Materials and Methods
3. MATERIALS AND METHODS

3.1 Collection of seaweeds

Seven species of seaweeds representing Chlorophyceae (*Ulva lactuca* Linnaeus, *U. reticulata* Forsskal, *Caulerpa scalpelliformis* (R.Brown ex Turner) C.Agardh and *C. racemosa* (Forsskal) J.Agardh), Phaeophyceae (*Padina tetrastromatica* Hauck and *Sargassum ilicifolium* (Turner) C. Agardh) and Rhodophyceae (*Gracilaria corticata* (J. Agardh) J.Agardh) were collected (Plate 1 and Plate 2) from the Hare Island, Tuticorin (Plate 2), Gulf of Mannar region. This island is largely sandy with outcrops of rocks extending from supralittoral to sublittoral.

3.2 Decomposition of seaweeds

The collected seaweeds were decomposed using the nylon net 'litter bag' method (De La Cruz, 1973).

3.2.1 Decomposition in coastal water ecosystem

Collected seaweeds of the chosen species were packed in nylon net bags (20 x 30 cm size with 1mm mesh) and were kept immersed in seawater at CMFRI experimental station (Plate 3). Samples of decomposed seaweeds were collected for analysis at weekly intervals from the bag.
a. *Ulva lactuca* Linnaeus

b. *Ulva reticulata* Forsskål

c. *Caulerpa scalpelliformis*  
(R.Brown ex Turner) C.Agardh

d. *Caulerpa racemosa*  
(Forsskal) J.Agardh
a. *Sargassum ilicifolium* (Turner) C.Agardh

c. *Gracilaria corticata* (J.Agardh )J.Agardh

b. *Padina tetrastromatica* Hauck

d. A view of the Hare Island, Tuticorin where the seaweeds were collected.
a. Experimental set up on the decomposition of seaweeds in coastal water (Field).

b. Experiments on the decomposition of seaweeds in Laboratory conditions.
3.2.2  **Aerobic decomposition in the laboratory using fresh water**

Samples of the seaweeds were kept in seven different troughs containing ten litres of fresh water (43 cm diameter; 15 cm height). Sustained aeration was given to accomplish aerobic decomposition. The experiment was conducted in a dark place to prevent photosynthesis (Plate 3). The water in the troughs was changed weekly. Samples were collected at weekly intervals for analysis.

3.3  **Preservation of the decomposed seaweeds**

The decomposed seaweeds collected from two different experiments were dried separately in hot air oven at 60°C. The dried seaweed samples were powdered, packed in airtight HDPE bags and stored at room temperature.

3.4  **Biochemical analysis**

**Chemicals:**

The chemicals used were of BDH (AR) or sigma chemical company, St. Louis M.O., U.S.A., throughout the study unless otherwise specified.

3.4.1  **Estimation of Protein (Lowry et al., 1951)**

**Procedure**

500mg of dry seaweed powdered sample was ground well with 15ml of phosphate buffer (0.2M, pH 7.2). The extract was centrifuged for 10 minutes at 3000 rpm. The lower layer was discarded and the supernatant was taken. To the supernatant, an equal amount of cold 5% TCA was added. It was left for 30 minutes in an ice bath. The precipitated protein was taken and again centrifuged. The supernatant was discarded and the pellet was dissolved in 25 ml of 0.2 N NaOH.
From this 1 ml was taken and mixed with 4 ml of alkaline copper reagent. It was shaken well and was allowed to stand for 10 minutes at room temperature. Then 0.1 ml of diluted Folin phenol reagent was added and mixed well. After 20 minutes, the O.D was read at 650nm using Spectrophotometer (SYSTRONICS 104). Bovine Serum Albumin was used as the standard.

### 3.4.2 Estimation of Carbohydrate (Seifter et al., 1950)

**Procedure**

100 mg of the dry seaweed powder was ground with 5 ml of 80 % methanol. The extract was centrifuged at 5000 rpm for 15 minutes. The supernatant was collected and made up to 10 ml with 80 % of methanol. To this 10 ml of petroleum ether was added and mixed well. The lower layer was taken for carbohydrate estimation.

