CHAPTER-III

Materials and Methods

3.1 The Study Area:

The fisheries, ponds, beels including river of the Kokrajhar district constitute the study area of present work. The districts Kokrajhar covers an area of 3,169.2 Km^2 having a population of 8,86,999 according to census 2011 and population density of 280 per Km^2. Kokrajhar Town is the head quarter of the Bodoland Territorial Council (BTC) located in the extreme north on the north bank of the Brahmaputra river in the state of Assam, in north east region of India, by the foothills of Bhutan. Kokrajhar district is Located at Latitude-26.4^0, Longitude-90.2^0 and having elevation/altitude of 50-43 metres above the sea level. The district shares its border with Bongaigaon District to the East , Dhubri District to the South, Coachbehar District to the west . It is sharing Border with West Bengal State to the west.

Amongst the Tribal population Bodos, Rabhas and less quantity of Garos are the inhabitants in this area. The other communities like Rajbangshis, Sarania, Tea communities, Santhal, Oraon etc. are also resident of BTC. Moreover, other general communities like Bengali, Assamese, Nepali and few numbers of Hindi speaking people are also found in the area.

3.2 Survey on small fish species consumed by the Bodo communities

Some of the Bodo dominated areas of Kokrajhar district were surveyed to know about the small fish species which are popularly consumed by them as food. A set of questionnaires (Appendix-I) had been carried out by interactions with some of the villagers of different age groups. Same types of questions were asked to all of them to get steady information about the highly consumed small fishes.

