Chapter- 3

MATERIALS AND METHODS

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3.1 Study Site (Kalpani Beel)

Kalpani Beel an ox-bow lake formed by the river Manas, a tributary originated in Bhutan of Eastern Himalayan Biodiversity hotspot. Kalpani Beel is located between 26.54800° and 26.55037° N and 90.80825° to 90.82676° E in the eastern part of the Chirang district of Assam. The Beel is the only fresh water Beel (wetland) of the district. Total area of the Beel covers 56.1655 hectares. The Beel has an outlet channels with the river Manas and it is an important breeding site of the fishes and other aquatic animals.

3.2 Assessment of Seasonal Variation in Water Quality

3.2.1 Collection of Water Samples

The water samples were collected for physical and chemical analysis from the four different sampling sites i.e. Monakocha (Station-1), Goraimari (Station-2), Bishpani (Station-3) and Bedlangkhaiti (Station-4). Monthly water samples were collected once in every month from April, 2016 to March, 2017 for a period of one year. The samples were collected in two litter polythene bottle during morning hours between 8.00 A.M. to 10.00 A.M. and tightly sealed and labeled the polythene bottles after collection in the field. Samples were brought to the laboratory in the minimum period of time for analysis.The parameters air temperature, water temperature and p^H was recorded on the spot.While the other parameters were determined in the laboratory within 24 hours of sampling.

3.2.2 Physical and Chemical Parameters of Water Samples

For the analysis of various physical and chemical parameters of water samples, “The standard Methods for Examination of water and waste water (APHA, 2005)” was adapted. “Handbook of Methods in Environmental Analysis Water, Soil and Air” (Gupta, 2009) was followed for water sample analysis and compared with the values as guided by WHO (1971) and BIS (1993).

3.2.2.1 Air Temperature (AT) (APHA, 2005)

Air temperature was measured by a mercury filled centigrade thermometer (0 to 100°C range).The readings were made by exposing the thermometer in the air.

3.2.2.2 Water Temperature (WT) (APHA, 2005)

The surface water temperature was measured by a mercury thermometer of 0 to 50°Crange and with 0.2°C least count. Surface water was taken in a plastic container and its temperature was recorded immediately by dipping the thermometer for about one minute.

3.2.2.3 p^H

p^H of water was measured with the help of digital p^H (KRMA, p^H-035) meter.The reading has been made by dipping the p^H meter in the water samples.

3.2.2.4 Free CO_2 (Gupta, 2009)

Free carbon dioxide was determined in water immediately after taking the sample. 50 ml of water sample was taken in a conical flask and added 2-3 drops of phenolphthalein indicator. Appearance of pink color indicated the absence of free CO_2. The colourless sample was titratedagainst sodium hydroxide solution until pink colour appears.

Calculation:

$$\text{Free } CO_2 \text{ (mg/L)} = \frac{\text{A x N of NaOH x 44 x 1000}}{\text{Sample taken for determination(ml)}}$$

Where, A = NaOH used for titration for sample (ml)

N = Normality for NaOH.

3.2.2.5 Total Alkalinity (T.A.) (APHA, 2005)

Total alkalinity as carbonates and bicarbonates was estimated by titrating the sample with a strong acid (HCI or H_2SO_4) to p^H between 4.2 and 5.4 with methyl orange or mixed indicator. The value is called as total alkalinity (TA).

Calculation:

$$\text{Total alkalinity (mg / L) as } CaCO_3 = \frac{\text{Volume of HCl x N of HCl x 1000 x 50}}{\text{Water sample (ml)}}$$

Where, N= Normality of HCl

3.2.2.6 Total Hardness (T.H.) (APHA, 2005)

Calcium is the major component of hardness in water and usually is in the range of 5 to 500 mg/l as $CaCO_3$, (2 to 200 mg/l as Ca). 5 ml of water sample was taken in a porcelain dish. It was diluted to about 25 ml with distilled water, added one ml of ammonium chloride-hydroxide buffer and 3 to 4 drops of eriochrome black T indicator and titrated with the standard versenate solution. The colour was changed from wine red to blue or bluish green. At the end point no tinge of the red colour remains.