To 0.1 ml of the extract, 4.9 ml of anthrone reagent (0.2 % w/v in conc H$_2$SO$_4$) freshly prepared was added and kept in a boiling water bath for 10 minutes. After cooling, the O.D was read at 625 nm using Spectrophotometer (SYSTRONICS 104). Glucose was used as the standard.

### 3.4.3 Estimation of Lipid (Bligh and Dyer, 1959)

**Procedure**

1 g of dry seaweed powder was ground with 10 ml of distilled water. The pulp was transferred to a conical flask and 30 ml of chloroform-methanol mixture (2:1 v/v) was added and mixed well. It was kept in the dark at room temperature overnight. After this period, a further addition of 20 ml chloroform and 20 ml of water was
made. The resulting solution was subjected to centrifugation, when 3 layers were seen. A clear lower layer of chloroform containing all the lipids, a coloured aqueous layer of methanol with all water soluble materials and a thick pasty interface were seen.

The lower layer was carefully collected in a preweighed beaker and evaporated. When the solution was free of organic solvents, the weight was determined again. The difference in weight gave the weight of the lipid.

3.4.4 Estimation of Amino acid (Rosen, 1957)

Procedure

500 mg of the dry seaweed powder was ground with 5 ml of 80 % methanol. The extract was centrifuged at 3000 rpm for 15 minutes. To the supernatant, an equal volume of petroleum ether was added. Lower methanol phase was used for amino acid estimation. To 1 ml of aliquot, 4 ml of 0.2 % ninhydrin (w/v in acetone) was added, mixed well and kept in boiling water bath for 15 minutes. After cooling, 1 ml of 60% ethanol was added. OD was measured at 550nm using Spectrophotometer (SYSTRONICS 104). Glycine was used as the standard.

3.4.5 Estimation of Nitrogen (Humphries, 1956)

Procedure

Step I (Digestion process)

100 mg of the dry powdered seaweed sample was heated in a digestion flask having 2 ml salycyclic acid (5%, w/v in conc H₂SO₄), 300 mg powdered sodium thiosulphate, a pinch of the catalyst (CuSO₄, K₂SO₄, SeO₂, 1:8:1) and 1 ml of
concentrated H₂SO₄ at 100°C till the appearance of apple green colour of the digest. After cooling, the digest was made up to 50 ml with distilled water

**Step II (Distillation process)**

10 ml aliquot was transferred to a micro kjeldahl distillation flask. To this was added 10 ml NaOH (40 % w/v) solution and 2 ml glass distilled water and the flask was heated. The liberated ammonia was collected in 10 ml of 2% (w/v) boric acid solution containing a drop of the double indicator, a mixture of bromocresol green solution and methyl red solution. Distillation was stopped when the reddish colour changed into peacock blue. This solution was then titrated against 0.02N (v/v) H₂SO₄. The end point was the reappearance of the reddish colour. The volume of H₂SO₄ consumed was noted (Titre value).

Amount of Nitrogen (mg/g) = Titre value × 2.8 × 10⁻³

**3.4.6 Estimation of Carbon (Walkley and Black, 1943)**

**Procedure**

100mg of dried sample powder was taken in a conical flask. To this was added 4 ml of 1 N potassium dichromate solution and 2 ml of concentrated sulphuric acid. The flask was kept undisturbed for about 30 minutes.

After the reaction, the content was diluted with 20 ml of distilled water. To this 2 ml of phosphoric acid (85 % v/v) was added followed by 1 ml of diphenylamine indicator. This was then titrated against 0.4 N ferrous ammonium sulphate. The end point was a change into brilliant green.

The amount of carbon was calculated using the following formula.
Organic carbon (%) = \[ \frac{3.951}{g} \left( 1 - \frac{T}{S} \right) \]

where \( g \) = weight of the sample in gram

\( S \) = ml of ferrous solution consumed in blank titration

\( T \) = ml of ferrous solution consumed in sample titration

3.4.7 Estimation of Minerals (K, Ca, Mg and Na) (AOAC, 1970)

**Procedure (Triple acid digestion)**

500 mg of dry seaweed powder was mixed with 10 ml of concentrated nitric acid, 4 ml of 60 % (v/v) perchloric acid and 1 ml of concentrated sulphuric acid. The mixture was left undisturbed overnight. After that it was heated on a hot plate containing concentrated sulphuric acid in a beaker until brown fumes ceased to come out and then was allowed to cool. After cooling, it was filtered through Whatmann No. 42 filter paper. The filtrate was made upto to 100 ml with glass distilled water.

