Chapter 4: Methods and Materials

This chapter includes the descriptions of the methods, materials, procedures etc that are followed in this work. They are

1. Habitat Survey
2. Collection and identification of sample
3. Climatic parameter of habitat
4. Food spectrum analysis
5. Biotic Component Analysis
6. Analysis of physico chemical parameters: test of pH, Dissolved O2,
   Free CO2, Chloride, Nitrate, phosphate, Alkalinity, Transparency,
   Total Hardness
7. Elemental determination
   Determination of elements in water
   Determination of elements in soil
   Determination of elements in various tissues
8. Estimation of phospholipids
9. Determination of inorganic phosphate
10. Ultra structure of stomach, duodenum, Liver and intestine
    by SEM and TEM
11. Ex-situ hatching
12. Statistical analysis
1. Habitat survey:

Habitat survey was done following the active search and trial guided by local people as well as fisherman. The catch areas were identified on the basis of information about the availability and distribution of various turtle species Viz. *A hurum*, *Nilssonia nigricans*, *P tecta*, *P.smithi* and *P. tentoria*. Survey period was selected based on the egg laying season (1st week of October to early December of a year) and egg hatching season (Late May to early July of a year).

(A) *Lahori Char*, Nagaon District, Assam with an area between about 20 square kilometer during egg hatching season (EHS) to about 100 square kilometer during egg laying season (ELS).

**Latitude;** 26°27'37.33" N, **Longitude;** 092°24'12.57" E  **Altitude;** 179 ft from MSL

(B) *Sitalmari char*, Nagaon District, Assam with an area of about 15 square kilometer during egg laying season (ELS) to about 80 square kilometer during egg hatching season (EHS).

**Latitude;** 26°33'y 13.32" N  **Longitude;** 92°29'12.57" E  **Altitude;** 179 ft from MSL

The two chars are nearby chars and the distance between them is about 20 kilometers. The distance of the nearest land point at Lahori char from the bank of the river Brahmaputra at Moirabari of undivided Nagaon District is about 40 kilometers and it takes about 4 hours to reach by a motorized boat.

The following items were considered during the survey period. Soil thermometer, GPS, Balance, Digital pH meter (Systronic), Seechi disk, and volumetric flask, Vernier calipers, Slide calipers, Scale, Fishing net, Video camera, Boat (normal and motorized), Chemicals and accessories. Major survey was repeated for eight times during egg hatching and egg laying season. Seasonal survey was marked for climatic parameters of the habitat site at least 2 days in every month during the experimental period.
2. Collection and identification of the species:

Identification of the species was performed after Das (1995). Live specimen were collected without causing injury, each specimen was measured with the help of vernier caliper for straight carapace length (SCL) carved carapace width (SCW) and shell height (SH). Scute and bone terminology of the shell were noted after Zangeri (1969). The dead specimen of the species were collected from the study site and preserved as well as measured for curved carapace length (CCL), straight carapace length (SCL), curved carapace width (CCW), shell height (SH), body weight.

3. Climatic parameters of habitat:

Rainfall: The total amount of rainfall over a given period is expressed as the depth of water which would cover a horizontal area if there is no run-off, infiltration and evaporation. This is generally expressed in millimeters. Accuracy of rainfall measurement is mainly affected by wind, by the height of gauge and exposure. Wind and exposure errors can be very large, even more than 50%. The catch of rainfall is a function of the height of the gauge; the more open the location, the greater will be the difference in the catch with height. Measurements were taken at the same time each morning. If it was raining at the time of observation, measurement were taken quickly to avoid loss of catch. The rain gauge was used to measure the rainfall on 24 hours basis.

Temperature: Air and water temperature was measured with the help of maximum and minimum thermometer in °C. Soil temperature was determined by soil thermometer at a depth of 8-10 cm.

Relative humidity: Digital Hygrometer was used to record min-max relative humidity in 24 hours time.


The mouth of a turtle is held open and a plastic tube is inserted into the esophagus. The jaws of more pugnacious turtles can be partly immobilized with a rubber stopper
of appropriate size placed near the angulis oris. Slight twisting of the tube during insertion insures smooth entry without doubling over. Resistance can be felt when the tube reaches the pyloric part. Injection of some water during insertion seems to lubricate to passage of the tube.