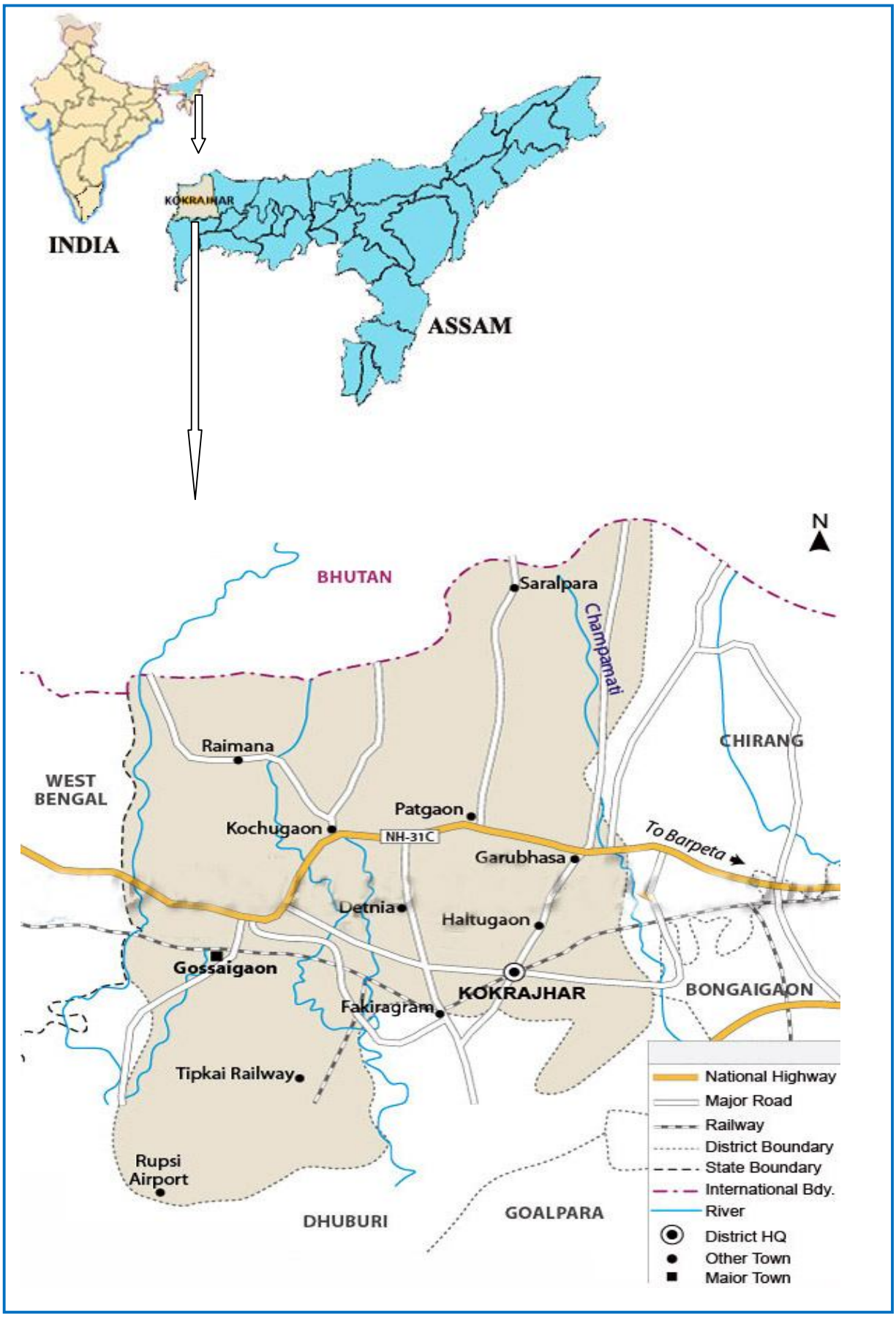

Fig.: 1.3 Map of Kokrajhar district

3.3 Description of the selected species:

i. **Boroli (*Barilius vagra)*:** The colour of this fish with beautiful combination of different shades, is very attractive. It is fish of small size, growing up to 12.5 cm. This species is widely distributed in Asian countries, Afghanistan, Pakistan, India, Nepal, Bangladesh and Sri Lanka. Adults of this fish species live in hill streams with gravelly and rocky bottom (Talwar and Jhingran , 1991.)

ii. **Bothia (*Neoeucirrhichthys maydelli*):** This species have a quite variable colour pattern. It has dark spots on the caudal fin which are quite distinctive. A dark, white-ringed spot can be seen at the centre of the caudal fin. *N. maydelli* also have a truncate or rounded tail (Hamilton, 1822)

This species are distributed in India, Nepal and Bangladesh. In India it's known from the Teesta river system in North Bengal and the Garo Hills in Meghalaya.

iii. **Chanda (*Chanda nama*):** The elongate glassy perchlet, *Chanda nama* is a native to an area of south Asia from Pakistan to Burma, in the Indo-Malaya ecozone. It reaches a maximum overall length of 11 cm. The species inhabits canals, ponds, streams and flooded rice paddies, in both fresh and blackish water and is found in particular abaundance during the rainy season.

iv. **Cheng (*Channa gachua*):** It is a dwarf snakehead fish attains up to 20 cm. The species has an elongated lateral body. The dorsal of this species is greenish and the anal is dirty pale bluish-green; both are edged with black. The upper edge has a red lateral margin and the lower a white sideward band. The caudal fin is rounded and marginally edged, first with black and then red with blue-green interspaces. This species is found in Asian countries from Pakistan to Indonesia. It is found in almost all wetlands in canals, rivers and lakes and especially in muddy water.

v. **Darikana (*Rasbora daniconius*):** The species, *Rasbora daniconius* has an elongated body, oblong and compressed with small mouth (Talwar and Jhingran 1991.). This species is widely distributed in Asia mainly in Mekong, Chao Phraya and Salween basins, northern Malay Peninsula, westwards to the Indus and Sri Lanka (Rainboth, 1996). The species is found in a variety of habitats like ponds, canals, haors, streams, rivers and inundated fields.

vi. **Goroi (*Channa punctatus*):** It is commonly known as spotted snake head. *Channa Punctatus* are small fishes with smaller eyes allocated on the anterior of head. Fish body is slight brown on the back which is faded beneath.

vii. **Kholihona (*Trichogaster fasciata*):** The fish body is strongly compressed dorsal and abdominal profile is equally convex. It is one of the most colourful fish species of the region. Available in both lentic and lotic systems.