The filtrate was used for the estimation of K, Ca, Mg and Na using a flame photometer with KCl, CaCl₂, MgCl₂ and NaCl employed as standards for calibration.

3.4.8 Estimation of crude fibre (AOAC, 1970; Sadasivam and Manickam, 1992)

**Procedure**

Dried sample powder of 1 g was defatted by using petroleum ether in a soxhlet apparatus for 16 h or till the petroleum ether extractant was colourless. Petroleum ether was evaporated and this defatted residue was used for crude fibre estimation.
The residue was taken in a 50 ml beaker and successively digested with 25 ml of 0.255N H₂SO₄ followed by 25 ml of 0.313N NaOH solution till all the liquid got evaporated. The digested sample was transferred to a cheese cloth placed in funnel inserted in a 250 ml flask. The sample was washed with boiling water followed by absolute ethanol, till the washing appeared clear. Then the digested washed residue was transferred to an ashing dish (preweighed dish W₁). The residue was dried for 2h at 130 ± 2°C. The dish was cooled in a desiccator and weighed (W₂). Then it was ignited for 30 min at 600 ± 15°C. Once again it was cooled in a desiccator and re-weighed (W₃). The crude fibre was calculated as follows:

\[
\text{Crude fibre (\%)} = \frac{\text{Loss in weight on ignition}(W₂ - W₁) - (W₃ - W₁)}{\text{Weight of the sample}} \times 100
\]

3.4.9 Estimation of Ash (AOAC, 1970)

2.0 g of dried seaweed sample powder was weighed in a preweighed porcelain crucible (W₁). Then the crucible with the sample was placed in a muffle furnace set at 600°C and maintained for 24h. The contents of the crucible were cooled in a desiccator and weighed immediately (W₂). The difference in weight of the crucible gave the ash content.

3.4.10 Estimation of Energy content (Karzinkin and Tarkovskaya, 1964)

**Procedure**

Sample weighing 20mg was placed in a round bottomed flask. To the flask was added 3 ml of 5% (w/v) potassium iodate solution and 20ml of concentrated sulphuric acid. The control flask contained 3 ml of potassium iodate (5% w/v) and 20
ml of concentrated sulphuric acid. The flasks were connected to the reflex condenser without shaking and heated on heating mantles. Boiling was continued for an hour.

After combustion, the experimental and control flasks were cooled and 50ml of distilled water was added separately. The liquid was mixed well and the flasks were heated (not boiled) until the complete disappearance of its pink colour and the smell of iodine. The contents were cooled and each flask was diluted with 250 ml of distilled water and 10 ml of potassium iodide was added after transferring them into a separate 500 ml conical flask.

Then the flasks were titrated against 0.1N sodium thiosulphate using starch (1% w/v) as indicator. The end point was the disappearance of blue colour. The energy content was calculated using the following formula:

\[
\text{Energy value (KJg}^{-1}\text{dry wt)} = \frac{\text{control titre value} - \text{Experimental value} \times 0.6667 \times 4.184 \times 3.38}{\text{Sample weight}}
\]

3.4.11 Statistical Analysis

All the biochemical parameters were estimated on triplicate determinations. Standard deviation, Correlation – Regression analysis between the duration of decomposition and values of biochemical parameters of decomposed seaweeds and Student’s ‘t’ test analysis were carried out.

(i) Standard deviation

\[
\text{SD} = \sqrt{\frac{\sum d^2}{N - 1}}
\]
where $d$ refers to the deviation of each score from mean and $N$ the total number of observations.

(ii) Students ‘t’ test

Student’s ‘t’ test was used to compare two means by applying the formula:

\[
t = \frac{\bar{x}_1 + \bar{x}_2}{\sqrt{SE_1^2 + SE_2^2}}
\]

where $\bar{x}_1$ and $\bar{x}_2$ represent the means compared and $SE_1$ and $SE_2$ their respective standard errors.

