwater runoff. Maximum distance of penetration of water of $0.9 \times 10^{-3}$ salinity is reported to a distance of about 65 km away from the mouth during the month of May. It gets reduced to a minimum of about 20 km during June-July following the onset of monsoon. The tidal influence has been recorded upto 41 km.

2.1.3 Station Position:

Six stations, three in Zuari estuary (Z1, Z2, Z3) two in Mandovi (M1, M2) and an offshore station, A, were selected based on the salinity and sediment characteristics [Fig. 2(a)]. The positions of the six stations were determined by Global Positioning System (GPS) (Magellan GPS NAV 5000™, USA).

Station A: is located at $15° 28' .655$ N; $073° 44' .024$E. it is the offshore station having a mean depth of 15 m and lies at the converging area of Mandovi and
Zuari estuaries in the Arabian Sea. The salinity on an average remains 34.45 psu (± 0.83) due to its location in the marine environment. The sediment here is clayey-silt.

**Station M1:** is located at 15° 30' .123N; 073° 49' .126E. This station remains saline most of the time due to its proximity to the sea, with salinity ranging from 9.1 – 32.5 psu (± 10.15). Mean depth at this station is 3.5 m and the sediment is mostly sandy, sometimes an admixture of sand and mud.

**Station M2:** is situated further upstream at 15° 30' .323N; 073° 52' .430E. This station also, like M1 remains saline most of the time, with a salinity range of 1.1-33.0 psu (± 12.79). The mean depth at this station is 3 m and the substratum is an admixture of sand and mud.

**Station Z1:** this station is located at the mouth of the Zuari estuary at 15° 25' .107N; 073° 51' .472E. This station remains saline most of the time with an average salinity of 32.79 psu (± 1.32). The substratum is predominately sandy having a mixture of silt for most of the year. There is an island (St. Jacinto) near this station from where the terrigeneous material also gets deposited. The average depth at this station is 3.5 m.

**Station Z2:** lies upstream from the mouth of the Zuari estuary at 15°25' .107 N; 073°51' .472 E and has a mean depth of 3.5 m. The salinity at this station varies from 20.5 – 33.0 psu (± 3.78). The substratum varies from sand to silty sand.

**Station Z3:** located at 15° 24' .640N; 073° 53' 680E. Salinity varies from 3.0 – 33.0 psu (± 10.34) throughout the year. The bottom is composed of mostly silt with varying combinations of sand and clay to give either sand-silt-clay or sandy-
silt or clayey-silt. A considerable amount of detritus is also found in the sediment that gets from the mangrove swamps. Mean depth at his station is 3 m.

2.1.4 Sampling:

The sampling program was carried out from October 1997 to September 1998. This was designed to cover the three seasons: postmonsoon, premonsoon and monsoon in a complete annual cycle. Monthly sampling was planned at each station. Six stations were sampled monthly except that stations A and Z1 were not sampled in June, July and August due to stormy weather.

There are numerous reports, which illustrate the methodology of benthic sampling and analytical techniques (Holme and McIntyre, 1971; Swartz, 1978), processing and interpretation of benthic data (Vilenkin, 1965; Clifford and Stephenson, 1975; Elliot, 1977) and environmental study (La Fond and Prasad Rao, 1968; Strickland and Parsons, 1972). In the present investigation benthic sampling and environmental parameters were studied using standard methods, which are being widely used.
2.2. DATA ANALYSES

2.2.1 Objective 1:

To study the influence of environmental variables on benthic macrofauna.

