Chapter 2

Materials and methods

2.1. STUDY AREA

Materials for the present study were collected from Indian EEZ and adjoining areas (shallow waters of northwest coast of India and Cochin backwaters). Environmental parameters, zooplankton and water samples were collected from all areas.

2.1.1. Indian EEZ (Arabian Sea, Bay of Bengal and the Andaman sea)

The data used for this study is based on samples collected during 12 cruises carried out in the Arabian sea, Bay of Bengal and the Andaman Sea (Indian EEZ) by the research vessel, FORV Sagar Sampada (Plate 2.1) as part of a multidisciplinary project entitled “Marine Research- Living Resources (MR-LR) Assessment Programme” funded by Ministry of earth science (Formerly, Department of Ocean development), New Delhi. The project was designed to assess and evaluate the environmental parameters and the marine living resources (primary and secondary), of the Indian EEZ by the simultaneous collection of physical, chemical and biological oceanographic parameters from the Seas around India.

Seasons followed were inter monsoon spring (March - May), summer or southwest monsoon (June - September), inter monsoon fall (October) and winter or northeast monsoon (November - February) as per the Protocol under Joint Global Ocean Flux Studies (JGOFS, 1996). Hydrobiological samples were collected from pre fixed stations during 12 cruises from Arabian Sea - 4, Bay of Bengal - 4 and Andaman Sea - 4 cruises.

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Plate 2.1. Research vessel FORV Sagar Sampada

Figure 2.1. Sampling locations in the Indian EEZ
From 105 stations, a total of 336 surface samples and 1325 stratified samples were analyzed. Station positions are shown in Fig. 2.1.

2.1.2. Shallow coastal waters of northwest coast of India

Materials were collected during many biological and environmental investigations in and around coastal waters of Gujarat and Maharashtra carried out for the environmental impact assessment of marine ecosystem. Samples were collected from 10 areas (Fig. 2.2).

2.1.3. Cochin backwaters

The material examined was collected as part of the studies on “Ecosystem modeling of Cochin backwaters” during March 2003 to February 2004, funded by ICMAM, Chennai. During the period of study, two types of sampling were done, weekly sampling from 3 stations (Thevara, WS1; Fort Cochin, WS2 and Bolghatty, WS3) for one year and two seasonal (premonsoon and postmonsoon) time series sampling (24 hour) from five stations (TS1, TS2 TS3, TS4 and TS5) (Fig. 2.3).

2.2. SAMPLING METHODOLOGY

2.2.1. Physico-chemical parameters.

In the Arabian Sea, Bay of Bengal and Andaman Sea, surface temperature (SST) was measured using bucket thermometer. A Sea-Bird electronic CTD (SBE 911 Plus, USA) (Plate 2.2) was used to obtain the temperature and salinity profiles. Salinity values from the CTD were corrected using the values obtained from the Autosal (Guildline - Model 8400A) and the values are represented in psu (practical salinity unit). Mixed layer depth (MLD) was computed as the depth at which density rises by 0.2 units from the surfaces. This density difference is equivalent to a 1°C change in temperature, if salinity is constant.
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Figure 2.3. Sampling locations in the Cochin backwaters

Plate 2.2. CTD rosette with Niskin bottles
Water samples were collected for chemical analysis (dissolved oxygen and pH) from different depths (0, 10, 20, 30, 50, 75, 100, 150, 200, 300, 500, 750 and 1000 m. Dissolved Oxygen was estimated by the Winkler titration method.

In shallow coastal areas, water samples were collected using a clean polyethylene bucket and Niskin sampler with a closing mechanism was used for obtaining subsurface water samples. Sampling at the surface and bottom (1 m above the bed) was done where the station depth exceeded 5 m. For shallow regions, only surface samples were collected.