Standard error was calculated using the formula:

\[
SE = \frac{SD}{\sqrt{n-1}}
\]

The level of significance for the ‘t’ at corresponding degrees of freedom ($Df = N-2$) was read from the probability table given in Zar (1974), where ‘N’ is the number of scores in both the experiments.

(iii) Simple correlation coefficients (r):

The simple correlation coefficient ‘r’ was determined from the formula.

\[
r = \frac{\sum xy}{\sum x^2 \sum y^2}
\]

iv) Simple regression

The regression equation was computed using the least square method. The basic formula followed was:
\[ Y = a + bX \]

where \( Y \) is the dependant variable, \( X \) the independent variable, \( a \) the intercept on \( Y \) and \( b \) the slope. The formulae used to derive the values \( a \) and \( b \) are:

\[
b = \frac{\sum xy}{\sum x^2}
\]

\[
a = \bar{Y} - b\bar{X}
\]

where \( \bar{Y} \) and \( \bar{X} \) denote the means of \( Y \) and \( X \), \( \sum xy \) and \( \sum x^2 \) are derived as follows:

\[
\sum xy = \sum XY - \frac{(\sum X)(\sum Y)}{N}
\]

\[
\sum x^2 = \sum X^2 - \frac{(\sum X)^2}{N}
\]

\[
\sum y^2 = \sum Y^2 - \frac{(\sum Y)^2}{N}
\]

Capital \( X \) and \( Y \) denote the raw scores and small \( x \) and \( y \), the deviation scores.

3.5 Preparation and determination of fatty acids (Venkatesalu et al., 2004)

Freshly collected species of Ulva and Caulerpa having more lipid content viz., Ulva lactuca and Caulerpa racemosa, Gracilaria corticata, P. tetrastromatica and S. silicifolium and their decomposed (upto 8th week in coastal water) counterparts were used for fatty acid analysis. Two grams of seaweed powder was refluxed with a mixture of dry methanol: benzene: concentrated sulphuric acid (20:10:1, v/v) for two hours. The methyl esters thus obtained were extracted three times with hexane and a small amount of sodium sulphate was added and filtered. The extract so obtained was evaporated under
vacuum. The residue was dissolved in hexane and analysed by gas chromatography (Varian GC # 1). The capillary column used to separate the fatty acids was CP-wax 58 (chrompack) (50m × 0.20m). The temperature conditions for GC were injector 210°C and detector 220°C. The temperature of the oven was programmed from 180°C and the carrier gas was N₂, H₂ and zero air and attenuation was 3⁻¹ⁱ amp / mv. Individual fatty acid methyl esters (FAME) were identified by comparing the relative retention times (RRT).

3.6 Bacteriological Analysis of decomposed seaweed

Sample:

The ten week old laboratory decomposed seaweed, Ulva lactuca was collected in a sterile polythene bag and transferred immediately to the laboratory for microbiological analysis. The time taken for collection of samples and processing the sample never exceeded four hours.

Preparation of serial dilution:

One gram of seaweed sample was homogenized with a known volume of sterile 1.0 % peptone distilled water by employing sterile homogenizer. The homogenized sample was transferred to 100 ml of sterile 1.0 % peptone water. Further serial dilution of sample was done by using 9 ml of sterile 1% peptone water blanks.

Plating and enumeration of total viable bacterial population

The total viable heterotrophic bacterial population of decomposed seaweed Ulva lactuca was enumerated using pour plate method by employing nutrient agar
medium. 1.0 ml of aliquots of appropriate dilution was poured into sterile petri dishes. 20 ml of sterile molten agar was aseptically poured into petri plates and the petri plates were rotated clockwise and anticlockwise for complete mixing of the sample and was allowed to dry. Replicate plates were also maintained for each dilution. The inoculated plates were incubated at 37°C for 48-72 hrs. After the incubation period the bacterial colonies were counted using bacteriological colony counter. The petri plates containing 30-300 colonies were selected for enumeration of bacterial population and the bacterial density was expressed as the number of colony forming units per gram (CFUg⁻¹) of the sample analyzed (Plate 4).