viii. **Kokila (*Xenentodon cancila*):** *Xenentodon cancila* (freshwater garfish) is a species of needlefish. This species has an elongate body with long, beak-like jaws filled with teeth. The dorsal and anal fins are positioned far back along the body close to the tail. *(Sterba, 1962)*. The body is silvery-green, darker above and lighter below with a dark band running horizontally along the flank. The male fish often having anal and dorsal fins with a black edge. It can reach upto a length of 40 cm

The freshwater garfish is widely distributed across South and Southeast Asia from India and Sri Lanka to the Malaysian Peninsula *(Riehl, R; Baensch, H 1996)*. The species eats animals such as fish and frogs.

ix. **Mola (*Amblypharyngodon mola*):** It is known as Indian Carpet. The body of *Amblypharyngodon mola* is laterally compressed and dorsal. Their caudal fin is deeply forked with pointed lobes. Dark markings are traced in dorsal and anal fins. The body colour of the fish is light greenish on black and silvery at sides and beneath. Maximum body length is reported to be 8cm (Bhuiyan, 1964) 20 cm (Talwar and Jhingran, 1991) 9.2 cm (Hussain, 1999). There is a conspicuous silvery lateral bound running from gill covers to base of caudal fin.

x. **Turi (*Macrognathus pancalus*):** It is commonly known as Stripped spiny eel. The species inhabits in slow and shallow water of rivers of plains and estuaries. It is a common food fish in the region and also a potential aquarium fish.

3.4 Study of Fish diversity:

A detailed survey of various water bodies of the Kokrajhar district was carried out for one year. Locally used fish traps like *Jekhai*, *Sen*, *Khoka* etc. as well as several fishing gears such as, gill nets, cast net, hooks and lines were used to collect various small fishes from their

habitats. All of the species were photographed in fresh condition and then preserved in 10% formalin solution. The fish species were identified following Talwar and Jhingran (1991) and Vishwanath et al. (2007). The vernacular names of the species were collected by questionnaires with the local people and fisherman and also from the Department. of Fisheries, Kokrajhar branch. Mean total length, breadth and somatic weight of the species were noted down (Table 1.2).

3.5 Preparation of samples:

The muscle for selected fishes was separated from their body just after washing. A sharp blade of stainless steel was used for dressing the fish species except *A. mola* due to the smaller size of the species. *A. Mola* was used in whole for the parameters of samples. The samples were dressed, beheaded, deskinned and filtered as per manual followed by thorough washing with distilled water. The fillet of the fish samples were minced, homogenized, packaged, labelled and stored frozen until analysis. Fishes were thawed and the bone and skin were separated from the flesh to carry on the proximate analysis.

3.6 Proximate composition:

The chemical composition of fish varies significantly between species and also among the individual fishes within the same species with the variation of age, sex, and season. Protein and ash content do not show much variation. The detailed protocols applied in the present study for the analysis of proximate composition of the studied fish species are given below

3.6.1 Moisture:

The moisture content of the fish species was determined by FSSI Lab. Manual.

Procedure: The ground fish sample was taken in a clean dry petri dish and kept in an oven at 105ºC for 2 hours. It was then cooled in a desiccators and weighed (w1).About 10 gm portion of the sample (w2) was taken in the preweighed petri dish and then kept in the oven at 105˚C overnight .The dish was cooled in a desiccator and weighed again (w3).Once again the petri dish was kept in the oven for half an hour and cooled as before and finally weighed to obtain the reproducible weights .

$$\text{Moisture content (\%)} = \frac{(W_2 - W_3)}{(W_2 - W_1)} \text{X } 100$$

3.6.2 Ash:

The ash content of the fish species was determined by FSSI Lab. Manual.

Procedure: Silica crucible was heated to 600 ºC in a muffle furnace for one hour, cooled in a desiccator and weighed (W_1). 2g of dried sample was weighed accurately in to a crucible and heated at low flame by keeping on a clay triangle to char the organic matter (W_2). The charred material was then placed inside the previously set (600 ºC) muffle furnace and heated for 6-8 hrs which gave a grayish white ash. The crucible was cooled in a desiccator and weighed (W_3). The crucible was heated again for further 30 mins to confirm completion of ashing, cooled and weighed again.

