CHAPTER II

MATERIALS AND METHODS
DESCRIPTION OF STUDY AREA:

A semi-enclosed area, in the Dona Paula bay, located at the mouth of the river Zuari (15°27' N lat. and 73°47' E long.) was chosen as the study site (Fig. 1). The Zuari river, originates in Dighi ghat of the Sahayadri mountain ranges (Western Ghat) in Karnataka, and after a prolonged flow of 67 kms, empties into the Arabian sea at Dona Paula. The average runoff of Zuari estuary is $3 \times 10^9$ km³ annum⁻¹ (NIO, Tech. report, 1979). Dona Paula bay is protected towards north, east and the south by rocky promontories and is less than 2.0 kms upstream of the opening of Zuari river in the Arabian sea.

The tidal influence in the study area has been reported by Singbal (1973) to be comparatively low in monsoon (June - September) upto 2.0 m (Dehadrai, 1970). Similarly, the wave action is reported to be minimum, thus facilitating protection and steady growth of culture animals round the year (Parulekar et al., 1982). The bottom deposits in Dona Paula bay (Parulekar et al., 1974) are firm, thereby allowing proper anchorage and maintaining the steady position of the raft. Low siltation and clear water in this region except during monsoon make it an ideal site for off-bottom culture experimental trials in the field. In general, this culture site was found to be ecologically and biologically suitable for experimental and partially controlled field studies.

DETAILS OF RAFT FABRICATION:

Raft used for the experimental culture of green mussel Perna
Fig. 1. Map showing site of raft culture
**viridis** L. was fabricated indigenously after assembling the material near the culture site. Wooden poles were used for fabrication of frame (4.0 x 4.0 m) (plate I). The bamboo poles were placed equidistant from each other, from which, ropes with mussel seed were suspended in the water column. The bamboo poles were tied using nylon ropes (5.0 mm diameter).

The fabricated raft was mounted on four (Poly Vinyl Chloride (PVC) drums (200 L capacity) which were tied to the frame with the help of nylon ropes. The gross weight of the raft of dimension 4.0 x 4.0 m inclusive of PVC drums was about 0.75 tonnes and covered 16.0 m surface area. The raft was towed to the site at a desired depth where human interference was minimum. The raft was then moored by using two cement anchors weighing about 70.0 kg each, with a thick nylon rope (40.0 mm diameter). The average depth at the culture site was 7.0 m. The raft was in good condition till the termination of experimental period of 12 months from October 1987 to September 1988.

**SURVEY OF MUSSEL SEED / COLLECTION OF SPAT:**

The hatchery system of mass production of seed, confers specific advantages, like production of fast growing and disease resistant strains through cross breeding and immunization. However, for large scale production, the application of this technique was not felt necessary as spats are available in abundance along the rocky shores of west coast of India. A survey for identifying the spatfall area in Goa and Karnataka was undertaken during August-September, 1987, which coincides with
Plate I. Raft used in present study
the spawning season of green mussel in these areas. Along the Goa coast, major rocky shore areas like Anjuna, Baga, Chapora and Arambol, which are supposed to support large population of mussel seed (Qasim et al., 1977) were surveyed. But during the present survey no settlement of spat was observed in these areas. Dense settlement of spat was observed at Keni in Karnataka (14 40' N lat. 74 25' E long.; Plate II and III) on a rocky shore in between the mid tide and the low tide marks. The size of mussel seed ranged from 3.0-8.0 mm when the spat was about 2-3 weeks old. The density of the mussel spat was approximately 1200 m$^{-2}$. This seed was removed by scraping with the help of an iron chisel and scraper, then washed with seawater. The mussel seed was then transported live to the culture site. During transportation the water was changed twice.