In Cochin backwaters, surface water samples were collected using a clean plastic bucket. Samples for dissolved oxygen were collected in 125 ml stoppered glass bottles taking care that no air bubbles are getting trapped in the sample. The samples were fixed immediately with manganous chloride solution (Winkler A) followed by alkaline potassium iodide (Winkler B) solution. Water samples for the analyses of salinity were collected in pre-cleaned polyethylene bottles. Temperature and pH of the water samples were measured in the field. For the estimation of Chl a, one litre of surface water was collected in clean plastic bottle.

2.2.2. Zooplankton collection

In oceanic waters, the zooplankton samples were collected from five different depth strata - surface to top of thermocline, top of thermocline to bottom of thermocline, bottom of thermocline to 300 m, 300-500 m and 500-1000 m) using a Multiple plankton net (MPN) (Plate 2.3) (Hydrobios-mesh size 0.2 mm and mouth area 0.25 m²) and surface samples using Bongo net (Hydrobios - mesh size 0.3 mm; filtering cone length 250 cm; ring diameter 60 cm) (Plate 2.4).
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Plate 2.3. Multiple plankton Net

Plate 2.4. Bongo Net
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The Multi Plankton Sampler has to be operated with an electrical connection (single or multi conductor cable) between Deck Command Unit and Underwater Unit. The net was hauled vertically with a speed of 1m/s.

The depths of hauls were fixed based on the thermocline besides two standard depths (1000 - 500 m, 500 - 300 m). The thermocline depths were 300 m - Base of thermocline (BT), Base of thermocline - Top of thermocline (BT-TT or thermocline layer), and Top of thermocline - surface (TT- 0 m or mixed layer). The depth of occurrence of the thermocline was taken as the depth where the temperature falls by 1°C from the surface and 15°C isotherm was considered as the bottom of the thermocline (Kesavadas, 1992). The number of strata sampled varies according to station depth and hydrographic conditions. At coastal stations two shallow depth intervals were invariably sampled. The volume of water filtered was calculated as the vertical distance (m) covered by the net’s mouth area (0.25 m²).

The Bongo net was towed horizontally for 10 minutes while the ship’s speed was 2 knots.

Volume of water filtered was calculated using the following formula,

\[ \text{Volume cubic meters} = 3.14 \times \left( \frac{\text{Net diameter}}{4} \right)^2 \times \text{Distance} \]

Distance in meters = Difference in flow meter reading \( \times \) Rotor constant

Rotor constant = 26873

For the collection of zooplankton from coastal waters, a Heron Tranter Net (Plate 2.5) (Tranter et al., 1972). The mouth area of the net is 0.25 m² and the total length is about 1.8 m (mesh size 0.33mm). Oblique hauls of 6 minutes duration were taken.
Plate 2.5. Heron Tranter Net

Plate 2.6. Working Party Net
Volume of water filtered (V) through the net was calculated by adopting the calibration formula provided with the flow-meter,

\[ V = [(0.157XN) - 0.003] \times A \]

Where, \( N \)-number of revolution, \( A \)= mouth area of net.

In Cochin backwaters, the surface zooplankton was collected using W.P net (Working Party net) mesh size 200\( \mu \)m and mouth area 0.6\( m^2 \) (Plate 2.6).

2.3. ANALYTICAL METHODS

(a) Physico-chemical factors

Temperature

The temperature of water samples was measured by a good quality centigrade thermometer immediately after collection.

\( p\text{H} \)

\( p\text{H} \) was measured using a digital \( p\text{H} \) meter (ECIL No. 5652) after standardizing with standard buffers of \( p\text{H} \) 4,7 and 9 just before the use.

Salinity

The estimation of salinity for the estuarine and coastal waters was carried out in the laboratory using precalibrated Salinometer (Digi Auto3G, accuracy ± 0.001)

Dissolved oxygen

Dissolved oxygen (DO) was determined by Winkler method (Grasshoff, 1976). Water samples were collected in 125 ml DO bottles
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without trapping air bubbles. Immediately the sample was fixed by adding 1 ml each of manganous sulphate solution (Winkler A) and alkaline iodine solution (Winkler B). The precipitate formed was dissolved in 1 ml concentrated sulphuric acid. The iodine liberated was estimated by titration using starch as indicator. Dissolved oxygen values are represented as µM.