2.2.1(i) Macro fauna:

Duplicate samples were obtained at each station with 0.04 m² van Veen grab, having a penetration of 15 cm. The sediment samples from the grab were preserved in 10% Rose Bengal-seawater formalin. Later these sediment samples were sieved through 0.5 mm mesh sieve. Fauna retained on the sieve were transferred into a white enamel tray, half-filled with fresh water. All the stained animals were picked up by means of forceps and stored in transparent plastic bottles containing 5% formalin. Macrofauna were identified upto species level using the available key for polychaetes (Fauvel, 1953), molluscs (Satyamurti, 1956 & Kundu, 1965 a & b), crustaceans and other groups (Barnard, 1935; Gosner, 1971). Food and feeding habits of soft–bottom macroinverterbrates were ascertained from the literature (Fauchald and Jumars, 1979). Numerical abundance of each species was recorded under stereozoom microscope. Population density was converted into nos/m².

2.2.1(ii) Biomass estimation of macrofauna

The animals of different size groups were weighed on a microbalance (Sartorius BP 221 S, Germany). The shells of molluscs were removed and crustacean forms were treated with dilute hydrochloric acid (10%) until
effervescence. The animals were then removed and placed on a tissue paper until all the water was absorbed, and then weighed. Due care was taken for incorporating the weight of different size groups while extrapolating the total biomass.

2.2.1(iii) Grain size analysis:

About 25 gm of dried sediment samples was weighed accurately and transferred into a clear 250 ml beaker. The samples were made salt free by repeated washing using distilled water. Approximately 5 ml of 10% sodium hexametaphosphate solution was added to this salt free sediment and disposed overnight. Subsequently, the samples were wet sieved through a 62μm sieve. The sand fraction (62μm) retained on the sieve was dried and weighed. This was later used for separating the different sand fractions on a mechanical shaker. While the mud fraction was collected in a 1000ml beaker, transferred to a 1000ml measuring glass cylinder and subjected to pipette analysis (Folk, 1968), which is based on the classic formula for settling velocities provided by Stokes' law. Percentage distribution of sand, silt and clay fractions in each sample was determined and textured classification was made based on the grain size variation (Folk, 1968). Sand samples were further analysed for medium to very fine sand (Buchanan, 1984). The conventional phi notations (Φ) were used instead of the Wentworth scale. The Wentworth scale can be converted to the phi notation using the formula:

Φ = - log 2 of the particle diameter in mm
The mean grain size and standard deviation (sorting) were calculated by the graphical method (Folk, 1968) using the formulae:

Graphic mean = $16\,\Phi + 50\,\Phi + 84\,\Phi$

\[ \text{Graphic S.D} = \frac{84\,\Phi - 16\,\Phi}{3} + \frac{95\,\Phi - 5\,\Phi}{6.6} \]

(Sorting coefficient)

The analysis was carried out at monthly intervals.

2.2.1(iv) Total Organic Carbon:

Sediment samples were collected at every station and organic carbon was determined using auto analyzer, NCS 2500, Italy. 1 g of the dried (<60° C) and finely powdered sample was treated with dilute hydrochloric acid (1N) to remove all inorganic carbon. This treated sample was used for the analysis. Total organic carbon was expressed as $\mu$g/g.

2.2.1(v) Total Organic Nitrogen:

Similarly organic nitrogen was determined using auto analyzer, NCS 2500, Italy, on the same sediment samples used for organic carbon, simultaneously. Total organic nitrogen was expressed as $\mu$g/g.

2.2.1(vi) Chlorophyll a:

Approximately 1 g of the sediment was used to extract chlorophyll a (chl-a) by adding 90% acetone in the dark. The fluorescence was measured on a Turner fluorometer, USA. The fluorometer was previously calibrated with standard chl-a obtained from Sigma chemicals. Phaeopigments of the samples
were also estimated by acidification with dilute hydrochloric acid (1N). Similarly chl-a from the surface and bottom water was also estimated. 500 ml of seawater was filtered on Millipore GF/F filter paper using suction pump. The filter paper along with the retained material (filtrate was transferred to glass vials and covered with Al-foil and stored in refrigerator for extraction, for a period of around 18 hours. (Lorenzen, 1966). The results obtained are expressed in µg/g for sediment. Further, microphytobenthic carbon was calculated by converting chl-a concentrations to carbon content (C-chl-a) using a conversion factor 40 (De Jonge, 1980).