When the tube is in the stomach the turtle is held vertically or at 45° angle over a square of gauze placed on a sieve and a continuous stream of water is pumped into the stomach. Turtles up to 200-250 mm carapace length can be easily held with one hand. Drinking water or water from local habitat is used for injection. Water injection has been accomplished thus far with a continuous pump syringe. This consists of an ordinary 10 cm³ hypodermic syringe fitted with the following: 2 metal finger rings on the barrel and 1 thumb ring on the plunger, permitting one-handed operation for filling and emptying it through another.

Small whole animals (larvae, insects, crustaceans, etc.) are normally flash up first in a loose suspension. Vegetable material, especially algal, is more likely to come out as a solidly-packed mass. In a successful stomach flushing, the last food material to emerge is usually in a mucus-coated mass (and is a reliable indicator that the stomach had been completely flushed. Materials flushed from the stomach were loosely wrapped in the gauze square, placed in a marked vial and preserved in 10% Formalin solution. Preliminary notes on the stomach contents were usually made at the time of flushing.

5. Biotic Component analysis:

Plankton i.e. zooplankton and phytoplankton samples were collected from the water bodies of Pangshura tentoria habitats by towing into bolting silk plankton net and were preserved in 5% formaldehyde solution. For identification available standard procedure and literature was followed (Verlecar and Desai, 2004).
6. Analysis of physico-chemical parameters:

All the chemicals used were E-Mark or Hi- Media grade.

**Test of pH:** The pH of a solution denotes the intensity of the acidity or alkalinity of a solution and is defined by the concentration of hydrogen ion in gram ion per liter. The pH scales runs from 0 to 14 with 7.0 being neutral. From 7.0 to 0, the solution becomes more and more acidic and from 7.0 to 14, the solution becomes more and more alkaline. Much surface water has pH between 6.0 - 8.0.

Apparatus used: Systronic digital pH meter.

**Dissolved oxygen (DO):** DO was estimated using modified Winkler’s method (Trivedy et al., 1987). It was allowed to react with Ionized Iodine and I2 which was then titrated against standard sodium thiosulphate solution (0.025N) using starch as indicator. The interference due to high organic matter and chloride content was removed by use of sodium oxide in the Winkers reagent.

\[
\text{MnSO}_4 + 2\text{KOH} = \text{MnO(OH)}_2 + \text{K}_2\text{SO}_4
\]

\[
\text{Mn} (\text{OH})_2 + \text{O} = \text{MnO(OH)}_2
\]

\[
\text{MnO(OH)}_2 + 2\text{H}_2\text{SO}_4 + 2\text{KI} - \text{MnSO}_4 + \text{K}_2\text{SO}_4 + 3\text{H}_2\text{O} + \text{I}_2
\]

**Calculation**

Since 1ml of 0.025N Sodium thiosulphate is equivalent to 0.2 mg Oxygen, therefore,

\[
\text{DO (mg/L)} = \left[ \frac{\text{ml.N of Sodium thiosulphate}}{V_2xV_1 - V} \right] x 8 x 1000
\]

Where

- \(V_1\) = Volume of the sample (ml)
- \(V_2\) = Volume of the titrate use
- \(V\) = Volume of MnSO4 and KI added.
- \(N\) = Normality of the titrate.
Free CO₂: Free Carbon dioxide (FCO₂) was calculated by titration method followed after Jhingran and Pullin (1988). FCO₂ reacts with NaOH to form Na₂CO₃, which was then titrated with N/4 sodium hydroxide near the PH 8.3, which developed pink colour. The development of pink colour indicates the completion of the reaction. The reading of titrate was noted down and FCO₂ was calculated as

\[ FCO₂ = \frac{\nu \times 1000}{V} \]

Where \( \nu = \) Volume of titrates, \( V = \) Volume of the sample taken.

Chloride:
Reagent required:
A. Potassium chromate indicator solution: 50 g of K₂CrO₄ was dissolved in distilled water. AgNO₃ solution was then added until a definite red precipitate was formed.