**TRANSPLANTATION OF MUSSEL SEED:**

The mussel seed was transplanted on the coir rope with the help of a mosquito cloth strip. The length of the coir rope used was 6.0 m whereas mosquito cloth strip was 5.0 m and the width 0.25 m. The mussel seed were spread along the length of the rope leaving half a meter on free ends (Plate IV and V). The density of the mussel seed per metre rope was about 300. The mosquito net cloth was stitched around the rope with mussel seed in it and a thin twine was wrapped around the stitched mosquito net clothing. The mussel seed gets attached to rope within few days (Plate VI). For each rope a sinker was tied to the end so that the ropes with mussel seed should not entangle when submerged from the raft due
Plate II Natural bed of mussel spat
Plate III. Collection of mussel spat from the natural bed.
Plate IV. Mosquito clothing with rope ready for transplanting spat.
Plate V. Mussel spat on mosquito clothing with rope
Plate VI. Mussel spat attached to rope
to the influence of wave action and currents.

As soon as the ropes with mussel seed were ready, they were transferred for submergence from the wooden raft. About 0.5 m of the top part of the rope was left without seeds to enable to tie it to the raft. The ropes containing the mussel seed were submerged up to 5.0 m deep water column which form a part of euphotic zone. These seed got attached to the coir rope with byssus threads within 48 hrs after transplantation. The mosquito net covering the mussel seed took around 10-15 days to disintegrate. The spat transplanted on ropes began growing immediately after settlement. During the growth period, mortality observed was negligible (about 5.0 %).

**SAMPLING:**

Water samples at the site of culture were collected from October 1987 to September 1988, covering three seasons viz. post monsoon (October 1987 - January 1988), pre-monsoon (February 1988 - May 1988) and monsoon (June 1988 - September 1988) at fortnightly interval for hydrobiological parameters at three depths (0, 4.0 and 6.0 m). Niskin bottle of 5.0 litre capacity was used for water sampling. These samples were transported to the laboratory immediately for chemical analysis. Details of sampling dates and time of collection are shown in Table 1.

**MUSSELS:**

Mussel samples were also collected fortnightly for observations on growth, morphometry, biochemical composition and
Table 1. Sampling programme at the site of raft culture during the period of study

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>DATE</th>
<th>TIME (HRS)</th>
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<tbody>
<tr>
<td>1.</td>
<td>8TH OCTOBER</td>
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<td>26TH DECEMBER</td>
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<td>25TH JANUARY</td>
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<td>9.</td>
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<td>10TH SEPTEMBER</td>
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trace metals analysis. Mussels were removed manually from ropes hung from the raft. During each sampling, 30-35 mussels were collected. The sampled mussels were cleaned externally to remove mud, dirt particles and any fouling organisms attached to the shell.

**MORPHOMETRIC CHARACTERISTICS:**

25 mussels were taken randomly from the fortnightly collections, and were examined for various morphological characters. The mussel were measured for shell length (anterior - posterior axis), breadth (lateral axis) and height (dorso-ventral axis) with the help of a Vernier calliper to 0.1 mm. The terminology used is illustrated in Fig. 2. Total weight was determined to the accuracy of 0.1 mg after removal of excess water. Thereafter, each organism, was immersed separately in boiling water for 30 secs., to separate the meat from the shell. The meat from each mussel was placed on blotting paper to remove excess water and then weighed to record soft tissue wet weight (STWW). The shell weight (SW) was determined by drying each shell at 40-50 C for about 30 minutes. After recording STWW, the soft tissue of individual mussel was allowed to dry separately in an oven at 80 C as followed by Borrero and Hilbish (1988). The drying was continued until the difference between two successive weighings remained near 0.1 mg. The volume of the mantle cavity was determined using displacement method.

