Chapter 2

MATERIALS and METHODS
A brief description of the Cochin estuarine system and the various methods employed in the present study are given in this chapter.

**The study area**

The Cochin estuary located along the south west coast of India extends between 9°40’N and 10°12’N and 76°10’E and 76°30’E. It has a length of about 70 km and a width which varies between a few hundred meters to about 6 km and covers an area of about 250 km². The Cochin estuarine system is connected to the sea through a permanent opening, the Cochin barmouth, which is about 450 m wide and 10 to 13 m deep. Here the depth is maintained by dredging as this opening is used for navigational activities. This barmouth is also responsible for the tidal flux of the Cochin estuary. Though less important, two other openings also exist, which are only seasonal in nature, one at Azhikode (northern region) and the other one at Andhakaranazhi (southern region).
Two major rivers discharge freshwater into this estuarine system; the river Periyar and the river Muvattupuzha joining the northern and the southern parts of this estuary respectively. A multitude of industrial concerns punctuate the banks of the estuary. Saline water intrusion to southern parts of the estuary is regulated by the Thanneermukkam bund, a salt water barrier commissioned in 1975. The depth of the estuary varies moderately around 3m except for the shipping channel which is dredged periodically to a depth of about 13m. The tides are of semi-diurnal type with an average range of about 90cm.

The study area and the location of the sampling sites are depicted in Fig.1. The stations selected were clam habitats located in diverse environmental characteristics. The hydrographical parameters (pH, temperature, salinity, dissolved oxygen and suspended solids) determined by standard methods (Grasshoff, 1983 a and b) are presented in Table 1.pH was determined by using a Philips portable pH meter (model PP - 9046 with a glass electrode).

**Sampling procedure**

Monthly field collections spread over a period of 15 months (from October 1988 to December 1989) were carried out at all stations except Station 5 and 6 (located at the northern end of
Table 1. Hydrographical parameters of the sampling sites

<table>
<thead>
<tr>
<th>STATIONS</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature °C</td>
<td>29.4 ± 1.2</td>
<td>29.5 ± 1.3</td>
<td>29.8 ± 1.3</td>
<td>30.2 ± 1.3</td>
<td>30.1 ± 0.7</td>
<td>31.2 ± 1.1</td>
</tr>
<tr>
<td>Salinity %</td>
<td>7.2 ± 6.6</td>
<td>8.6 ± 7.4</td>
<td>5.4 ± 4.7</td>
<td>2.0 ± 1.7</td>
<td>23.0 ± 11.3</td>
<td>4.2 ± 4.3</td>
</tr>
<tr>
<td>Dissolved Oxygen ml l⁻¹</td>
<td>3.2 ± 0.5</td>
<td>3.8 ± 1.3</td>
<td>4.9 ± 1.6</td>
<td>4.9 ± 1.0</td>
<td>4.2 ± 0.6</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td>pH</td>
<td>7.0 ± 0.2</td>
<td>7.0 ± 0.2</td>
<td>7.0 ± 0.2</td>
<td>7.0 ± 0.2</td>
<td>7.3 ± 0.4</td>
<td>7.0 ± 0.0</td>
</tr>
<tr>
<td>Suspended Solids mg l⁻¹</td>
<td>23.7 ± 9.0</td>
<td>24.0 ± 14.2</td>
<td>16.1 ± 7.0</td>
<td>14.5 ± 8.9</td>
<td>23.8 ± 12.8</td>
<td>14.8 ± 7.9</td>
</tr>
<tr>
<td></td>
<td>10.4 ± 5.0</td>
<td>10.2 ± 5.6</td>
<td>4.2 ± 2.7</td>
<td>5.0 ± 3.6</td>
<td>11.3 ± 4.3</td>
<td>6.1 ± 3.4</td>
</tr>
</tbody>
</table>
Fig. 1 Map of Cochin estuary showing location of stations (1 - 6)
the estuary), where, owing to practical difficulties, only six monthly collections (between November 1988 and September 1989) were carried out. While Villorita cyprinoides var. cochinensis (Hanley) was sampled from all the stations except Station 5, Meritrix casta (Chemnitz) was the species sampled from Station 5. The clams were collected from the beds using a van Veen grab and were washed free of epiphytes and adhering sediments and were transported to the laboratory. Bottom water samples were collected from these stations using a pre-cleaned teflon Hytech water sampler and stored in pre-cleaned, acid-washed polyethylene bottles. A stainless steel, plastic-lined van Veen grab was used to collect sediment samples and aliquots were carefully transferred to polyethylene bags which were stored at -5°C till analyses were performed.

Chemical analyses

All glassware used for the analyses were soaked in 5N nitric acid and thoroughly washed with distilled water before use. All the reagents used were of BDH-AnalaR grade, unless otherwise specified. Deionised, double-distilled water was used for the chemical analyses.

Water

The water samples were filtered using acid washed, 0.45 \mu m
Whatman membrane filters. The filtrate was used for the analyses of dissolved metals while the residue was used to estimate the particulate metal concentrations.

The dissolved metal concentration was estimated by the method described by Danielsson et al. (1978; 1982). The filtered water samples were subjected to solvent extraction using ammonium-1-pyrrolidine dithiocarbamate / diethylammonium diethyl dithiocarbamate / chloroform (APDC / DDDC / Chloroform) mixture. The extract was acidified with concentrated nitric acid and the metals (copper, cadmium, zinc, lead, and nickel) were brought into the aqueous phase by equilibration with a definite volume of water and then analysed on an atomic absorption spectrophotometer.