**Chlorophyll a**

Collected water sample filtered under low vacuum through GF/F (normal pore size 0.7 m) filters, added one or two drops of magnesium carbonate solution and kept in refrigerator (Strickland and Parsons, 1972). The filter paper was extracted in 90% acetone, centrifuged and made up to 10ml using 90% acetone and the absorbance was measured using a spectrophotometer using 1cm cuvette against 90% acetone as blank at different wave lengths of 750, 664, 647, and 630nm. The amount of plant pigment in the water sample was calculated using the equation (SCOR/UNESCO).

\[
\text{Chl- } a = 11.85 \times 10^{-6} 665-1.54 \times 10^{-6} 645-0.08 \times 10^{-6} 630
\]

\[
\text{mg Chlorophyll/m}^3 = C/V \times 10
\]

Where, 
C = value obtained from the formula given

V = volume of water filtered in liters

10 = volume of 90% acetone

**b) Zooplankton**

Zooplankton sampling and analysis were done following the standard method (UNESCO, 1968; IOBC, 1969). Samples were preserved in 5% buffered formalin.
The classification of Mysidacea adopted in the present work is that of Hansen (1910) in which the more primitive forms are considered first and the more complex ones later according to their degree of specialization.

Generic definitions have been adopted mainly from the monographs of Tattersall and Tattersall (1951), Hansen (1910) and Li (1964) and specific identifications are mainly based on the literature by Tattersall (1908, 1914, 1915, 1922), Hansen (1910), O.S. Tattersall (1951) and Pillai (1957, 1961, 1964, 1973).

Mysids were divided mostly on the degree of development of the secondary sexual characteristics into the following classes (1) secondary sexual characteristics not developed - juveniles (2) immature male (3) mature male (4) females with marsupium developing but no eggs or young present yet - immature females (5) mature females, females with eggs or young present in the marsupium (Mauchline, 1971). Immature individuals were distinguished from juveniles by the presence of rudimentary oostegites (female) and longer fourth pleopod (male). Mature females were categorised according to the reproductive conditions. The eggs and larvae in the marsupium were enumerated and the developmental stages of the brood were determined. The development within the marsupium of ovigerous females which did not have a damaged or ruptured marsupium can be divided into three stages as eggs, eyeless larvae and eyed larvae.

All figures have been drawn with the aid of a Camera Lucida. Total length of all species has been measured from the tip of the rostral plate along the dorsal surface to the apex of the telson. The measurement have been made with an eye piece micrometer. Characteristics described refer to both sexes unless other wise stated. Holotypes of the new species and where possible paratypes and allotypes have been deposited in the Reference Collection of IOBC (RC, NIO, Cochin).
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2.4. DNA BARCODING

DNA Isolation from Mysid

DNA from the mysid genome was extracted as per standard Proteinase K digestion method (Sambrook et al., 1989). Sample tissues were first washed in 0.85% saline several times, then suspended in TEN buffer having 1% SDS. Proteinase-K was then added to a final concentration of 200 µg/ml and mixed gently. The suspension of lysed cells was incubated in a water bath for 3 hours at 50°C. The solution was cooled to room temperature and an equal volume of phenol equilibrated with 0.5M Tris-HCl (pH 8.0) was added and gently mixed by slowly turning over the tube for 10 minutes. The two phases were separated by centrifugation at 5,000rpm for 15 minutes at room temperature. The viscous aqueous phases were transferred to a clean centrifuge tube and the extraction with phenol was repeated twice. A third extraction with a 24:1 mixture of chloroform and iso-amyl alcohol was given to the sample and the aqueous phase was collected carefully. DNA samples in solution were precipitated after the addition of 0.1 volume of 3.0M Sodium acetate (pH 5.2) and 0.6 volumes of iso-propanol. The precipitated DNA was pelleted by centrifugation at 10,000 rpm for 15 minutes at room temperature. The excess salt was removed by washing the DNA pellets three times in 70% ethanol. The DNA samples were dried under vacuum and dissolved in 100 µl volume of TE.