Calculation:

$$\text{Total hardness as } CaCO_3 \text{ (mg/l)} = \frac{\text{EDTA used (ml) x N of EDTA x B x 1000}}{\text{Sample (ml)}}$$

Where, N= Normality

B= mg $CaCO_3$ equivalent to 1.00 ml EDTA titrant

3.2.2.7. Phosphate (PO_4^-) (APHA, 2005)

In surface water phosphorus is usually present as phosphate (PO_4^-).50 ml of water sample was taken in a conical flask and to it 4 ml of ammonium molybdate with 5 drops of stannous chloride ($SnCl_2$) was added. Then its absorbance was recorded by Spectrophotometer at 690 nm

Calculation:

$$\text{Phosphate mg/L} = \frac{\text{P (mg) (in approx.104.5 ml final volume)}}{\text{Sample (ml)}} \times 1000$$

3.2.2.8. Chloride (Cl^-) (APHA, 2005)

5 ml of water sample was taken in a porcelain dish and dilute it to about 25 ml with water, added 5 to 6 drops of K_2CrO_4 (Potassium chromate indicator) and titrated with the standard $AgNO_3$ solution (with stirring) till the first brick red tinge appears.

Calculation:

$$\text{Chloride (mg/L)} = \frac{(\text{ml x N}) \text{ of } AgNO_3 \text{ x } 1000\text{x } 35.5)}{\text{Sample used (ml)}}$$

Where, N= Normality

3.2.2.9 Nitrate (NO_3^-) (APHA, 2005)

25 ml of water sample was taken in a porcelain dish (50 ml capacity) and evaporated it to dryness on a hot water bath. 3 ml of phenol disulphonic acid was added to the residue and dissolved the latter by rotating the dish. After ten minutes, 15 ml of distilled water was added and stirred with a glass road. On cooling, the contents were washed down into 100 ml volumetric flask. Then ammonia (1:1) was added slowly with mixing till the solution was alkaline as indicated by the development of yellow colour due to the presence of nitrate. Another 2 ml of ammonia was added and the volume was made up (100 ml) with distilled water. The intensity of yellow colour was read in the colorimeter at 420 nm (blue filter).5 ml of distilled water and 1.5 ml of potassium hydroxide solution was added. Standard curve of nitrate was prepared. A stock solution containing 100 ppm nitrate-nitrogen (NO_3^-) was prepared by dissolving 0.7215 g of AR grade potassium nitrate (oven dried and cooled) in distilled water and made the volume to one litre. This was diluted ten times to give a 10 ppm NO_3^- solution. Aliquots (2, 5,10,15,20 and 25ml) were evaporated on boiling water bath to dryness in small porcelain dishes (or beakers). When cool, 3 ml of phenol disulphonic acid was added and yellow colour was developed and read (described above). A blank

(without nitrate) run and correction was made by adjusting the colorimeter to zero with blank. A calibration curve was drowning between concentration of NO_3^- and colorimeter reading.

Calculation:

$$NO_3^- \text{ (mg) / Litre} = \frac{\mu\text{mg of } NO_3^- \text{ from Standard Curve}}{\text{Sample (ml)}}$$

3.2.2.10 Nitrite (NO_2^-) (Gupta, 2009)

50 ml of filtered sample was taken in an Erlenmeyer flask and 1 ml of each EDTA solution, sulphanilic acid and α-naphthylamine hydrochloride solution was added one after the other. An appearance of wine red colour indicated the presence of nitrites. The absorbance of this solution on spectrophotometer was recorded at 520 nm and a blank with distilled water. Similar way standard nitrite solution was used and recorded the absorbance in different concentrations. Plotted a standard curve between absorbance and concentrations of standard solutions and deduced the nitrite-nitrogen content of sample by comparing its absorbance with the standard curve. The result was expressed as mg NO_2-N/L.

3.2.2.11 Ammonia (NH_3) (Gupta, 2009)

40 ml water sample was taken in a 50 ml volumetric flask. 4 ml each of phenolnitroprusside solution and alkaline hypochlorite solution was added. Made up the volume of contents to 50 ml by adding ammonia free distilled water and kept it in a dark place at 25°C for about 1 hour. The absorbance on Spectrophotometer at 635 nm was recorded and distilled water used as blank. Process had done the standard ammonium chloride solutions of different concentrations in similar way and recorded the absorbance for each. Plotted the concentration of ammonium ions in sample in mg NH_4^+-N/L against absorbance and had prepared standard curve. Calculation of concentration of unknown was done with the help of curve.