The solution was then allowed to stand for 12 hours, filtered and diluted to 1 liter.
B. Standard silver nitrate solution, 0.0282 N: 4.790 g of AgNO₃ was dissolve in distilled water and diluted to 1 liter. The solution was then standardized against 0.0282 N NaCl solutions.
C. Standard sodium chloride, 0.0282 N: 1.648 g of NaCl (dried at 140°C) was dissolved in distilled water and diluted to 1 liter.

Procedure: 10 ml of sample was diluted to 100 ml. The sample was then titrated in the pH range of 7-10. The pH of the sample was adjusted with NaOH. Then 1.0 ml of K₂CrO₄ indicator solution was added. Then, the solution was titrated with standard AgNO₃ titrant to pinkish -yellow end point. AgNO₃ titrant was standardized and reagent blank value was established by titration method. A blank of 0.3 ml was prepared.

Calculation;

\[ Chloride (mg/L) = \frac{(A - B) \times 1000}{ml \ of \ sample} \]
Where
A = ml of AgNO$_3$ required for sample;
B = ml of AgNO$_3$ required for blank.

Nitrate:
Nitrate reacts with phenol disulphonic acid to produce nitro-derivative that in alkaline solution rearranges its structure to form a yellow colour compound that obeys Beer’s Law. The intensity of the yellow colour produced is directly related to nitrate concentration. The colour developed in an unknown sample is compared to that developed by known concentration using Nessler’s tubes.

Apparatus used: Nessler’s tube of 100 ml capacity.

Reagent required:
A. Phenoldisulphonic acid
B. Potassium hydroxide (KOH) solution.
C. Nitrate solution.

The table used for measuring the Nitrate as N in mg/L

<table>
<thead>
<tr>
<th>Nessler’s Tube (50ml) No.</th>
<th>Standard Nitrate solution (ml)</th>
<th>Nitrate Nitrogen added (ml)</th>
<th>Equivalent as N content based on 100 ml of sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>0.001</td>
<td>0.01</td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
<td>0.002</td>
<td>0.02</td>
</tr>
<tr>
<td>4</td>
<td>0.4</td>
<td>0.004</td>
<td>0.04</td>
</tr>
<tr>
<td>5</td>
<td>0.6</td>
<td>0.006</td>
<td>0.06</td>
</tr>
<tr>
<td>6</td>
<td>0.8</td>
<td>0.008</td>
<td>0.08</td>
</tr>
<tr>
<td>7</td>
<td>1.0</td>
<td>0.01</td>
<td>0.10</td>
</tr>
<tr>
<td>8</td>
<td>2.0</td>
<td>0.02</td>
<td>0.20</td>
</tr>
<tr>
<td>9</td>
<td>4.0</td>
<td>0.04</td>
<td>0.40</td>
</tr>
<tr>
<td>10</td>
<td>6.0</td>
<td>0.06</td>
<td>0.60</td>
</tr>
<tr>
<td>11</td>
<td>8.0</td>
<td>0.08</td>
<td>0.80</td>
</tr>
<tr>
<td>12</td>
<td>10.0</td>
<td>0.10</td>
<td>1.00</td>
</tr>
<tr>
<td>13</td>
<td>20.0</td>
<td>0.20</td>
<td>2.00</td>
</tr>
<tr>
<td>14</td>
<td>30.0</td>
<td>0.30</td>
<td>3.00</td>
</tr>
<tr>
<td>15</td>
<td>40.0</td>
<td>0.40</td>
<td>4.00</td>
</tr>
</tbody>
</table>
Procedure: 100 ml of the sample was taken with pH adjusted to 7.0 and it was evaporated to dryness on water bath in a china dish. The residue was then dissolved in 2ml phenol disulphonic acid and the solution was mixed thoroughly with the glass rod. The solution was then diluted and transferred to Nessler’s tube, KOH solution was added drop wise with stirring until the maximum colour developed. The colour of the sample was matched visually against that of the nitrate comparison standards. NO₂ was recorded as N in mg/L.

Phosphate:
Phosphate occurs in natural water and in waste water almost solely in the form of various types of phosphate. These forms are commonly classified into Orthophosphates, condensed phosphates (pyro-, meta- and polyphosphates) and organically bound phosphates. These forms of phosphates may occur in the soluble forms, in particles of detritus or in the bodies of aquatic organisms.