**Generic composition of bacterial strains**

Morphologically dissimilar well isolated colonies were randomly selected and restreaked on nutrient agar for getting pure colonies. The pure colonies were again streaked on nutrient agar slants and stored at 4°C in a refrigerator to keep the microbial culture viable and subculture of the stored strains was done once in a month. The isolated microbial strains were identified upto generic level following the schemes of Simidu and Aiso (1962), Shewan (1961) and Gilmour et al. (1976) and the Bergey’s manual of determinative bacteriology (Buchanan and Gibbons, 1984) was also referred in the identification procedures.

**3.7 Formulation and preparation of experimental feeds**

Feed trials were conducted using feeds prepared with four species of seaweeds viz. Ulva reticulata, Gracilaria corticata, Sargassum ilicifolium and Padina tetrastromatica. The decomposed seaweeds with higher protein content were incorporated as one of the ingredients (Protein source). Control feed was also
a. Control.

b. Total viable heterotrophic bacterial population of the decomposed *Ulva lactuca* in 10⁻³ dilution (Original).

c. Total viable heterotrophic bacterial population of the decomposed *Ulva lactuca* in 10⁻³ dilution (Replicate).

d. Total viable heterotrophic bacterial population of the decomposed *Ulva lactuca* in 10⁻⁴ dilution (Original).
prepared using groundnut oil cake as protein source for comparative studies. The details of the percentage composition of the different ingredients of the feeds prepared for the experiment are given in Table 1.

To assess the utility of decomposed seaweeds as fish feed, a total of 5 feeds were formulated and prepared. Feeds were prepared by adopting the method of Santhanam et al. (1990).

The ingredients used in all the feeds were dried, powdered and sieved through a 300µ mesh sieve and the required quantities of the ingredients pertaining to different feeds were mixed well. To these, water was added at the rate of 500ml/Kg of feed to make a dough and cooked in an autoclave for 15 minutes at 15 lb pressure. After cooking, the dough was allowed to cool to room temperature and extruded through a hand pelletizer with a die hole of 2 mm diameter. The extruded pellets were sun dried. The dried pellets were broken into pieces of 2 - 3 cm size and stored at room temperature.

3.8 Experimental methods

In order to conduct feed trials common carp, *Cyprinus carpio* (Linnaeus) (Plate 5) and catla, *Catla catla* (Hamilton) (Plate 5) collected from Manimuthar dam and procured from state fisheries department, Tirunelveli, were kept in an aerated tank for acclimatization. Uniform sized animals were taken for feed trial experiments. The test animals were starved for 24 hours to ensure complete evacuation of their alimentary canal. Feeding experiments were conducted in circular plastic troughs of 20 L capacity. Sixteen liters of fresh water was maintained in all the troughs throughout the study period of 21 days. For both the animals, each treatment (feed)
Table 1: Percentage composition of the different ingredients in the control and experimental feeds prepared.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Feed Type</th>
<th>Ingredients</th>
<th>Percentage composition</th>
<th>Protein Content (%)</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Calculated value</td>
</tr>
<tr>
<td>1</td>
<td>CF</td>
<td>Groundnut oil cake</td>
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<td></td>
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<tr>
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<td></td>
<td>Tapioca</td>
<td>4.48</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rice Bran</td>
<td>4.48</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fish Meal</td>
<td>5.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin Mix</td>
<td>1.20</td>
<td></td>
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<tr>
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<td></td>
<td>Sunflower oil</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>DGF</td>
<td>Groundnut oil cake</td>
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<td></td>
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</tr>
<tr>
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<td></td>
<td>Rice Bran</td>
<td>8.89</td>
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<tr>
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<td></td>
<td>Fish Meal</td>
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<td></td>
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<td>Sunflower oil</td>
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<td>3</td>
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<td>Groundnut oil cake</td>
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<td>16.28</td>
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<td>Fish Meal</td>
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<td></td>
<td>Rice Bran</td>
<td>11.63</td>
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<td>Fish Meal</td>
<td>5.01</td>
<td></td>
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<td></td>
<td>Vitamin Mix</td>
<td>1.20</td>
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<td>Sunflower oil</td>
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<td>Fish Meal</td>
<td>5.01</td>
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<td></td>
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<td>Vitamin Mix</td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sunflower oil</td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>
a. *Cyprinus carpio* (Linnaeus)  
b. *Catla catla* (Hamilton)  
c. Operation of feeding trial experiment with *Cyprinus carpio*  
d. Operation of feeding trial experiment with *Catla catla*
was triplicated. In total 15 troughs representing 5 feeds including control for each animal were used (Plate 5).