3.2.2.12 Iron (Fe) (APHA, 2005)

Iron usually exists in natural water both in ferric and ferrous forms. Ferrous iron has tendency to oxidize readily to ferric state. 5 ml of water sample was taken in a beaker and 2 to 3 drops of alpha-alpha-Bipyridyl solution was added which reacts immediately with the ferrous iron to form to a red colour compound. The red colour compound indicated the presence of iron. This compound is stable. The water of the sample was evaporated on boiling water bath to completely dried in beaker. Then its weight was recorded by digital balance.

Calculation:

Iron (mg/L) = (Beaker and its dried content – Clean beaker)

3.3 Statistical Analysis

Statistical calculations were done with the help of Ipsen and Feigl (1970) methods

1. Mean ($\bar{X}$) = $\frac{\sum x}{N}$

2. Standard Deviation (S.D.)

$$S.D. = \sqrt{\frac{\sum (X - \bar{X})^2}{N}}$$

3. Standard Error of Means (S.E.)

$$S.E. = \frac{SD}{\sqrt{N-1}}$$

$\sum$ = Summation

N = Number of observations

X = Individual observations

$\bar{X}$ = Mean of observations

$X - \bar{X}$ = Deviation from the mean.

3.4 Fish Diversity Study

3.4.1 Collection and preservation of fish samples

Collection of fish species was accomplished by the taking help of local fishermen. The market survey was performed during 8.00 a.m. to 11.00 a.m. in the morning and from 3.00 p.m. to 6.00 p.m. in the evening in markets located in the vicinity of the Beelsite. Digital Camera (Sony DSC-W 830/ PCE 32) was used to capture the photograph of fish species for registering the specimens. The secondary data were collected all the way through by observation and interaction with the local people and fishermen of the areas.

3.4.2 Fish identification

Fish samples collected were preserved in 8% of formalin for detailed examination and authentication. Taxonomic identification and authentification was done with the help of skilled experts from Zoological Survey of India (ZSI) in Shillong, vide letter no.F.4/2015-16/Tech./6033 dated 27 Sept. 2016 and also by using standard literature (Sen, 1985; Nath and Dey, 1989; Sarkar and Ponniah, 2000; Barooah and Sarma, 2016). Information on fish species and their local name were gathered from fishermen, Mahaldar and local informants. The present status of authenticated fish species were determined by comparing the categories specified by IUCN in 2011 for threatened fish species (www.iucnredlist.org).

3.5 Macrophyte Diversity Study

3.5.1 Collection, preservation and identification of samples

The study on aquatic macrophytes was carried out from February, 2017 to January, 2018. During the study period the photograph of aquatic macrophyte were captured in digital camera (Sony DSC- W830/PCE32) to register the specimen. The macrophytes in shallow waters were collected directly while those from deeper water with the help of long handled hook. The collected plants were washed thoroughly under tap water, excess water soaked with filter paper, kept in polythene bags lined with filter paper and brought to the laboratory and preserved in 10% formalin and observed. The specimens were identified up to species level using standard literature (Cook, 1996; Kodarkar, 1996 and Biswas *et al.*, 1984).

Information on aquatic macrophyte species and their local name were collected from fishermen and local informants.

3.5.2 Classification

The macrophyte species were categorized into six categories such as free floating (FF) submerged suspended (SS), submerged anchored (SA), rooted with floating leaves (RFL), emergent anchored (EA), swampy and marshy (SM) following the system of Weaver and Clement (1929) and Daubenmire (1947).

3.6 Mineral Content Analysis of Aquatic Plant

3.6.1 Sample collection and identification

The plant samples used in this study were collected from their natural habitat with the help of fisherman from Kalpani Beel of Assam, India. The samples were taxonomically authenticated by taking help of experts from Botanical survey of India, Shillong.

3.6.2 Sample preparation

The plant samples were washed thoroughly under tap water to remove contaminants and air dried under shade for 2 weeks. Subsequently, the samples were oven dried for 24 hours at 40°C. The dried plant material was grounded to form fine powder and filtered through a 345-micron pore sieve. The powder was stored in an air-tight container until further analysis.