The particulate matter separated above was digested according to the APHA (1985) procedure. The dry residue in the membrane filter was leached with 10 ml of an acid solution (HClO₄, HNO₃ and HCl in the ratio 1:1:3) at 90°C for 6 hours. The resultant solution was centrifuged and then made up to 10 ml with 0.1N HCl for analysis on the AAS.

Sediment

Trace metals in the sediment were extracted according to the scheme depicted in the Fig.2. The extractants, the sequence
Fig. 2  Sequential extraction scheme
and the procedure followed were adapted from methods employed by Tessier et al. (1979; 1984) and Nair et al. (1991). The different metal species studied are:

- **Fraction 1** - Exchangeable cation fraction
- **Fraction 2** - Carbonate bound fraction
- **Fraction 3** - Easily reducible fraction (metals combined with Fe/Mn oxides)
- **Fraction 4** - Organically bound fraction
- **Fraction 5** - Residual fraction

Eight to ten gram aliquots of the wet sediment samples were weighed out into 250ml Erlenmeyer flasks and allowed to equilibrate with 50 ml of the extractant. The phases were separated by centrifugation. The supernatant liquid separated was analysed for trace metals (by AAS), whereas the residue was carefully washed back into the flask with the next extractant of the sequence and the operation repeated. The percentage of moisture in the samples were determined separately and were used to recalculate and express the metal concentrations obtained above on a dry weight basis. The total metal concentrations referred to (in Chapters 4 and 5) are the summation of the metal concentrations in the sequentially extracted fractions.
Bivalves

Sample pre-treatment

The individual clam collected from each of the Stations was washed free of attached epiphytes and adhering sediments and their dimensions noted. The shells were then opened and the soft tissue was separated from the shell and the shell-weight \( W_s \) recorded. Each shell was heated to 110°C overnight and the final weights were noted \( W_{sf} \).

Analyses of shells

The analyses of shells were carried out according to two schemes explained below:

(i) For the first part of the studies reported in Chapter 3 (pages 28 to 32) viz. attempts to ascertain the relationship between the metal concentration in shells and their respective shell weights, shells were analysed individually. The individual shell was warmed at 60°C for one hour in a 40% solution of 30 v/v hydrogen peroxide (Glaxo) so as to completely dissolve away the periostracum and any other attached organic matter. They were then washed well with distilled water and allowed to remain in 0.05M HCl for 2 - 3hr to strip off any surface adsorbed elements, periostracum residue or any surface contamination. They were then dried to
constant weight at 110°C.

Each of these shells was dissolved in the minimum amount of 0.5 M HCl added drop-wise. Any excess acid present was evaporated off and the residue redissolved and made up to a definite volume (dependent on the weight of the shell) in deionised, double-distilled water for subsequent metal analyses.

(ii) When the results of the above individual shell analyses revealed that the trace metal concentrations of bivalve shells were totally independent of shell-weights, it was decided that, henceforth, all shell analyses be carried out on four composite samples of shells, each sample being a powdered mixture of a minimum of four individual shells of varying weights. The values of metal concentrations reported in the latter half of Chapter 3 (i.e. for intercomparison of metal concentrations - Table 10) as well as in Chapters 4 and 5, are the mean of the concentrations of the four composite samples. For metal analyses, 0.5 g of each of these composite samples was dissolved in the minimum amount of 0.5M HCl added drop wise. The excess acid was evaporated off and the residue redissolved and made up to 25ml in deionised, double-distilled water.

Determination of valve thickness

The valve thicknesses of the shells were determined by the
method of Goldberg et al. (1978). An aluminum foil of uniform thickness and known weight per unit area was pressed over the shells and cut carefully along the edges of the shells. The aluminium foil was then weighed and the effective shell area calculated. The shell thickness was obtained as the ratio between the weight of the shell and the shell area.

Infrared spectra

Infrared spectra of the shell samples were recorded as KBr pellets on a Perkin-Elmer (model-183) infra-red spectrophotometer.

Electron paramagnetic resonance spectra

The Q- band EPR spectra of powdered shell samples were recorded at 35.5 GHz, at room temperature (RT) and at liquid nitrogen temperatures (LNT) using a Varian E112 X/Q band EPR Spectrometer. The spectra were calibrated using diphenylpicryl hydrazide as the field marker.

Analysis of soft tissue

The soft tissue of each composite sample (obtained as described on page 19) were dried at -80°C for 24 hrs. (For the investigations in Chapter 3 alone, the soft tissues were grouped according to weights of the bivalve shells for reasons indicated on page 38). The dried tissues were powdered and a
definite weight digested in a Kjeldahl's flask (Martincic et al., 1984). To about 0.1 - 1.0 g of sample, 5 - 10 ml of conc. HNO₃ and 0.5 - 1.0 ml of conc. HClO₄ were added. After pre-heating, the samples were digested for about 3 - 6 hrs. The solutions were cooled and made up to a specific volume for analyses on the AAS.

Analysis of trace metals using AAS

A Perkin Elmer Atomic Absorption Spectrophotometer (model 2380) was used for the analyses of the trace metals. The sample solutions were directly aspirated into the flame (Air - Acetylene) and the concentration in the digest was measured. For the analysis of shells, the standards were prepared in approximately 2% solution of CaCO₃ (BDH - ARISTAR) dissolved in 0.5M HCl. Blanks were also prepared and read wherever necessary.

Analysis of data

All data were statistically processed wherever necessary. Regression analyses were performed between various biological factors and the environmental variables referred to in Chapter 4. In Chapter 5, Chi-square analysis was done to find out the level of significance between the observed and expected environmental concentrations of the trace metals.