Each replicate of common carp contained 5 animals whereas that of the catla contained 2 animals. Before the commencement, the animals were carefully wiped in a blotting paper and weighed in an electronic mono-pan balance. The weighed animals in each replicate of the respective treatment were fed on weighed quantities of the diet viz;

<table>
<thead>
<tr>
<th>Feed Code</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF</td>
<td>Control feed</td>
</tr>
<tr>
<td>DGF</td>
<td>Feed from decomposed <em>Gracilara corticata</em></td>
</tr>
<tr>
<td>DSF</td>
<td>Feed from decomposed <em>Sargassum ilicifolium</em></td>
</tr>
<tr>
<td>DPF</td>
<td>Feed from decomposed <em>Padina tetrastromatica</em></td>
</tr>
<tr>
<td>DUF</td>
<td>Feed from decomposed <em>Ulva reticulata</em></td>
</tr>
</tbody>
</table>

twice a day at 8.00 hour and 18.00 hour for a period of 1 hour each. The unutilized food was completely removed from trough, transferred to petri dishes and dried at 60° C in a hot air oven to estimate the actual amount of feed consumed in each treatment. Throughout the course of the experiment, the medium was changed once in two days.

To estimate the growth of the fish at the commencement and termination of the experiment, "Sacrifice method" (Maynard and Loosli, 1962) was followed. At the end of the experiment, the test animals were starved for 24 hours to ensure complete evacuation of the gut and they were killed and dried to estimate the growth of the
animals. All weighings were made in an electronic mono-pan balance to an accuracy of 1 mg.

Food consumption was estimated gravimetrically in terms of dry weight by subtracting the weight of unconsumed / unutilized from that of the food offered. Production or conversion was estimated as the difference between the dry weight of the fish at the commencement and at the termination of the experiment. Rates of feeding and conversion were calculated by dividing the respective quantities by the product of the initial wet weight of the fish and duration of the experiment. Gross conversion efficiency was calculated as the quantum of production to consumption.

**Consumption rate**

or

**feeding rate (mg g⁻¹ day⁻¹)**

\[
\text{feeding rate} = \frac{\text{Total dry food consumed (mg)}}{\text{Initial live weight of the fish (g) \times number of days}}
\]

**Production rate**

or

**Conversion rate (mg g⁻¹ day⁻¹)**

\[
\text{conversion rate} = \frac{\text{Increase in dry weight (mg)}}{\text{Initial live weight of the fish (g) \times number of days}}
\]

**Gross conversion efficiency (%)**

\[
\text{Gross conversion efficiency} = \frac{\text{Increase in dry weight (mg)}}{\text{Total dry food consumed (mg)}} \times 100
\]

**Specific Growth Rate (SGR) (%)**

\[
\text{Specific Growth Rate} = \frac{\text{Final wet weight (g) - Initial wet weight (g)}}{\text{Number of Days}} \times 100
\]
Gain in live weight (g) 

Protein Efficiency Ratio (PER) = \frac{\text{Gain in live weight (g)}}{\text{Total Protein Consumed (g)}}

Weight of Protein gained (g) 

Protein Conversion Ratio (PCR) = \frac{\text{Weight of Protein gained (g)}}{\text{Weight of Protein Consumed (g)}}

Gain in calorific value (KJg\(^{-1}\)dry weight) = \text{Final calorific value of fish} - \text{Initial calorific value of fish}

The data collected on the different growth parameters were statistically treated. The above feed trial experiments were conducted as per the procedure of Ramadhas and Sumitra Vijayaraghavan (1979), Santhanam (1981) and Rath (1993).