4. METERIAL AND METHODS:

4.1. STUDY AREA:

Haldi River is a tributary of Hooghly River flowing through PurbaMedinipur district of West Bengal. It is 24 kilometers long. It is the last major river to flow into the Haldi and Hooghly estuaries before the latter flows into the sea. The Haldi river joins the Hooghly river at the industrial town of Haldia. Its coordinates are 22°0′56″N to 22°04′18″N latitude and 87°00′57″E to 88°08′40″E longitude. The major industries located in Haldia includes petrochemical and refining sector of Indian Oil Corporation (IOC), Haldia Petrochemicals Pvt. Ltd., Mitsubishi Chemical Corporation Pvt. Ltd, South Asian Petrochemicals Ltd. and some oil and gas terminals. Apart from these units, there is a detergent unit of HLL, Tata Chemicals Ltd, pesticide unit of Shawallace Pvt. Ltd., Exide Industries Ltd., vegetable oil units, etc. Although these industries have recycled their waste products but lot of waste including heavy metals discharge in the mouth of Haldi River. Besides these untreated sewage originated from domestic use and discharge to the said river. As result water become polluted and causes severe harm to aquatic biomass. Conservative waste like heavy metals, halogenated hydrocarbon etc. are not subject to microbial attack and therefore, exist over a long duration and cause harm to plant and animals. Polluted water is also unfit for drinking, recreation and agriculture.

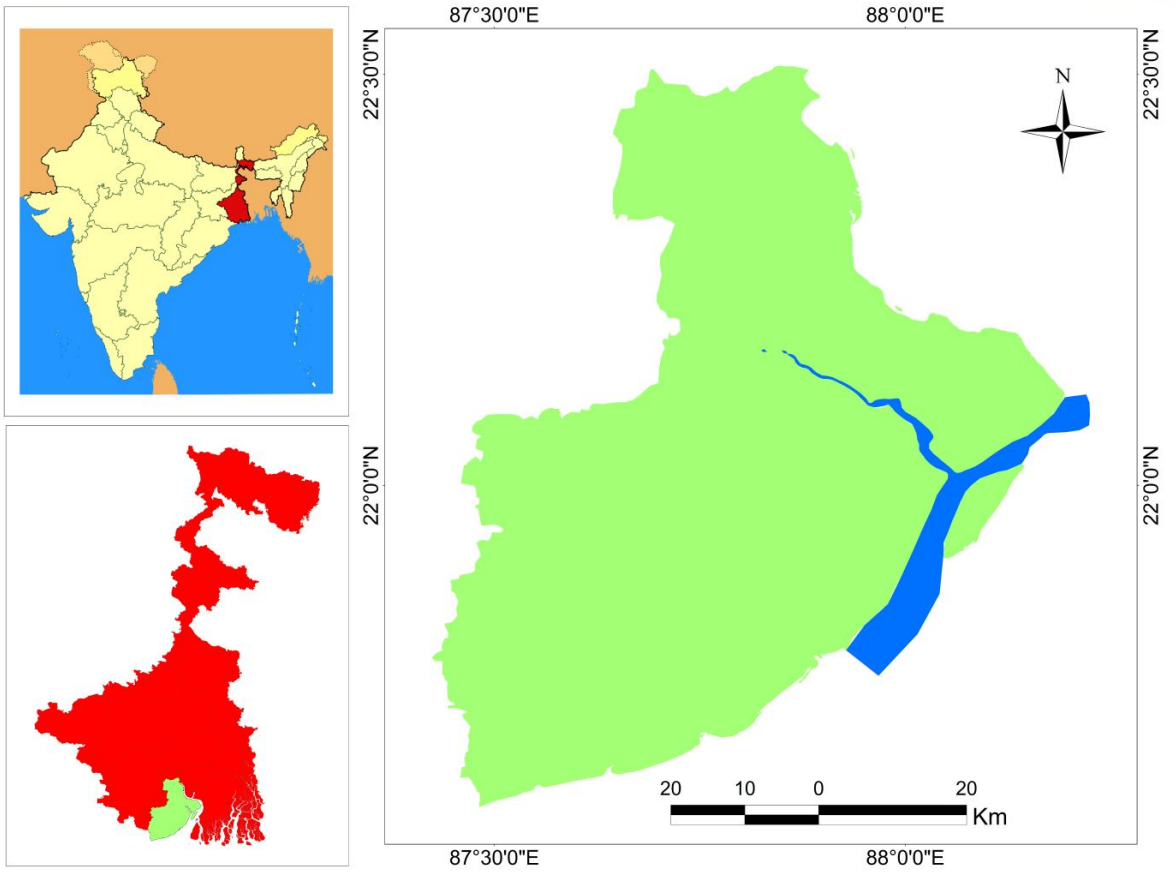
The mouth of the Haldi river has been divided into three separate zones based on their effluent released and contaminated the water and soil;