Nucleic acid quantification

The DNA was quantified spectrophotometrically using a nanodrop spectrophotometer (ND1000) by measuring the optical density both at 260 and 280nm. The reading at 260 nm gives the concentration of the nucleic acid in the sample. The ratio between readings at 260 nm and 280 nm (OD\textsubscript{260}/OD\textsubscript{280}) provides an estimate of the purity of the nucleic acid. The integrity of DNA samples was estimated by visualizing samples on a 0.8% agarose gel stained
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Polymerase chain reaction (PCR)

PCR was carried out on the extracted DNA from mysids. The universal primer designed by Folmer et al., (1994) used for the amplification of COI gene of mysid.

HCO2198 TAAACTTCAGGGTGACCAAAAAATCA
LCO1490 GGTCAACAAATCATAAAGATATTGG

All the PCRs were performed in 50 µl of reaction volume consisting of 1x PCR buffer, 50 pico moles each of forward and reverse primers, 200 mM concentrations of each of dNTPs and 1 U of Taq polymerase (Bangalore Genei, Bangalore). 100 ng of DNA was used as template. The thermocycling conditions consisted of an initial denaturation at 94 °C for 5 min followed by 35 cycles of 30 s denaturation at 94 °C, 50 s annealing at 50°C and 2 min extension at 72 °C. The final extension of flush ends was carried out at 72 °C for 5 min. All PCRs were performed in a EPPENDORF thermocycler. The products of PCR were separated on a 1.8 % agarose gel, stained with ethidium bromide (0.5 g/ml) and photographed using gel documentation system (Kodak Gel logic1500 imaging system). PCR products were cleaned using QIAquick® PCR Purification Kit (QIAGEN, USA). The PCR product were sequenced using the same primers at Bangalore Genei, Bangalore, by an ABI Prism 3700 sequencer (Applied Biosystems).

2.5. BIOCHEMICAL COMPOSITION

Samples for biochemical studies were collected from Cochin backwater during premonsoon period at weekly basis (2nd March, 16th March and 31st March, 2007) using Working Party net. After collecting zooplankton, samples
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were immediately taken to lab, the organisms were classified (*Mesopodopsis orientalis*, *Mesopodopsis zeylanica* and *Rhopalophthalmus indicus*) and separated into mature male, immature male, spent female, female with embryo (brooding female), immature female and juveniles then individual specimens rinsed briefly with small amount of chilled distilled water, blotted on filter paper. Samples from each group were lyophilized, weighed, and kept at -30°C.

Protein quantification was conducted according to the Folin- Phenol method described by Lowry *et al.*, (1951) using bovine albumin as standard. The quantification of total carbohydrate was performed according to the colorimetric method using phenol and sulphuric acid, described by Dubois *et al.*, (1956) using glucose as standard. Lipid extraction was carried out according to Bligh and Dyer (1959) by direct elution with chloroform and methanol (1:2 v:v). The extracted lipid were dried at 80°C (20min.) and determined spectrophotometrically after carbonization at 18° C in concentrated sulphuric acid according to Marsh and Weinstein (1966); tripalmitine solution were used as standard.

2.6. STATISTICAL ANALYSIS

Linear Multiple regression Analysis

Multiple regression analysis was employed to assess the predictability of mysid population density in relation to physicochemical variables in the Cochin backwaters.

The model used for the purpose was

\[ Y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_4 x_4 + b_5 x_5 \]

Where \( y \) = population density, \( x_1 \) = chlorophyll, \( x_2 \) = dissolved oxygen, \( x_3 \) = salinity, \( x_4 \) = pH, \( x_5 \) = water temperature. The significance of the fitted regression was tested using ANOVA.
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Two-factor ANOVA

To test whether there is significant difference between species and between stages with respect to protein carbohydrate and lipid, the experimental data were subjected to statistical analysis using Two-factor ANOVA. Wherever, the treatment effects were found to be significant, least significant difference at 5% level were worked out and significant treatment effects were separated.