2.2.1(vii) Proteins:

Proteins were analyzed using the method of Lowry et al., (1951). Appropriate blank and standards (bovine serum albumin) were similarly treated and the absorbance was measured at 750 nm. The concentration was expressed in mg/g.

2.2.1(viii) Carbohydrates:

Carbohydrates were estimated following the method of Kochert, (1978). Appropriate blank and standards (glucose) were similarly treated. The absorbance was measured at 485 nm and the concentration was expressed as mg/g.

2.2.1(ix) Lipids:

Lipids were analyzed using the method of Parsons et al., (1984). Appropriate blank and standards (stearic acid) were treated similarly and the absorbance measured at 440 nm. The concentration was expressed in mg/g.
The principle of this method depends upon the oxidation of lipids by acid dichromate. The oxidation reaction is followed by a decrease in the dichromate colour.

Proteins, carbohydrates and lipids were converted into their carbon equivalents using the conversion factors 0.49, 0.40 and 0.70 respectively (Fabiano et al., 1995). To further investigate the nature of organic nitrogen, protein concentrations were converted to organic nitrogen (N-PRT) by using the conversion factor 6.25 (Fabiano et al., 1995) and were expressed as percentages of ON (N-PRT: ON).

2.2.1(x) Salinity of seawater:

The electrical conductivity ratio of seawater samples was measured in a Guildline “Autosal” model 8400A salinometer (measurement range: 0.005 – 42 psu). The salinity of the sample was calculated using the equation for conversion of conductivity ratio to salinity (Fofonoff, 1983). Low pressurized air forces the saline sample from the sample bottle and through the sampling element, which is called conductivity cell. The sample passes as a continuous flow through the conductivity cell and electrodes implanted in the cell initiate signals that are proportional to the sample’s conductivity. Using an internal preset electrical reference to produce an error signal, the instrument provides a numerical radiant, which corresponds in magnitude and direction to the error signal. The display reading provides a valid measurement value when the internal reference has been preset or standardized against a known external reference.
2.2.1(xi) pH of seawater:

Similarly, water samples from the surface and bottom layers were collected and the pH estimated using a pH meter, LI612, Elico Pvt. Ltd., India.

2.2.1(xii) Dissolved oxygen of seawater:

Bottom water was collected using a Niskin sampler and the oxygen estimated using the Winkler Method (Strickland and Parsons, 1972). Water was carefully collected in standard bottles with Winkler A and B in the field and subsequently titrated with sodium thiosulphate solution in the laboratory using starch as the indicator. The results are expressed in ml/l. A divalent manganese solution (manganous sulphate), i.e. Winkler A followed by a strong alkali (potassium iodide + sodium hydroxide), i.e. Winkler B is added to the sample. The precipitated manganous hydroxide is dispersed evenly throughout the seawater sample that completely fills a stoppered glass bottle. Any dissolved oxygen rapidly oxidizes and equivalent amount of divalent manganese to basic hydroxides of higher valency states. When the solution is acidified in the presence of iodide the oxidized manganese again reverts to the divalent state and iodine, equivalent to the original dissolved oxygen content of water, is liberated. This liberated iodine is titrated with standardized thiosulphate solution.

2.2.1(xiii) Statistical Analyses:

The data obtained from the 12-month study was subjected to statistical analyses. The mean values of various parameters were calculated along with the standard deviations as given in Elliot (1977).
2.2.1 (xiii) (a) Mean:

\[ \text{Mean (X)} = \frac{x}{n} \]

2.2.1 (xiii) (b) Standard Deviation:

\[ \text{Standard deviation (S.D)} = \frac{(x - x)^2}{n - 1} \]

Where ‘x’ is the observation and ‘n’ is the number of observations.