Reagents required:
A. Ethyl alcohol (95%)
B. Sulphuric acid solution, (5N): 10ml H₂SO₄ was diluted with 500ml distilled water.
C. Antimony Potassium Tartrate: 4.3888g K (SbO) C₄H₄O₆. 1/2H₂O was dissolved in 200ml distilled water and the solution was stored in a bottle at 4°C.
D. Procedure: 20ml of sample was pipetted into a clean, dry test tube. 1ml ethyl alcohol was added. The solution was then mixed thoroughly. Then 1ml of combined reagent was added and mixed thoroughly. The solution was then allowed to stand for 10 mins for colour development before reading in a Spectrophotometer at a wave length of 880 nm.

Calculation:

\[ PO₄ (mg/L) = \frac{mg \cdot P \cdot x \cdot 1000}{ml \cdot of \cdot sample} \cdot 3.06 \]
Alkalinity (AT):

Total alkalinity is determined by the titration method of phenolphthalein indicator (Hooda and Kaur, 1999). Therefore its alkalinity, bicarbonate, carbonates and hydroxides are considered as the main bases in natural water. If water contains bases then it turns yellow in presence of methyl orange indicator.

If water sample contains carbonate then it turns pink in addition of phenolphthalein indicator (PH about 8.4) and measurement of alkalinity is done by titrating the sample with phenolphthalein indicator. In the first stage, the water sample when titrate with phenolphthalein indicator, its carbonate content is converted into bicarbonate.

\[ CO_3^- + \text{H}^+ = \text{HCO}_3^- \]

In the second stage of titration the methyl orange was added to the above sample and the titration continued till the end point achieved and here all the bicarbonate are converted into CO2 and \( \text{H}_2\text{O} \).

\[ \text{HCO}_3^- + \text{H}^+ = \text{H}_2\text{O} + \text{CO}_2 \]

The titrate value of alkalinity is calculated with the following formula.

Phenolphthalein alkalinity in mg/L

\[ \text{CaCO}_3 = \frac{\text{ml of titrant for M} \times N \times 50 \times 1000}{\text{Sample volume(ml)}} \]

Where N is the normality of the titrant (HCl)

Transparency:

Transparency of water was determined by using Seechi’s disc (Hooda and Kaur 1999). The average transparency is calculated as

\[ \text{Transparency(cm)} = \frac{A + B}{2} \]

Where, \( A = \text{Depth of disappearance} \) and \( B = \text{Depth of reappearance} \)
Total hardness:

Total hardness was estimated following the procedure of Hooda and Kaur (1999). Total hardness of the water is the concentration of alkaline earth cations (e.g., Ca$^{++}$, Fe and Mg$^{++}$). Eriochrome black T forms wine red complex compound with metal ions (Ca$^{++}$ and Mg$^{++}$). The ethylene diamine tetra acetic acid di sodium salt (EDTA) extracts the metal ion from the dye metal ion complex as colorless chelae complex leaving a blue colored aqueous solution of the dye.

$$Ca^{2+} + Mg^{2+} + Ca and Mg eri chrome (wine red).$$

Black T + EDTA Ca $\rightarrow$ EDTA + Mg EDTA + Eri chrome black – T (Blue).

Calculation:

$$Total\perp hardness(mg/L\ per\ CaCO_3) = \frac{ml.\ of\ nitrate\ used\ x\ .1000}{ml.\ of\ sample}$$

The total hardness of river water had been recorded seasonally and the volume was recorded as mg/L.

7. Element determination:

Elemental estimation was carried out with the help of Atomic Absorption Spectrophotometer (Perkin Elmer 3110) at Sophisticated Analytical Instrumentation Facility (SAIF), North Eastern Hill University, Shillong, India.

(A) Water: Element estimation in water was followed after Fishman and Downs (1966). Samples were collected in plastic container and the slandered solutions were prepared, except calcium and manganese, by diluting the stock solution with 5% Lanthanum solution and 1N HCl. Each sample was filtered through 0.45 micron micro pore filter aspirated directly. The concentration of the element was determined
by using routine procedure except calcium and manganese, where the results were corrected by using a reagent blank.