Calculation:

$$\text{Ash content (g/100g)} = \frac{W_3 - W_1}{W_2 - W_1} \times 100$$

Where,

W_1= Weight of crucible,

W_2 = Weight of dry matter and crucible,

W_3 = Weight of crucible after ashing

3.6.3 Whole Protein:

The protein content of the fish species was determined by method IS:7219:1973 (RA 2005)

Principle: The nitrogenous compounds in the sample are converted in ammonium sulfate by boiling with concentrated sulfuric acid. Upon distillation with excess alkali, the ammonia is liberated which is estimated by titration with standardized sulfuric acid.

Procedure:

Exactly 0.1-0.2 g of wet sample was weighed in to a Kjeldahl flask. A pinch of digestion mixture (copper sulphate and potassium sulphate were mixed in the ratio 1:8 and finely powdered) and 10 ml of concentrated sulphuric acid was added. It was then digested over a sand bath by heating slowly till the solution starts boiling and then vigorously until the solution becomes colourless. The sample was then cooled and made up to the desired volume (100ml).

A conical flask containing 10 ml of boric acid with few drops of boric acid indicator (pink in colour) was placed at the receiving end of the distillation apparatus in such a way that the tip of the condenser is slightly immersed in boric acid. 5ml of the made up sample was pipetted out in to the distillation apparatus. 10ml of 40% NaOH as shown excess by phenolphthalein indicator was added in to the distillation unit followed by rinsing with little

distilled water. The unit was made air tight. The content was steam distilled for 5minutes. The colour of the solution turns green. The flask was lowered and the condenser tip was washed with little water.

The green solution in the receiving flask is green at this stage. The content was titrated against N/100 sulphuric acid until the original pink colour was restored. The volume of acid used for titration was noted. The distillation and titration process was repeated to get concordant value.

Calculation

1000ml 1N H_2SO_4 =14g N_2

1ml 1 N H_2SO_4 = 0.014g N_2

1ml 0.01 N N/100 H_2SO_4= 0.00014g nitrogen or (0.14/1000)

$$\text{Protein content (\%)} = \frac{\text{(Sample T.V - Blank T.V) x 1.4007 Normality x Protein factor}}{\text{Wt. of Sample}}$$

3.6.4 Crude Lipid:

The crude fat content of the fish species was determined by FSSI Lab mannual.

Procedure:

10 gm of dried fish sample (W_1) was placed in a thumble placed in a soxhlet apparatus and approximately 200ml ether was added and distilled for 16 hrs. After cooling the apparatus, the solvent was filtered in to a pre-weighed conical flask (W_2). The ether was then removed by evaporation and the flask with lipid was dried at 80-100 ºC, cooled in a desicator and weighed (W_3).

Calculation:

The fat content was then calculated using the formula

Fat content (g/100g) = $(W_3\text{-}W_2)/ W_1$ x 100

3.6.5 Carbohydrates:

The carbohydrate content was determined according to AOAC (1995) and it was calculated using the formula:

Carbohydrate content = 100 – (protein (%) + moisture (%) + fat (%) + ash(%))

3.7 Amino acid analysis:

High Performance Liquid Chromatography (HPLC) (method QA.16.5.10/AOAC 19th edition) was employed for determination of amino acid contents of the fish samples.

Preparation of hydrolysed amino acid Sample: About 100 mg of homogenized fish mince was weighed in to a test tube filled with nitrogen and digested at 120 º C for 24 hrs in an oven. The contents of the test tube was cooled and filtered using Whatman No 1 filter paper. The filtrate was then evaporated in a vacuum flash evaporator. The contents were made acid free by repeated washing with distilled water and subsequent evaporation.