2.2.1 (xiii) (c) Species Diversity:

Species diversity was computed using the Shannon-Wiener Index (Pielou, 1975). This was originally proposed by Shannon (Shannon and Weaver, 1963).

\[(\text{Species diversity}) \ H' = - \pi \log_2 \pi \]

Where ‘\( \pi \)’ is the proportion of individuals belonging to ‘i’ th species and ‘s’ is the number of species. Species diversity has species evenness and a species richness component.

2.2.1 (xiii) (d) Species evenness:

Evenness (J) was computed as \( J = \frac{H}{\log_2 s} \) (Pielou, 1966)

Where \( H = \frac{H'}{H' \text{ max}} \) and ‘s’ the number of species.
Such method has been used by several authors like Sanders (1968), Heip and Engels (1974) and Parulekar et al. (1980).

2.2.1 (xiii) (e) Species Richness:

Species richness was calculated as suggested by Margalef (1958).

\[
\text{(Species richness) } SR = \frac{(s - 1)}{\ln N}
\]

Where 's' is the number of species and "N" is the total number of individuals in a collection.

2.2.1 (xiii) (f) Index of Dominance:

This was calculated as, Dominance (D) = J (evenness) - 1.

2.2.1 (xiii) (g) Two-way analysis of variance:

Two-way ANOVA tests were carried out to see any significant differences between stations and seasons. This was computed using MINITAB-Release 8.3 (MINITAB Inc., 1991). Further, one-way Tukey's HSD multiple comparison was used when significance was detected (P<0.05).

2.2.1 (xiii) (h) Linear Multiple Regression:

The best multiple linear regression models (Draper and Smith, 1981; Wiesberg, 1985) were used to assess the relative significant influencing environmental parameters on the benthic community structure such as species diversity, total wet biomass and total population and to construct the predictive
models. The three types of regression methods available; forward, backward and stepwise do not always agree on the best model, especially when multicollinearity exists. The regression explaining the greatest amount of variation with all the parameters coefficient significant were presented as the best fit based on maximum $R^2$ and minimum Mallows' Cp (Helsel and Hirsch, 1992). The total data of four sites were first anlaysed to give regression model. However, it did not give any significant regression and variation was very low. Moreover, ANOVA revealed significant differences of environmental parameters among the sites. Hence, it was decided to perform this analysis on the data set of each site separately.

All the raw data were log transformed into log $10(x+1)$ and percentage values were transformed into arcsine (Bakus, 1990) before any statistical analyses.

2.2.1 (xiii) (i) Cluster Analysis (CNESS):

A new faunal distance matrix, Chord Normalised Expected Species Shared (CNESS) was used for clustering and to study the species succession. The statistical programme COMPAH 96 (Combinatorial Polythetic Agglomerative Hierarchical Clustering) (Gallagher, 1996) was used for the purpose. The results were expressed in the form of dendrograms.
2.2.1(xiii) (j) Principal Component Analysis - Hypergeometric (PCA-H):

Principal Component Analysis – Hypergeometric (PCA-H) was used to examine the relationship among the macrobenthos and other parameters. Species versus month matrix was arranged for each sites as there was significant difference in environmental parameters and community structure such as Shannon-Weiner's diversity index, total population and wet biomass. Actual mean density data and species which has contributed more than 2% of the samples were considered for the analysis (Trueblood et al., 1994). Sample size (m) was determined as half of the total sample population.

2.2.2 Objective 2:

Evaluation of changes in benthic fauna due to organic enrichment

A section of a beach near site M1 if the Mandovi estuary, receiving domestic sewage through a ‘nullah’ was sampled from March 2000 – May 2000. Sampling areas each covering an area of 1 m² was sub sampled at 5 points (4 at the corners and 1 at the center of each point). The sampling areas were placed around 10m apart from each other from the low tide to the high tide zone (i.e. along the organic enrichment gradient). A total of 4 sampling areas were covered: 3 sample sites and 1 reference site.
2.2.2 (i) Macrofauna:

Macrofauna was sampled using a PVC core having an internal diameter of 20 cm and penetration depth of 15 cm. The sediment was preserved with 10% seawater Rose Bengal-formalin. Later the sediment was washed and the fauna retained on the sieve was transferred to an enamel tray from where they were picked and transferred to vials containing 5% formalin. This fauna was later identified using the keys for the literature mentioned in the earlier section of this chapter.

