Materials and Methods
(a) Selection of study site:

The study was conducted at Uttar Gobindapur village located within Kakdwip region of Indian Sundarbans (21°52′35.6″N and 88°11′55.6″E) for a duration of 240 DOC (Fig. 1). *Macrobrachium rosenbergii* farming was done in grow-out ponds through application of formulated feeds incorporated with selected floral ingredients. The site selection criteria was mainly based on (i) availability of cultivable ponds (ii) easy transportation from Kolkata city (iii) year round availability of fish feed ingredients (iv) ready fish market (v) adjacent estuary and mangrove belt and (vi) economic profile of the local people.

(b) Collection of floral ingredients and proximate analysis:

Kakdwip region was selected for collection floral ingredients *viz.* green seaweed (*Enteromorpha intestinalis*), salt-marsh grass (*Porteresia coarctata*) and mangrove litter in context to prawn feed preparation. They were collected from mangrove belt adjacent to Kakdwip Research Centre of ICAR- Central Institute of Brackishwater Aquaculture (21°51′15.1″N and 88°10′12.9″E) and Uttar Gobindapur village (21°52′35.6″N and 88°11′55.6″E) located at the bank of Kalnagini river. A brief description about all the floral ingredients are given below:
**Green seaweed (Enteromorpha intestinalis)**

<table>
<thead>
<tr>
<th>Systematic Position:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Division – Chlorophyta</td>
</tr>
<tr>
<td>Class – Chlorophyceae</td>
</tr>
<tr>
<td>Order – Ulvales</td>
</tr>
<tr>
<td>Family – Ulvaceae</td>
</tr>
<tr>
<td>Genus – Enteromorpha</td>
</tr>
<tr>
<td>Species – intestinalis (Link)</td>
</tr>
</tbody>
</table>

**Figure 2. Enteromorpha intestinalis**

**Salient features:**

- Plant body is tubular, more or less compressed, constricted, and coiled in the form of intestine.
- Thallus dark green in colour and found attached to the substratum with the help of primary attaching cell.
- Presence of numerous multinucleated rhizoids growing from lower cell of the thallus.
- Cells of the thallus are small and elongated.

**Importance:**

- Rich in astaxanthin and hence used in fish feed preparation.
- Rich in protein (10-19%).
- Accumulator of heavy metal (Zn, Cu and Pb).
- Used as manure due to its rich content of trace elements.
Salt-marsh grass (*Porteresia coarctata*)

**Systematic Position:**
- Kingdom: Plantae
- Phylum: Magnoliophyta
- Class: Liliopsida
- Order: Cyperales
- Family: Poaceae
- Genus: *Porteresia*
- Species: *coarctata* (Roxb.)

**Figure 3. Porteresia coarctata**

**Salient features:**
- Perennial grass found growing on mudflats of lower Gangetic delta.
- This species can tolerate high range of salinity.
- Leaves linear, leathery with spinulose margins.

**Importance:**
- Pioneer species of mangrove ecological succession.
- Efficient in binding soil particles and prevents soil erosion.
- Protein content ranges between 10-12%, traditionally used as animal food and fodder.
- Rich in iodine.
Mangrove litter

Mangroves are very productive coastal ecosystems of the tropics. Fallen leaves supply "green manure" to the ambient water that promote natural food webs in the integrated system (Fitzgerald, 2000). Wild shrimp juveniles are highly dependent on this mangrove detritus, which account for 84% carbon in the shrimp body (Chong et al., 2001). The degrading leaf litter forms a high protein material that is consumed by a wide variety of organisms and release nutrients to the mangrove ecosystem for recycling as well. Such detritus derived from mangroves is of considerable ecological significance, since it serves as a major food source for many estuarine organisms (Odum and Heald, 1975). The nutritive value of this litter depends on the biochemical composition of the fresh leaves and the subsequent changes in it during decay.
The plant materials were collected during low tide condition, washed in ambient water and then with freshwater to remove epiphytes and other extraneous matter, dried in hot air oven under 55°C to preserve the biochemical constituents and finally processed into powdered form. The proximate analysis was done for protein, carbohydrate, lipid, ash and moisture contents. The protocols are as follows:

**Analysis of protein content:** The protein content was analyzed by using folin-ciocalteau phenol reagent following the method outlined by Lowry *et al.*, (1951). Protein present in the samples reacts with folin-ciocalteau reagent to give a coloured complex, produced by the reduction of phospho-molybdate by tyrosine and tryptophan liberated from the sample protein under the action of alkaline copper. The intensity of the colour depends on the amount which was measured spectrophotometrically against blank and working standards and finally expressed as % DW.