i. Station/Site -1: major industrial effluent out fall at Patikhali in Hooghly river

ii. Station/Site -2: 5 km down stretch from station 1

iii. Station/Site -3: 5 km above confluence on river Haldi

Plate- 1: Location map of the mouth of Haldi river



4.2. COLLECTION AND PRESERVATION OF SAMPLES

The mouth of Haldi river water samples for physico-chemical analysis were collected every month (July 2014 to June 2016) in clean polyethylene cans or glass bottles. The sediment samples were collected in clean polyethylene bags. The sample were stored in ice pail till they were transported in the laboratory and preserved at 5-10 ˚C. All the physico-chemical analyses were done with in 24 hr. of sample collection. Samples for oxygen analysis were fixed immediately after collection , using modified Winkler's reagents (APHA, 1989). The samples for COD analysis were fixed with 1 ml of Conc. H_2SO_4 (EPA, 1974). For the analysis of the remaining parameters the water and soil samples were transported to Kolkata Scientific Research Laboratory(Analytical & Environmental Engineering Laboratory).

4.3. PHYSICO-CHEMICAL CHARACTERISTICS OF SOIL:

4.3.1. pH (Hanlon, E. A., CIR1081):

Procedure:

- One scoop of soil to a 3-oz plastic cup using a 20-cc (~ 25g) scoop.
- Add 40ml of water (distilled or de-ionized water) to each cup using an automatic pipette or suitable volumetric container. Stir with a glass rod and let the sample sit for 30 min. (Remember to use the same type of water each time you test!)
- Calibrate the pH meter according to the instructions with meter. It is best to calibrate with at least two buffer solutions (pH 4.0 and pH 7.0).
- Stir the sample again immediately before measuring the 1 pH. Do not place the electrode(s) directly in the sand layer at the bottom of the cup. The electrode(s) should be

positioned in the solution just above the sand layer. Sometimes measurements must be repeated three times to ensure accurate results.

- Record pH to the nearest 0.1 pH unit.
- Properly triple rinse electrode(s) with distilled or de-ionized water after each use and before testing another sample.

4.3.2. Organic Carbon (Walkey-Black methods 1934):

Procedure:

- Take 1g of soil in a 500 ml conical flask.
- Add 10ml of 1N $K_2Cr_2O_7$ solution and shake to mix it.
- Then add 20ml con. H_2SO_4 and swirl the flask 2 or 3 times.
- Allow the flask to stand for 30 minutes on an asbestos sheet for the reaction to complete.
- .Pour 200ml of water to the flask to dilute the suspension. Filter if it is expected that the end point of the titration is not to be clear.
- Add 10ml of 85% H_3PO_4 and 1 ml of Diphenylamine indicator and back titrate the solution with 0.5 N Ferrous Ammonium Sulphate, till the colourflashes from violet through blue to bright green. H_3PO_4 gives sharper endpoint, by making the colour change, distinct through a flocculating effect.
- Note the volume of Ferrous Ammonium Sulphate.
- Carryout blank titration (without soil) in a similar manner.