2.2.2 (ii) Biomass of fauna:

Wet weight of individual's species was estimated following the method described in the earlier sections.

2.2.2 (iii) Water characteristics:

Interstitial water was collected from the depressions formed as a result of the core insertion in the sediment for obtaining macrofauna. Water was analysed for salinity, pH, dissolved oxygen and BOD₅. All the parameters were analysed using standard techniques. Biological oxygen demand was estimated after 5 days in order to check the biological activity. Oxygen bottles were filled with seawater from the surface, capped and covered with a black paper or cloth and kept in a cool environment. After 5 days the oxygen from these bottles was fixed by adding sodium thiosulphate and was estimated by Winkler's method. The
difference in the amount of dissolved oxygen obtained was a result of the biological activity.

2.2.2 (iv) Sediment characteristics:

Sediment parameters such as temperature, grain size, organic carbon, chlorophyll a, were estimated using the standard methods explained earlier.

The data thus obtained was analysed for the Abundance Biomass Comparison (ABC) Curves (Warwick, 1986) and dendrograms using the statistical programme PRIMER-v5 (Plymouth Routines In Multivariate Ecological Research) (Clarke and Gorley, 2001). Also the Species Abundance Biomass (SAB) Curves (Pearson and Rosenberg, 1978) were drawn to summarise the changes in the basic faunal parameters occurring along the gradient of organic enrichment.

2.2.3 Objective 3:

Assessment of bioturbation activities

2.2.3 (i) Vertical trend of chlorophyll a in the sediment:

Station A was selected for the study as these stations had more of clayey substratum. This enabled us to obtain the core easily. Core samples were obtained by operating a gravity corer having a length of 50 cm and an internal diameter of 4.5-cm. After retrieval of the core, the temperature of the sediment...
was recorded and subsequently the core was sectioned at every 2 cm interval. All the samples were stored frozen until extraction. Later the sections were analyzed for chlorophyll a using the method explained in the previous sections of this chapter.

2.2.3 (ii) Nutrient flux experiments:

Microcosm experiments to compare the nutrient values in sediment-overlying waters were carried out following the method of Kristensen and Blackburn, 1987 to suit our experiments. The uppermost ~ 5 cm of the sediment surface was collected and sieved through a 500 micron mesh in order to remove the macrofauna and larger particles (shells and gravel). Simultaneously soldier crabs, *Dotilla myctiroides* having a carapace length of 120mm were collected (form Zuari estuary) and kept separate from the sediment. Tanks containing the sieved sediment to a depth of ~ 15 cm and the crabs were transported to the laboratory for further treatment. Since the objective of the study was to examine the flux rates, meiofaunal organisms present in the sediment were killed before the start of the experiment. These small animals were killed by freezing the sediment for 48 hours at -20°C before further use. Microscopy on sediment samples before and after the freezing procedure revealed that live larvae and meiofauna had disappeared following the treatment. After thawing, the sediment was homogenised by handmixing in the transport-tanks and compaction was allowed to proceed. Aquarium tanks of size 40x 25x 25 cms were used for the study. 8 individuals each were kept in two tanks, one containing sediment and
water (35 psu) and other containing only water. Similar tanks only with water, but without the animals, were kept as controls. Nitrate was estimated from water column (Grasshoff, 1983) after definite intervals of time.

2.2.3 (iii) Sediment reworking rate:

To study the sediment-reworking rate of the soldier crab, *Dotilla myctiroides*, an intertidal area of a small beach was selected. The crabs were monitored and the reworking rate (*in vivo*) was worked out according to Rowden and Jones (1993).
Fig. 2 (a) Map showing the six different study sites in the two estuaries, Mandovi and Zuari