**Analysis of carbohydrate content:** The carbohydrate content was analyzed according to phenol-sulphuric acid method (Dubois *et al.*, 1956). The samples were allowed to react with a solution of anthrone in sulfuric acid. This gives green coloured products with an absorption maximum at 630 nm. The extinction of the resulting green solutions were measured spectrophotometrically against blank and working standards and finally expressed as % DW.

**Analysis of lipid content:** The lipid content was determined by Soxhlet method as described by Folch *et al.*, (1957). Initial weight was recorded before placing the samples within the soxhlet flask prior to extraction process. Samples were extracted
with petroleum ether for 6-8 hours at room temperature and final weight were recorded after the completion of the extraction process. Lipid content was calculated as the ratio between the sample weight differences (initial and final) with that of total sample weight and finally expressed as % DW.

**Analysis of ash content:** The ash contents were determined by heating the samples overnight in furnace at 525°C (AOAC, 1990).

**Analysis of moisture content:** Moisture contents were determined by weight difference method keeping the samples at 100°C overnight (AOAC, 1990).

**(c) Feed preparation and proximate analysis:**

The experimental feeds were prepared according to the nutritional requirements of prawn (Mukhopadhyay *et al.*, 2003). The floral ingredients were incorporated at a level of 5% within the feed by reducing fishmeal along with other ingredients. Three types of feeds were prepared *viz.* ENT feed (feed incorporated with *E. intestinalis*), POT feed (feed incorporated with *P. coarctata*) and ML feed (feed incorporated with mangrove litter). A control feed was also prepared with 0% reduction of fishmeal (Table 3). The feed ingredients (fishmeal, soybean oil cake, mustard oil cake, rice polish, wheat flour, oyster shell dust and shark oil) were chosen on the basis of their nutritional status, price and year round availability in the local market. The ingredients were weighed properly and taken in a container to make a dough. The resulting dough was steam cooked, cooled at room temperature and finally pressed through a manual feed pelletizer. The pellets were dried in well aerated place under
the shade for 2 days until it became sufficiently dry. Finally they were packed and stored for further use. The proximate analysis of the feeds were determined by the following methods described as follows:

**Analysis of protein content:** The protein content was analyzed by using folin-ciocalteau phenol reagent following the method outlined by Lowry *et al.*, (1951). Protein present in the samples reacts with folin-ciocalteau reagent to give a coloured complex, produced by the reduction of phospho-molybdate by tyrosine and tryptophan liberated from the sample protein under the action of alkaline copper. The intensity of the colour depends on the amount which was measured spectrophotometrically against blank and working standards and finally expressed as % DW.

**Analysis of carbohydrate content:** The carbohydrate content was analyzed according to phenol-sulphuric acid method (Dubois *et al.*, 1956). The samples were allowed to react with a solution of anthrone in sulfuric acid. This gives green coloured products with an absorption maximum at 630 nm. The extinction of the resulting green solutions were measured spectrophotometrically against blank and working standards and finally expressed as % DW.

**Analysis of lipid content:** The lipid content was determined by Soxhlet method as described by Folch *et al.*, (1957). Initial weight was recorded before placing the samples within the soxhlet flask prior to extraction process. Samples were extracted with petroleum ether for 6-8 hours at room temperature and final weight were recorded after the completion of the extraction process. Lipid content was calculated
as the ratio between the sample weight differences (initial and final) with that of total sample weight and finally expressed as % DW.

**Analysis of ash content:** The ash contents were determined by heating the samples overnight in furnace at 525°C (AOAC, 1990).

**Analysis of moisture content:** Moisture contents were determined by weight difference method keeping the samples at 100°C overnight (AOAC, 1990).