3.6.3 Sample digestion

The samples for analysis of minerals were prepared by wet digestion method (Puwastein *et al.* 2011). A dry sample weighing 5.1 g was taken in a Teflon cup with screw cap. Concentrated nitric acid (HNO_3) (5 mL) and concentrated perchloric acid ($HClO_4$) (1 mL) (HNO_3:$HClO_4$ = 5:1) were added. The sample was predigested in a tightly closed cup overnight at room temperature. The cup was placed in an oven at 100°C for 5–8 hours, cooled to room temperature in a fume hood, transferred to a 50 mL volumetric flask, and diluted to mark with deionised (DI) water and mixed well. The obtained solution was filtered through Whatman filter paper No. 541 and transferred to Nalgene plastic bottles for mineral detection.

3.6.4 Sample analysis

3.6.4.1 Determination of Ca and Mg

An aliquot of the test solution prepared in Section 3.6.3 was added to a volumetric flask 1% w/v $LaCl_3$. The solution was diluted to an appropriate volume with DI water. The solution was then measured for calcium and magnesium using an atomic absorption spectrophotometer (AAS) (GF-Atomic Absorption Spectrophotometer (AAS), Model: Analytik Jena Vario-6). The specific wavelength for each mineral was: (Ca = 422.7 nm and Mg = 285.2 nm) (Puwastein *et al*. 2011).

Calculation:

$$\text{Ca or Mg (mg/100 g)} = \frac{(C_0) \times \text{Total volume (mL)} \times \text{dilution} \times 100}{\text{Weight of sample (g)} \times 1000}$$

Where:

Co = concentration of the sample in mg/L

1000 = conversion of mL to L

3.6.4.2 Determination of Na and K

An aliquot portion of the sample solution prepared in Section 3.6.3 was added to the $CsCl_2$ solution to prepare a final dilution of 1% w/v $CsCl_2$ prior to the analysis to an appropriate volume. Analysis of the elements was performed using the AAS at their respective element wavelengths. (Na = 589.0 nm for 0.5-1.5 ppm and 330.4 nm for 10-100 ppm), (K = 766.5 nm) (Puwastein *et al.* 2011).

Calculation:

$$\text{Na or K (mg/100 g)} = \frac{\text{Co} \times \text{V} \times \text{D} \times 100}{\text{W} \times 1000}$$

Where,

Co = concentration of samples in mg/L

V = total volume in mL

D = dilution factor

W = weight sample, g

1000 = conversion of mL to L

3.6.4.3Determination of Iron (Fe), Copper (Cu), Zinc (Zn), Cobalt (Co), Chromium (Cr), Manganese (Mn), and Molybdenum (Mo)

The sample was prepared by wet digestion method in the Section 3.6.3. An aliquot portion of the acidified sample was diluted to appropriate volume and analysed using AAS at relevant element wavelengths (Fe = 248.3 nm), (Cu = 324.7 nm), (Zn = 213.9 nm), (Co = 228.6 nm), (Cr = 267.7 nm), (Mn = 257.6 nm), and (Mo = 202.0 nm) (Puwastein *et al*. 2011).

Calculation:

$$\text{Trace element (mg/100 g sample)} = \frac{\text{Co} \times \text{V} \times \text{D} \times 100}{\text{W} \times \text{P} \times 1000}$$

Where,

Co = concentration of the sample in mg/L

V = total volume in mL

D = dilution factor

W = weight sample in g

P = sample solution taken, mL

1000 = conversion of ml to L

3.6.4.4 Statistical Analysis

Statistical analysis was performed with SPSS 26.0 version.

3.7 Photosynthetic Pigments of *Hydrilla verticillata* plant

The Chlorophyll is the essential components for photosynthetic and occurs in chloroplasts as green pigments in all photosynthetic plant tissues. They are bound loosely to proteins but are readily extracted in organic solvents such as acetone or ether. Chemically, each Chlorophyll molecule contains a porphyrin (tetrapyrole) muclens with a chelated magnesium atom at the centre and long chain hydrocarbon (Phytyl) side chain attached through a carboxylie acid group. There are at least five

types of Chlorophylls in plants. Chlorophyll a and b accrue in higher plants, ferns and mosses. Chlorophylls c, d and e are only found in algae and in certain bacteria.