**BIOCHEMICAL ANALYSIS:**

The dried meat samples were powdered with mortar and pestle.
Fig. 2. Terminology used in present study to describe mussel shell.
This powder was used for the analysis of biochemical components such as protein, carbohydrate, lipid and total organic carbon. Protein was estimated following the method described by Herbert et al. (1971). 5 mg of powdered sample was taken in a dried test tube and homogenized in 0.5 ml of 1N NaOH and 2.5 ml of distilled water. This was heated at 80°C in a water bath for 30 minutes to extract the protein. After cooling, 0.5 ml of 1N HCL was added to neutralize it and subsamples were transferred to other test tubes. 2.5 ml of mixed reagent (carbonate - tartrate - copper solution) was added, followed by 0.5 ml of 1N Folins-ciocalteau reagent. The solution was mixed well, allowed to stand for 30 minutes and then centrifuged. The absorbance was measured at 750 nm using spectronic 1001, spectrophotometer. Appropriate blanks and standards (bovine serum albumin) were similarly treated to prepare standard curves. All concentrations are expressed as mg/g dry weight of animal tissue.

Carbohydrate was estimated using Phenol-sulphuric acid method (Dubois et al., 1956), modified by Hitchcock (1977). To a 5 mg of dried sample, 2 ml of 80% sulphuric acid was added and digested for 18 hrs at room temperature. To this 2 ml of phenol reagent (5%) and 5.0 ml of concentrated sulphuric acid were added. The mixture was centrifuged and the absorbance measured at 490 nm. Standard curve was drawn with the help of analar grade D-glucose. All concentrations are expressed as mg/g dry weight of animal tissue.

Lipid was estimated following the method described by
Parsons et al. (1984). The dry powdered sample (10 mg) was placed in homogenizer with 8 ml chloroform-methanol mixture (1:2:0.8 v/v of chloroform, methanol and distilled water respectively). This mixture after filtering through an ignited (450 °C for 3 hrs) GF/C filter paper, was transferred to a separating funnel. Extraction of lipid was made by 2 ml of distilled chloroform and 4 ml of distilled water by continuous shaking for 10 minutes. The chloroform layer was separated and evaporated to dryness in vacuum. 2 ml of 0.15% potassium dichromate reagent was added and the tubes were kept in boiling waterbath for 15 minutes. 4 ml of distilled water was added to this, and after cooling the tubes, absorbance was measured at 440 nm. Blanks and standards (stearic acid) were treated similarly and standard graph was plotted. All concentrations are expressed as mg/g dry weight of the animal tissue.

Total organic carbon in the animal tissue was determined by wet oxidation method as mentioned by Parsons et al. (1984). To a known quantity of the dried sample powder, 1 ml of phosphoric acid and 1 ml of distilled water were added in order to prevent the chloride interference and kept in a boiling waterbath for 30 minutes. Sulphuric acid dichromate reagent was added (10 ml) to these tubes and the samples were digested in a boiling waterbath for a period of 1 hour. Therefore, each sample was diluted with distilled water to 50 ml. Subsamples (approximately 10 ml each) were centrifuged at 4000 rpm for 20 minutes and absorbance was measured on a spectrophotometer at 440 nm. Blanks and standards (D-glucose) were treated similarly and the standard curve was
plotted. Concentrations are expressed as mgC/g dry weight of the animal tissue.

**TRACE METALS IN SEAWATER:**

**Collection and storage of samples:**

The essential criteria in the trace metal analysis is that the samples should be devoid of any external contamination. The sample containers themselves form one of the potential source of metal contamination. Much of the analytical accuracy will depend upon the choice of container materials and the procedure adopted to clean them. Adequate precautions for cleaning sample containers and other glasswares were taken. Polythene bottles used for storage of water samples were allowed to stand with analar grade HCL at room temperature for 3 days. These containers were then rinsed with distilled water and dried at 55°C. The collected water samples were then stored by reducing the pH below 4 using 1N HCL.