**(d) Freshwater prawn farming (Macrobrachium rosenbergii):**

**Pond preparation and stocking:** At the very initial stage of the experiment, attention was given on pond preparation properly. The ponds were dried sufficiently in order to decompose all organic matters, to oxidize different toxic compounds present in the pond bottom soil and also to eliminate undesirable filamentous algal mat and eggs of different predatory fishes, crabs etc. Lime was then applied accordingly to maintain soil pH and neutralize the organic acid, pyrite etc. present in the pond bottom. Slender twigs and concrete tubes were kept at different points of the ponds to provide shelter to the prawn juveniles. Prawn seeds were procured from a local hatchery and acclimated to the pond conditions prior to feeding trial. Seeds were stocked at a density of 2 individuals/ m² with average initial weight of 2.5 gm.
Feeding trial: The feeding trial was conducted in a grow-out ponds for 240 DOC. The experimental facility consisted of triplicate ponds for each feed treatment. Each pond was well connected to adjacent estuary so that possible hydrological variations affect all the ponds simultaneously. Prawns were fed twice daily at 0630 and 1800 hrs and the uneaten feed was checked at regular intervals. The detail description of the experimental ponds along with geographical location is given in Table 4.

Table 4. Detail description of the experimental ponds at Kakdwip region of Indian Sundarbans

<table>
<thead>
<tr>
<th>Ponds</th>
<th>Location</th>
<th>Co-ordinates</th>
<th>Pond area (ha)</th>
<th>Average Pond Salinity (psu)</th>
<th>Days of Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Uttar Gobindapur village</td>
<td>21°52'41.3&quot;N 88°11'50.1&quot;E</td>
<td>0.02</td>
<td>1.75 ± 1.38</td>
<td>240 (April to November 2012)</td>
</tr>
<tr>
<td>ML</td>
<td>Uttar Gobindapur village</td>
<td>21°52'41.6&quot;N 88°11'46.0&quot;E</td>
<td>0.03</td>
<td>1.52 ± 1.33</td>
<td>240 (April to November 2012)</td>
</tr>
<tr>
<td>ENT</td>
<td>Uttar Gobindapur village</td>
<td>21°52'37.4&quot;N 88°11'55.6&quot;E</td>
<td>0.05</td>
<td>1.86 ± 1.09</td>
<td>240 (April to November 2012)</td>
</tr>
<tr>
<td>POT</td>
<td>Uttar Gobindapur village</td>
<td>21°52'35.6&quot;N 88°11'55.0&quot;E</td>
<td>0.07</td>
<td>2.15 ± 1.39</td>
<td>240 (April to November 2012)</td>
</tr>
</tbody>
</table>

ML Pond= Ponds applied with mangrove litter incorporated feed.
ENT Pond= Ponds applied with Enteromorpha intestinalis incorporated feed.
POT Pond= Ponds applied with Porteresia coarctata incorporated feed.
e) Hydrological parameters:

Water samples for the hydrological parameters were collected from the surface by a clean bucket. Water samples for analyzing dissolved oxygen were directly taken into 150 ml BOD bottles without agitation and fixed immediately after collection. The temperature, pH, salinity were estimated in situ. The samples for other analysis were collected in several tarson bottles and transported at 4°C to the laboratory.

**Surface water temperature:** Measured by using a 0°–100°C mercury celsius thermometer.

**Surface water pH:** Measured by using a portable pH meter (sensitivity = ±0.02) which was calibrated with pH buffer 4.0 and 7.0 before every use.

**Surface water salinity:** Measured by means of an optical refractometer (Atago, Japan) and cross-checked in laboratory by employing Knudsens’ Argentometric method (Strickland and Parsons, 1972).

**Dissolved oxygen:** Measured by DO meter in the field and subsequently cross-checked in the laboratory by Winkler’s method (Strickland and Parsons, 1972).