3.7.1. Collection and identification of plant sample

Hydrilla verticillata (submerged aquatic plant) plants with leaf were collected from their natural habitat in the Kalpani Beel of Assam, India. The plant was indentified with the help of expert from Botanical Survey of India (BSI), Shillong, vide letter NO. BSI/ERC/Tech/2017/78 dated 16-05-2017.

3.7.2 Method

For the analysis of photosynthetic pigments of *Hydrilla verticillata* plant sample. The standard method for examination of photosynthetic pigments (Sadasivam and Manickam, 2015) was adopted

3.7.3. Procedure

1gm of plant sample was taken into a clean mortar and grinded the tissue to a fine pulp with the addition of 20 ml of 80% acetone. Thencentrifuged (5,000 rpm for 5 min) and transferred the supernatant to a 100 ml volumetric flask. Again, grinded the residue with 20 ml of 80% acetone, centrifuged and transfered the supernatant to the same volumetric flask. Repeated this procedure until the residue is colourless. The mortar and pestle were washed with 80% acetone and collected the clear washings in the volumetric flask and made up the volume to 100 ml with 80% acetone. Separation of the extract was achieved by red in spectrophotometer. Identification of the compound was done by UV- visible spectrometry (Analytic xena 4250, Germany Comp) and then comparing these date with the data present in the chemical library.

3.8 Effect of *Hydrilla verticillata* Formulated Fish Feed on Growth and Muscle Composition in *Labeo rohita* fingerlings

3.8.1 Experimental set up

Twenty-four fingerlings of *Labeo rohita* Ham.(Rohu) were obtained from a private fish farm. The weight of the individuals ranged from 9.31 g to 9.53 g. and length 7.50 cm to 7.80 cm. The fish were acclimated to experimental conditions for a fortnight prior to the start of the trail. During acclimatization period the fish were

fed with traditional feed (rice bran and mustard oil cake in ratio 1:1). After acclimatization the fingerlings were kept under starvation for two days prior to the commencement of the experiment.

3.8.2 Feed preparation and formulation

Hydrilla verticillata plants were collected from Kalpani Beel of Assasm. The samples were washed thoroughly under tap water and air dried under shade for two weeks. The dried plant material was pulverized to fine powder and filtered through 345-micron pore size sieve. Rice bran, mustard oil cake, dry fish, dry silk-worm pupae powder and dry meat powder were collected from local markets in Kokrajhar, dried, pulverized, sieved through 345-micron sieve and stored. Vitamin and mineral premix were obtained from Zincovit, manufactured in India by apex laboratories private limited, Tamil Nadu. Formulated feeds containing 30%, 40% and 50% *Hydrilla verticillta* powder were designated as experimental diets H30, H40 and H50 respectively. A control diet without *Hydrilla verticillta* powder was designated as CD. The ingredients and proportion of the formulated feeds mixture are presented in the Table 5 (Sivani *et al.* 2013; slight modified).

Table 5: Composition of formulated feed (g/100g)

Sl. No	Ingredients (g/100g)	Control diet(CD)	Experimental diets		
			H 30	H 40	H 50
1	Rice bran	39	24	19	14
2	Mustard oil cake	39	24	19	14
3	Soyabean meal	10	10	10	10
4	Dry fish meal	03	03	03	03
5	Silk-worm pupa powder (dry)	06	06	06	06
6	Dry meat powder (mutton)	02	02	02	02
7	*Hydrilla verticillata* powder	---	30	40	50
8	Vitamin &Mineral pre mix	01	01	01	01

The feed ingredients for each of the four diet plans were measured and mixed thoroughly using a food mixer. Sufficient quantity of water was added to the mixtures and hand kneaded to get the required soft consistency. Each of the four mixtures was cooked separately in a pressure cooker for 30 min. The cooked feed was cooled by spreading and air drying. After cooling the vitamin and mineral premix was added and mixed uniformly.