(B) Soil: Estimation for elements in soil was followed after the method of Pawluk (1967). Soil collected was made air dried and crushed. 2.5 g soil was weighted in 125 ml Erlenmeyer flask and to it, 25 ml of 1N NH₄OH (pH 7.0) was added. The sample was placed in a shaker for 15 minutes. The solution was filtered and was analyzed by AAS. Using the routine procedure, concentration of the element of interest was determined. Standards were prepared by suitable dilution of the stock with the extractant. The concentration of Ca and Mg was determined by flame atomic absorption.

(C) Tissue: Only matured female specimen were collected (adult female before egg laying, during August to September in a year) termed as (AH) and adult female after egg laying (during May to July) termed as (AL) for element determination. The specimens were found killed due to trapping by the local fisherman.

About 5gm accurately weighed formalinized tissue was placed in a conical flask, 10ml ml of 1:2 mixture of concentrated HNO₃ and HClO₄ were added to the tissue and the solution was boiled till the solution was clear. The volume of the digested solution was measured and diluted to 100 ml with deionized water. The final dilution with de-ionized water (100 ml) was adjusted to ensure that, the concentration falls within suitable absorption range (Kahnke, 1966).

8. Estimation of Phospholipids:

Phospholipids of blood, tissue and yolk of egg is found as Lecithin, Cephalin, Sphingomyelin, phosphatidylserine ect. which are obtained by extraction of the tissue with certain non aqueous solvents. The simplest procedure for the determination of phospholipids in the analysis of liquid containing extract for total phosphorous as described below i.e. isolation of phospholipids from non lipid phosphorous by
precipitation with acetone and magnesium chloride and determination of the phospholipids titrimetically after oxidation with chromic acid

**Principle:** The extracted lipids were oxidized with sulphuric acid and hydrogen peroxide and the phosphate present was determined photo metrically.

9. **Determination of inorganic phosphate:**

A: Standard phosphate solution: Dissolved exactly 0.351g of pure di mono potassium phosphate in water and transferred quantitatively to a one liter volumetric flask. 10ml of 1N sulphuric acid was diluted to the mark with water and mixed. This solution contained 0.4 mg of phosphorus in 5 ml and is stable indefinitely.

B: 10N Sulphuric acid solution: Carefully added 45 ml of concentrated sulphuric acid to 1300 ml of water. 10ml of this solution diluted to 100ml in a volumetric flask, mixed and titrated to a 10ml portion of standard 1N Sodium hydroxide. From titration the results adjusted the original solution, if necessary, to make it exactly 10N.

**Procedure:** Transfer 18ml of alcohol ether mixture to a wide mouthed test tube (15 by 20 mm ) graduated at 20 ml and dropped to it slowly 1ml of yolk extract, mixed and placed the tube in a boiling water bath. After five minutes the tube was removed and allowed it to cool at room temperature. The solution was marked up to 20 ml with alcohol ether mixture, mixed and filtered and transferred 8 ml filtrate to a 200 ml Pyrex test tube, with the addition of silica (from broken silica wire) placed in a wire rack containing a wire bottom over an electric hot plate and evaporated to dryness.

A 25ml of 5N Sulphuric acid was added to residue in the tube and allowed it to be digested over the hot plate with colour development at a final volume of 25 ml, a different was however, used containing only half as much inorganic phosphate i.e.
0.5ml of standard phosphate solution containing 0.04 mg of inorganic phosphate. The condition for photometric measurement is the same for acid soluble phosphate.

Calculation: Since 8ml of extract represent 0.4ml of original sample, calculation of result is similar to that for acid soluble phosphorous except that the value 0.04 replaces 0.08 in the calculation, corresponding to the use of half as strong a standard or calculate as acid soluble phosphorous and divide the result by 2 to obtain mg of lipid phosphorous per 100 ml of original sample.