**Dissolved inorganic nutrients:** Surface waters for nutrient concentrations were collected in clean tarson bottles and transported to the laboratory in ice-freezeed condition. Triplicate samples were collected to maintain the quality of the data. The standard spectrophotometric method of Strickland and Parsons (1972) was adopted to
determine the nutrient concentrations in surface water. **Nitrate** was analyzed by reducing it to nitrite by means of passing the sample with ammonium chloride buffer through a glass column packed with amalgamated cadmium fillings and finally treating the solution with sulphanilamide. The resultant diazonium ion was coupled with N - 1-naphthyl ethylene diamine to give an intensely pink azo dye. Determination of the **phosphate** was carried out by treatment of an aliquot of the sample with an acidic molybdate reagent containing ascorbic acid and a small proportion of potassium antimony tartarate. Dissolved **silicate** was determined by treating the sample with acidic molybdate reagent. The resultant silico-molybdic acid was reduced to molybdenum blue complex by ascorbic acid and incorporating oxalic acid prevented formation of similar blue complex by phosphate.

**Phytopigment (chlorophyll a) concentration:** For pigment analysis, 1 litre of surface water collected from each of the experimental ponds were filtered through a 0.45 µm millipore membrane fitted with a vacuum pump. After filtration, the membranes were dissolved in 90% acetone and kept in refrigerator for about 24 hours in order to facilitate the complete extraction of the pigment. The resulting solutions were centrifuged for about 20 minutes under 5000 rpm and the supernatant solutions were considered for the determination of the chlorophyll pigment by recording the optical densities at 750, 664, 647 and 630 nm with the help of spectrophotometer (Systronics- Type 117, No. 690). All the extinction values were corrected for a small turbidity blank by subtracting the 750 nm signal from all the optical densities, and finally the phytoplankton pigment was estimated as per the following expression of Jeffrey and Humphrey (1975):

\[
\text{Chlorophyll} \ a = 11.85 \ OD_{664} - 1.54 \ OD_{647} - 0.08 \ OD_{630}
\]
where, OD= Optical density

The values obtained from the equation were then multiplied by the volume of the extract (ml) and divided by the volume of the filtered water (m³) to express the chlorophyll content in mg m⁻³. All the analyses were done in triplicate in order to ensure the quality of the data.

**Soil organic carbon:** Organic carbon contents of pond bottom soil were determined following the modified version of Walkey and Black (1934). Sediment samples were collected and air-dried. Organic carbon in air-dried sediment samples were oxidized by dichromate-sulphuric acid and the amount of remaining dichromate was determined by titration with a standard ferrous solution. One gram sample from the experimental ponds were taken into clean, dry 500ml conical flasks. Exactly 10ml 1 (N) potassium dichromate and 20 ml conc. sulphuric acid was added and mixed by gentle swirling at first and then vigorously for a total time of 1 minute. The flasks were kept to react the mixture for about 30 minutes. After the reaction was over the contents were diluted with 200 ml distilled water followed by the addition of 10 ml conc. ortho-phosphoric acid and finally cooled. 1 ml of diphenylamine as redox indicator was added and titrated with 0.4(N) ferrous ammonium sulphate solution. At the end point colour changes from dull green through turbid blue to a brilliant green. A blank was run with same quantity of the chemicals but without sediment. Calculations were done by the following expression:

\[
\% C = 3.951/g \times (1-\frac{T}{S})
\]

Where, C= Organic carbon content
\(g = \) Weight of sample in gm
\(S=\) Volume of ferrous solution with blank titration
\(T=\) Volume of ferrous solution with sample titration
(f) Morphological characteristics of *Macrobrachium rosenbergii* (in terms of growth and production performance):

The culture species were monitored from the experimental ponds at every 30 days interval throughout the trial. The following response variables were determined from the experimental ponds:

**Fulton’s condition equation** was used to find out the condition factor (Chow and Sandifer, 1991) as follows:

\[ K = \frac{W}{(TL)^3} \times 100 \]

Where \( K \) is the condition factor, \( W \) is the average weight (g) and \( TL \) is the average total length (cm)

**Specific growth rate** (SGR, % d\(^{-1}\)) was calculated using the conventional equation:

\[ SGR = \frac{\left(\ln W_f - \ln W_i\right)}{t} \times 100 \]

Where \( W_f \) and \( W_i \) are the average final and initial weight in time \( t \).

**Length-weight relationship** (LWR) was calculated by using the conventional formula (Ricker, 1973) as given below:

\[ W = a \times TL^b \]

Where \( W \) is fish weight (g), \( TL \) is total length (cm), \( a \) is the proportionality constant and \( b \) is the isometric exponent.