3.8.3 Nutritional analysis of the formulated feed mixtures

Proximate composition of each formulated feed was analyzed by following standard methods. Moisture content, crude protein, crude fat, crude ash and crude fibers were investigated following AOAC (2005) analytical techniques. Total solids and carbohydrate obtained as nitrogen free extract- NFE were calculated by the formula of James (1995). Nutritive value (Total energy value in K Cal/g) was calculated by the formula of FAO, (2003).

3.8.3.1 Determination of moisture

A sample of 3 gm was spread uniformly and dried in a hot air oven for 3 hours at 105^0c. Sample was transferred to a desicator to be cooled and weighed. The differences in weight represented the loss of moisture and were expressed as a percentage of oven dried sample (AOAC 2005).

Calculation:

$$\text{Moisture (\%)} = \frac{W_1 - W_2}{W_1} \times 100$$

W_1= weight (gm) of sample before drying

W_2 =weight (gm) of sample after drying

3.8.3.2 Determination of total solids

Subtraction of the moisture percent from 100 represents the total solid. Total solids were estimated by subtracting the value of moisture from 100 (James, 1995).

Calculation:

Total solids (%) =100 - Percent of moisture

3.8.3.3 Determination of crude protein

Total nitrogen and crude protein was estimated using micro Kjeldahl method (AOAC 2005). 20 ml. of concentrated sulphuric acid was added to 1 g of sample in a digestion flask followed by 9.6 g potassium sulphate and 0.4 g copper sulphate and heated gently until frothing ceased. Solution was boiled briskly until clear, allowed to cool and added 60 ml. of distilled water. Immediately the flask was connected to digestion bulb on condenser with tip of condenser immersed in standard acid with 5-7 drops of mix indicator in receiver. The flask was heated until all ammonia was distilled. The receiver was removed and the tip of the condenser washed. The distilled excess standard acid was titrated against standard NaOH solution.

Calculation:

$$\text{Nitrogen \%} = \frac{\text{(Sample titre – black titre) x Normality of HCl x 14 x 100)}}{\text{Weight of sample x 1000}}$$

Protein % =Nitrogen % $\times$ 6.25

6.25 = The protein nitrogen conversation factor for fish and its by-product.

3.8.3.4 Determination of total fat

Total fat in the sample was estimated in a Soxhlet apparatus (AOAC 2005). In a filter paper 3 gm of sample was weighed and wrapped carefully. The wrapped sample was introduced into the extraction thimble of the Soxhlet. 250 ml of petroleum ether was filled into the round bottom flask and placed on the heating mantle. The sample was heated about 14 hours at a heat rate of 150 drop/min. The solvent was evaporated by using vacuum condenser. The bottle was incubated at 90^0c until solvent was completely evaporated and the bottle completely dried. The

bottle was transferred to a dessicator with partially covered lid and allowed to cool. The bottle and its dried content were reweighed.

Calculation:

$$\text{Fat (\%)} = \frac{\text{Weight of fat}}{\text{Weight of sample}} \times 1000$$

3.8.3.5 Determination of crude fibre

Fats from sample were extracted with petroleum ether and the sample dried. 2 g of dried sample was boiled with 200 ml sulphuric acid (H_2SO_4) for 30 minutes with bumping chips. It was then filtered through muslin cloth and washed with 25 ml of boiling H_2SO_4, three portions of 500 ml of water and 25ml of alcohol. The residue was transferred to an ashing dish (w_1). After drying the residue was reweighed (w_2) and ignited for 30 minutes at 600^0C, cooled and reweighed (w_3) (AOAC 2005).

Calculation:

Loss in weight= $(w_2\text{-}w_1) - (w_3 - w_1)$

$$\text{Fibers (\%)} = \frac{\text{On ignition}}{\text{Weight of sample (g)}} \times 100$$

3.8.3.6 Determination of ash

A muffle furnace was used to analyze a 5 g sample for total ash (AOAC 2005). The sample was heated in a silica crucible over low Bunsen flame with half covered lid. When fumes were no longer produced, the crucible and lid were placed in muffle furnace and heated at 550^0 c overnight. After complete heating the lid was placed on the crucible to prevent loss of fluffy ash and cooled in a dessicator. Weight was taken when the sample turned grey.

Calculation:

$$\text{Ash (\%)} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

3.8.3.7 Determination of available carbohydrates

Carbohydrate content of a sample was determined by calculating the percent remaining after all the other components had been measured (James, 1995).