HPLC analysis: 20 µL of the hydrolyzed sample was injected in HPLC (1260 Infinity) equipped with a C18 reverse phase (RP) column and a fluorescence detector. The amino acids were identified the concentration of each type of Amino acids were calculated using the formula-

$$\frac{\text{Area of Spl x Std. Conc. x Vol. x Dil x P}}{\text{Wt of Sample x Area of Std x 1000000}}$$

3.8 Fatty acid analysis:

Gas Chromatography- Mass Spectrometry (GC/MS) (method QA.996.06/AOAC 19th edition) was employed to determine the Fatty acid contents in the fish samples.

3.8.1 Preparation of fatty acid methyl ester (FAME):

5g of lipid was taken in a round bottom flask (125mL) and saponified with alcoholic KOH solution (50mL). The mixture was then refluxed for 45 minutes on a water bath. The reaction mixture was then allowed to cool and then neutralized by HCl (5N). Alcohol was removed from the neutralized solution by evaporation over a steam bath. The pH of the solution was adjusted by adding concentrated HCl. The acidified aqueous mixture was then extracted three times with 20 mL ether in a separating funnel. Ether was then removed from the extract to give a fatty acid mixture which was then esterified with methanolic solution of sulphuric acid (0.25 M) After esterification, the mixture was dissolved in ether (25mL) and washed with dilute sodium carbonate solution in a separating funnel until effervescence ceased. It was then washed

with water, dried over anhydrous Na_2SO_4 and finally ether was removed to give fatty acid methyl ester (FAME) mixture.

3.8.2 GC/MS Analysis of the methyl esters of lipids:

The FAMEs were quantified by injecting 1 µL (30:1 split ratio) into GC-MS. The fatty acids were identified and quantified using a GC (Trace GC Ultra, Thermo Scientific) The MS conditions were as follows; ionization voltage 70 eV, Mass range of 45-600 and the scan time equal to the GC run time.

The fatty acids in the mixture were identified by comparing its relative retention volume (Clark JM, 1964) The area of each chromatogram peak was determined by multiplying the height of the peak by width of the peak at one half of the height. The percentage of fatty acid contribution to each peak was calculated. After that response factor (R_i) for each fatty acid was calculated by the following equation.

$R_i = (P_{Si}/ Ps_{C11:0}) \times (W_{C11:0}/W_i)$

Where, P_{Si} = Peak area of individual fatty acid in mixed FAME standard solutions, $Ps_{C11:0}$ = peak area of $C_{11:0}$ fatty acid in mixed FAMEs standard solution, $W_{C11:0}$ = Weight of internal standard in mixed FAMEs standard solution and W_i = Weight of individual FAME in mixed FAMEs standard solution.

The amount of the individual triglycerides, W_{TG} of each samples were then calculated using the following formula.

$$W_{FAMEi} = \frac{Pt_i \times W_{C11:0} \times 1.6007}{Pt_{C11:0} \times R_i}$$

$W_{TGi} = W_{FAMEi} \times f_{TGi}$

Where, P_{ti} = Peak area of fatty acid I in test portion, $Wt_{C11:0}$ = weight of $C_{11:0}$ internal standard in test portion, 1.0067 = conversion of internal standard from TG to FAME, $Pt_{c11:0}$ = peak area of $C_{11:0}$ internal standard in test portion and f_{TG} = conversion factor for FAME to TGs for individual fatty acid.

Retention time of fatty acid of known standards and conversion factor for FAMEs to TGs for individual fatty acids were listed in Appendice-II.

3.9 Mineral Analysis

3.9.1 Analysis of Fe, Zn & Ca:

Atomic Absorption Spectroscopy (AAS) **(**method AOAC 19th edition) was employed for quantitative determination of Fe, Zn and Ca in the fish samples.

Procedure: Chemical analysis for the estimation of the trace elements (Fe, Zn and Ca) in fishes were performed with the Flame Atomic Absorption Spectrophotometer (AAS). The technique involved the following steps:

The stock standard solutions of 100 ppm of Fe, Ca, Zn, salts with deionized water were prepared. Standard solutions of these metal ions were prepared by suitable dilution of the stock standard solutions. The samples of the fish were diluted to a known volume and both the samples and the standard solutions were analysed by a Flame AAS (Model: AAS 280(000)).