**Final average body weight** (ABW, g), **feed conversion ratio** (FCR) and **survival** (%) were estimated after harvest by drag netting and dewatering the pond finally as:

FCR = \( \frac{\text{Total feed intake}}{\text{Total biomass gain}} \)

Survival = \( \frac{(\text{Number of fish harvested})}{(\text{Number of fish stocked})} \times 100 \)
(g) Biochemical characteristics of *Macrobrachium rosenbergii* muscle:

The samples were preserved at -20°C and were analyzed within 48 hours. The preserved samples were brought to normal room temperature, then excess water was soaked using Whatman filter paper. Samples were homogenized and used for biochemical analysis. The extinction values were measured by spectrophotometer (Systronics- Type 117, No. 690). The composition of the prawn muscle was determined by the following methods described as follows:

**Analysis of protein content:** The protein content was analyzed by using folin-ciocalteau phenol reagent following the method outlined by Lowry *et al.*, (1951). Protein present in the samples reacts with folin-ciocalteau reagent to give a coloured complex, produced by the reduction of phospho-molybdate by tyrosine and tryptophan liberated from the sample protein under the action of alkaline copper. The intensity of the colour depends on the amount which was measured spectrophotometrically against blank and working standards and finally expressed as % DW.

**Analysis of carbohydrate content:** The carbohydrate content was analyzed according to phenol-sulphuric acid method (Dubois *et al.*, 1956). The samples were allowed to react with a solution of anthrone in sulfuric acid. This gives green coloured products with an absorption maximum at 630 nm. The extinction of the resulting green solutions were measured spectrophotometrically against blank and working standards and finally expressed as % DW.
Analysis of lipid content: The lipid content was determined by Soxhlet method as described by Folch et al., (1957). Initial weight was recorded before placing the samples within the soxhlet flask prior to extraction process. Samples were extracted with petroleum ether for 6-8 hours at room temperature and final weight were recorded after the completion of the extraction process. Lipid content was calculated as the ratio between the sample weight differences (initial and final) with that of total sample weight and finally expressed as % DW.

(h) Histological characteristics of Macrobrachium rosenbergii muscle:

Sampling of prawn muscle tissue: Muscle samples of randomly selected individuals from the experimental ponds were collected from 60 DOC to 210 DOC. The exoskeleton was removed and about 1.0 gm of muscle tissue from second abdominal segment was dissected and fixed in Bouin’s solution.

Tissue processing and staining: The fixed tissues were washed in running tap water and then dehydrated gradually in different concentrations of ethanol. After dehydration, the tissue samples were immersed in cedar wood oil until they get totally transparent. The transparent tissues were impregnated in xylene: paraffin (1:1) mixture at 60ºC for 1 hour and then transferred to fresh paraffin for another 1 hour. The whole procedure was repeated twice. The tissues were embedded in paraffin blocks and then sectioned into 4μ thickness using a microtome and mounted on slides. After drying, the slides were immersed in xylene to remove the wax completely. The deparaffinized tissue sections were rehydrated and stained with haematoxylin and
counter stained with eosin. The sections were mounted using Distrene, Plasticiser, Xylene (DPX).

**Measurement of muscle fibre surface area:** The surface area of the muscle fibre were measured to the nearest millimetre by using a Planimeter from the cross-sectional images taken with the help of NIKON phase contrast research microscope fitted with an image analyzing system. The areas of ten randomly selected fibre were measured per slide and the average was estimated. A total of three slides were analyzed from each feed treatment.

**(i) Benefit - Cost ratio analysis:**

An economic analysis of the prawn culture experiment was performed to estimate the benefit-cost ratio (BCR) according to the method outlined by Biswas *et al.*, (2012). BCR was determined as:

\[
BCR = \frac{\text{Total income}}{\text{Total expenditure}}
\]

**(j) Statistical analysis:**

One way Analysis of Variance (ANOVA) was performed to assess whether morphological, biochemical and histological characteristics of *M. rosenbergii* varied significantly among the experimental ponds applied with different types of formulated feed; possibilities less than 0.01 (p < 0.01) were considered statistically significant. All statistical calculations were performed with SPSS 9.0 for Windows.