Calculation:

Available carbohydrates (%) = 100 – (protein + Ash + Moisture + Fat + Fiber)

3.8.3.8 Determination of nutritive value

The method described by FAO 2003 was followed to estimate the total energy value in Kcal /100 gm.

Calculation:

Nutritive value = 4 ×protein percent + 9 ×fat percent + 4 ×percentage of carbohydrate (FAO, 2003)

3.8.4 Feeding Regimes

Feeding trails were conducted for a period of 60 days starting from 5th October, 2019 to 5th December, 2019. Four groups of *L.rohita* fingerlingers, where each group consisted of six fishes were kept in four glass aquarium sized 2 ½ x 1 ½ x 1 ½ feet. Two third (2/3) of each aquarium were filled with water. Based on the four experimental diets prepared as presented in Table 5.The four groups were designated as H30, H40, H50 and CD. Initial body weight and body length of fingerlings of each unit (N=6) were recorded using an electronic compact scale (KERR, BL, 5002) and wooden scale at fortnight intervals. A single serving of *ad libitum* experimental diet was received by each group every morning at 8.00 am.

Leftover feeds were collected daily, dried and weighted. Water in the aquarium was constantly aerated and 25% of water was daily exchanged.

3.8.5 Growth parameters

After the period of 60days of feeding trial the fishes were collected from the aquarium with the help of fishing nets. The fishes were weighted and the number of survivors noted. The weight gain in experimental diet fed fishes were measured with the help of an electronic balance and calculated by the formulae specified by Hari and Kurup (2003).

3.8.5.1 Weight Gain (WG)

Calculation:

Weight gain (WG) = Average of final body weight–Average of initial body weight

3.8.5.2 Percentage Weight Gain (PWG)

Calculation:

$$\text{Weight gain (WG) (\%)} = \frac{\text{(Final body weight – Initial body weight)}}{\text{Initial body weight}} \times 100$$

3.8.5.3 Length Gain (LG)

Length gain by the fishes was measured using a wooden scale and calculated by the method of Hari and Kurup (2003) by using the formula:

Calculation:

$$\text{Length gain (LG) (\%)} = \frac{\text{(Final length – initial length)}}{\text{Initial length}} \times 100$$

3.8.5.4 Specific Growth Rate (SGR)

Specific growth rate (SGR) is important for minute monitoring of growth as it indicates the increase in cell mass per unit time. It was calculated by the formula given by Hari and Kurup (2003).

Calculation:

$$\text{Specific growth rate (SGR) (\%)} = \frac{(\text{Log } w_t - \text{Log } w_o)}{D_t} \times 100$$

Where, W_t= Final weight

W_o = Initial weight

D_t = Rearing period in days

3.8.5.5 Average Condition Factor (ACF)

Average condition Factor ACF is a qualitative measurement based on visual assessment of fishes. Changes in general shape of fishes including length, weight and its all-round appearance gives an idea of the condition of the fish. ACF can be calculated from the results of WG and LG Hari and Kurup (2003).

Calculation:

$$\text{Average condition factor (ACF)} = \frac{\text{Final weight}}{(\text{Final length})^3} \times 100$$

3.8.5.6 Percentage of Survival Rate (PSR)

Survival rate (SR) is an important parameter which screens out the effect of mortality from any cause. It is calculated by the formula:

$$\text{Survival rate (SR) (\%)} = \frac{\text{Number of fish survived}}{\text{Number of fish stocked}} \times 100$$

3.8.6 Cost analysis of the formulated feeds

The cost of production of *H.verticillata* formulated feeds were compared to the production cost of other commercial formulated fish feeds available in the market.

3.8.7 Proximate composition of fish muscle

For analysis of proximate composition in *Labeo rohita* muscle, the fishes were killed and muscles were detached from the bones. The muscles were dried, pulverized, sieved and analyzed for moisture (AOAC, 2005), total solids (James, 1995), proteins (AOAC, 2005), lipids (AOAC, 2005), ash (AOAC, 2005) nitrogen free extract (James,1995) and calorific value (FAO, 2003) by the same methods as followed in the section 3.8.3 (Nutritional analysis of the formulated feed mixtures).