10. Ultra structure of Stomach, Duodenum, Liver and Intestine:

A. Scanning Electron Micrograph:

The duodenum, stomach, liver and intestine were fixed in 2.5% glutaraldehyde prepared in 0.1N Sodium Cacodylate buffer at PH 7.2 to 7.4 at 4°C. Washed in buffer for overnight, post fixed in 1% buffered Osmium tetroxide for one hour and dehydrated through increasing concentration of acetone. The dehydrated samples were dried either in the critical point drier (Samdri Pvt Tousimis ) using acetone as the intermediate fluid and CO₂ as transitional fluid or by TMS drying technique (Day et al., 1989). In this technique the dehydrated samples were dipped in tetramethylesilame (TMS) at 4°C for 10 minutes and process was repeated for another 10 minutes. The samples from TMS were placed in a glass slide and dried at room temperature. The samples were then secured horizontally to brass stub (10mm to 12mm) with double coated adhesive tape connected via patch of silver paint to ensure change conduction. A conductive coating was applied to the sample using TFC 1100 (Jeol) ion sputter coater. A relative low vacuum (10-3 tor) was established in the sputtering chamber, and gold was used as the target material. The preparations were examined with SEM, JSM – 35 CF (Jeol) using the secondary electron emission made at an accelerating voltage of 15 KV (SAIF NEHU, Shillong)

B. Transmission Electron Micrograph:

The samples were fixed in 2.5 % gluteraldehyde prepared in 0.1M Sodium cacodylate buffer at PH 7.2 to 7.4 for 4 hours at 4°C which was collected instantly after dissection. After 4 hours, the samples were washed in 0.1M sodium cacodylate
buffer and post fixed in 1% Osmium tetra oxide (OSO₄) solution for 1 hour at 40°C. After post fixation, the samples were again washed in buffer solution for 30 minutes with two changes. Dehydration of the post fixed tissues was carried out gradually by using increasing concentration of acetone with 3 changes in each step. The dehydrated samples were subjected to toluene for 5 minutes. After cleaning the infiltration, embedding was done by using araldite liquid resin containing araldite cy212, dedecenyl succinic anhydrate (DDSA), tridimethyl amino -methyl phenol (DMP) and plasticizer. After infiltration, the tissue was then polymerized, initially at 500°C and gradually raised to 600°C. After 48 hours, the block was ready. The raised blocks were sectioned at 600 Å thicknesses using ultra microtome. Double staining of sections were done with uranyl acetate stain for 30 minutes at room temperature followed by lead citrate stain at different time intervals. The stain preparations were examined with Transmission Electron Microscope (TEM) 100.2 (Jeol).

11. Ex-situ hatching:

Methods:

Batch of egg clusters collected from the original nesting site(s) were examined, weighed and implanted at a depth of 8-10 cm in the sandy soils of the river Brahmaputra with proper marking. Occasional checking had been performed during the study period. The soil temperature was maintained at (naturally) 28°C±2°C. Soil moisture was also recorded.

On the other hand, collected eggs were implanted in plastic trough of at a depth of 20 cm, where coconut husks were placed (3-4 pieces; photograph -7e). Over the husk, sandy soil collected from the egg collection site(s) were kept to which the eggs were implanted at a distance of 10 cm apart. However, soil moisture, temperature was continuously monitored. For retention of moisture, the sandy soils were occasionally sprayed over by water spray. The process (as it is) was allowed to continue for 8
months 12 days (2.10.04 to 12.06.05, 10.10.05 to 11.06.06, 8.10.06 to 15.06.07). The same process was repeated for 3 consecutive years.

12. Statistical analysis:
Analysis of variation (ANOVA) was done for the element profile and critical difference (CD) was calculated at 5%. The experimental data expressed as mean values. Standard deviation (± SD) was analyzed by using fisher’s (1963) method of ANOVA (one way) and subjected to CD calculation (Raghurumulu, 1983). For the evaluation of significance the CD was calculated by using the following formula.

\[
CD = \frac{t^*}{\text{EMS}} \cdot \left(\frac{1}{K_1} + \frac{1}{K_2}\right)
\]

Where, \(t^*\) is the tabulated value of t at the desired level and at the error degree of freedom. EMS is the error means square; \(K_1\) and \(K_2\) are the number of replications in the group compared. CD evaluation was done by Microsoft origin.