**PREPARATION OF TISSUE FOR METAL ANALYSIS:**

The mussels, after collection, were cleaned and kept in filtered seawater in an aquarium for 48 hrs to defecate. The mussel meat was removed by using clean scissors and forceps under hygienic conditions so as to avoid any contamination. The scissors and forceps were cleaned with distilled water before and after use. The mussel meat was then kept in an oven and dried at 80°C for 24 hrs. The dried meat was stored in glass vials, previously cleaned with concentrated hydrochloric acid.
ANALYSIS OF WATER SAMPLES:

Trace metals were analysed by using the method of Brooks et al. (1967), modified by Sen Gupta et al. (1978). 500 ml of filtered seawater was taken and to this 10 ml of an aqueous 2% ammonium pyrrolidine dithiocarbonate (APDC) solution was added which acts as useful chelating agent for various metals and was also effective in wide pH range (Brooks et al., 1967). To this 25 ml of methyl isobutyl ketone (MIBK) was added and the samples were vigorously shaken for 10 minutes, and the phases were separated via separating funnel.

To prepare a standard working curve, the extracted seawater samples were re-extracted to ensure that they were free of trace metals. Aliquotes (500 ml each) were then taken and known concentration of trace metals were added (0, 1, 2 and 3 ppm) using micropipette. These standards were extracted in a similar way as mentioned above. All these samples were run into an Atomic Absorption Spectrophotometer (model Varian Spectra AA 30) and the concentration expressed as ppm.

TRACE METALS IN MUSSEL TISSUE:

Analysis of trace metals (Fe, Cu, Zn and Mn) and major elements (Ca and Mg) in the total mussel tissue were undertaken. The metals were estimated by the method described by Leonard (1971). A known weight of each dried powdered sample (pooled for each day of collection) was taken in a glass beaker. To this 70% nitric acid was added and subjected to digestion on an electric hot plate until the brownish fumes completely disappeared and the
residue turned whitish in colour. The residue was allowed to cool at room temperature. Then, 2 ml of perchloric acid was added and digested for about 10-15 minutes on an electric hot plate to dryness. The dried residue was treated with 10 ml dilute HCl and measured by atomic absorption spectrophotometry (AAS model Perkin and Elmer, 5000). Appropriate dilutions were made depending upon the sensitivity of detection in these samples. Reported concentrations of metals are expressed as ppm. Appropriate blanks and standards were also prepared by using the same method.

ANALYSIS OF HYDROGRAPHIC DATA:

Water samples were analysed for hydrographic parameters, such as temperature, salinity, dissolved oxygen, pH, total suspended matter, particulate organic carbon, chlorophyll a and phytoplankton cell count at fortnightly intervals.

Temperature of the water samples was measured with the help of a mercury thermometer immediately after the collection. The values are expressed in °C.

Salinity of the water samples was estimated following the method as described by Strickland and Parsons (1968). This involves determination of chlorinity by the Mohr Knudsen titration method with standard silver nitrate solution, using potassium chromate as an indicator. Salinity values are expressed as parts per thousand (%).

Dissolved oxygen concentration of water samples was
monitored using Winkler method. This method involves the fixation of dissolved oxygen using Winkler A and B reagent, followed by the titration against standard sodium thiosulfate solution using starch as indicator. The concentration was expressed as ml/litre of the sea water. For pH measurement digital pH meter (Phillips, PP 9046) was used.

The total suspended matter was determined by filtering 1 litre of water sample through preweighed millipore membrane filter paper (0.45 μ). The residue was dried in an oven at 70°C and weighed on an electric balance (Mettler AE 200). The concentrations are expressed as mg/litre (dry weight) of sea water. Particulate organic carbon was estimated following the method of wet oxidation using sulfuric acid-dichromate reagent as described by Parsons et al. (1984).

Chlorophyll a estimation was done spectrophotometrically (Spectronic 1001). About 500 ml to 1 litre water sample was filtered through a GF/C filter paper followed by an extraction with 90% acetone in dark bottles at low temperature i.e. <5°C (Yentsch and Menzel, 1963). The values are expressed as ug/litre of seawater.

Phytoplankton cell count was done by taking a known volume of water sample and enumerating under the microscope for dominant groups. The phytoplankton cell count is expressed as cells x 10^3 /litre.