Calculation :

The concentration of metal is detected in mg/l and

$$\text{Metal mg/100g} = \frac{\text{Concentration of metal in ppm x volume made}}{\text{weight of sample}}$$

3.9.2 Analysis of Phosphorous:

UV-Visible Spectrophotometer (UV-VIS) (Method IS:1482.8:200) was employed for quantitative determination of Phosphorous content in the fish samples.

Procedure: The ash obtained from the crude ash content analysis was mixed with 5 mL water and 5 mL HCl and boiled for 5 minutes on hot plate. The solution was then cooled to room temperature and brought to sign at 50 mL water. In parallel a procedural blank was also prepared. 10 mL of the prepared solution were transferred into 50 mL calibrated flask and 20 mL of molybdate-ascorbic acid solution was added. Five standard solution of Phosphorous were prepared and 20 mL of molybdate-ascorbic acid solution was added and the sample prepared along with the standard solutions were placed in a metal basket and it was immersed in a boiling water bath for 15 minutes. The flasks were then cooled to room temperature and the colour of the solutions turned blue.

Phosphorous calibration curve solutions

Standard solutions	Blank	Std 1	Std 2	Std 3	Std 4	Std 5
Concentration (mg/L)	0.0	5.0	10.0	20.0	40.0	50.0

The absorbance of the standard solutions and sample solutions were read at wavelength 430 nm in a UV-VIS Spectrophotometer (Cary 60 Model No 2.00)

The Phosphorous content were expressed in mg/100g and was calculated using the formula

Phosphorous content (mg/100g) = $(V_2/V_1)xC_{read}$ x 50 / m

Where, m = Weight of sample, V_1 = Volume of solution utilized for colour reaction, V_2= Volume where the ash was brought to 50 mL and C_{read}= Amount of phosphorous from calibration curve.

3.10 Vitamin Analysis:

High Performance Liquid Chromatography (HPLC) (Method QA:16.5.3/AOAC 19th edition) was employed for determination of Vitamin A and D in the fish samples.

Procedure: Oil extracted from fish meat as described (Folch et al., 1957) earlier under fatty acids was used for analysis of fat soluble vitamins. About 0.15 g fish oil was refluxed with 25 mL methanol and 150% potassium hydroxide (KOH) in water bath for 30 min. It was then extracted with 50 mL petroleum ether. The petroleum ether layer was collected, concentrated and dissolved in 5mL acetonitrile (ACN). 100 µL of sample is then injected in a HPLC (HPLC 1260 Infinity) equipped with C18 RP column and UV detector. The fat soluble vitamins A&D were identified and quantified by comparing retention times and peak area with those of vitamins standards respectively (R7632, C9756, T3251 and M5625, Sigma-Aldrich).

Then the concentration of each type of Vitamin were calculated using the formula-

$$\frac{\text{Area of Spl x Vol x Dil x Std. Conc. x 100}}{\text{Wt of Sample x Area of Standard}}$$

3.11 Analysis of some water quality paramaters:

3.11.1 Collection, pre-treatment and preservation of samples

Water samples were collected randomly from three beels and one river surrounding the Kokrajhar town during January-March of 2016. They were stored in pre-cleaned 250 ml polyethylene bottles and BOD bottles in triplicates and brought to laboratory for further analysis. The samples were collected from 12-15 cm beneath the water surface. All the precautionary measures were adopted during sampling.

3.10.2 Physico-chemical analysis

The collected samples were experimented to analyse different physic-chemical parameters such as pH, temperature, TDS, alkalinity, BOD, COD, DO, turbidity and salinity by following the standard protocols as per APHA 22nd Edition (2012). Samples were analyzed immediately for parameters like temperature, which need to be determined instantly.

A quality controlled procedure was steadily maintained throughout. The instruments were recalibrated. All chemicals and reagents used were of analytical grade. For all solutions distilled water was used. The standard solutions were made by diluting the stock solution.

3.11 Statistical analysis

Simple correlation and regression equations were applied for analysis of certain biological parameters. The statistical packages viz., MS Excel, Minitab 11 were used in the entire experiments of the present study.