Calculation:

% of Organic Carbon in soil (R) is

$$R = \frac{(V_1 - V_2) \times N \times 0.003 \times 100}{W} \times C$$

Where,

W- Weight of Sample

V_1- Blank Titrate value

V_2- Titrate Value of the Sample

N – Normality of $K_2Cr_2O_7$ (Here it is 1N)

C – Correction Factor (1.334, 1.724)

4.3.3. Available Nitrogen (APHA, 2000):

Procedure:

- Weigh 50g of processed soil sample in 500 ml Kjeldahl flask.
- Add 1 g $CuSO_4$, 10 g K_2SO_4 and 30 ml Con. H_2SO_4.
- Shake the contents of the flask until through mixing and allowing to stand for at least 30 minutes with frequent shaking or until complete solution results.
- Digest the content until greenish colour appears. K_2SO_4 raises the boiling point of the acid. So that the loss of acid volatile solution is prevented. $CuSO_4$ $5H_2O$ is digestion accelerator which catalyses the speed of digestion process.

- The reagents sometimes contain impurities so run a blank with the same quantities of reagents and subtract the blank value from the value of the soil digest.
- Digestion is effected on the Kjeldahl digestion rack withlow flame for the first 10-30 min until the frothing stops and then gradually more strongly until the sample is completely charred. The heat is gradually raised until the acid reaches approximately one third the way up the digestion-flask. The flame is not allowed to touch the flask above the part occurred by the liquid. Excessive boiling may cause volatilization of the acid before the organic matter is oxidized.
- Cool the content and dilute to about 100 ml with distilled water. Swirl the flask for about 2 minutes and transfer the fluid part to a 1000 ml distillation flask.
- Wash the residue left in the Kjeldahl flask with 4 or 5 lots of 50-60 ml distilled water, decanting the washings into the distillation flask.
- Add a few, glass bead to prevent bumping.
- Fit the flask with two neck joints to one neck dropping funnel is connected for adding 40% NaOH while to the other neck Kjeldahl trap, which is used to trap the NaOH coming with the the distillate. The trap is connected to the condenser with a delivery tube which dips into 50 ml of 0.1 N HCl contained in a conical flask, with one or two drops of methyl red indicator.
- Add about 125 ml (or 100 ml if bumping is a problem) of 40 % NaOH solution till the content are alkaline in reaction (about 5 times the volume of Con. H_2SO_4 used during the digestion). Heat the RB flask.

- Allow the ammonia formed to be absorbed in standard HCl. Wash down the end of the tube. 150 ml distilled water is added to the conical flask. When no more ammonia is received (test with a red litmus paper turning blue) stop the distillation.
- Titrate the excess of the acid with 0.1 n NaOH solution till the pink colour changes to yellow.
- From the titre value calculate the multi equivalence of the acid participating in the process of ammonia absorbing during digestion.

a) Calculation:

i. Blank:

Volume of HCl taken for blank = a ml

Volume of NaOH used = b ml

Volume of HCl consumed by liberated NH_3 present in blank = a – b = z ml

ii. Sample:

Volume of HCl taken for sample = v ml

Volume of NaOH used = u ml

Volume of HCl consumed by liberate NH_3 present in sample = v – u = w ml

Volume of HCl consumed for NH_3 liberated by sample only = w – z = y ml

1000 ml 1N HCl = 1000 ml 1N NH_3 = 17g NH_3 = 14g N

1 ml 1N HCl = 1 ml 1N NH_3 = 0.014g N

1 ml 0.1 N HCl = 1 ml 0.1N NH_3 = 0.0014g N

Weight of Nitrogen in 5g of Sample = y × 0.0014g N = q g N

$$\% \text{ of N in sample} = \frac{q \times 100}{5}$$

= p %

4.3.4. Available Potassium (Metson, 1956):

Procedure:

Take 5g soil in 100 ml conical flask and 25 ml of 1N NH_4OAc Soln. shake the content for 5 minutes and then filter through Whatman No. 1 filter paper. Potassium extract is measured by flamephotometer after calibration.

Calculation:

$$\text{Available K (kg ha}^{-1}) = \frac{R \times F \times 25 \times 100 \times 20 \times 1.121}{5 \times 1000}$$

= R×F× 11.217

4.3.5. Available Phosphorous (Bray and Kurtz, 1945)

Procedure:

1g of air dried soil sample was taken in a 250 ml reagent bottle. 200ml 0.002N H_2SO_4 (pH 3) was added, the mixture was shook for 30 minutes in a mechanical shaker. The sample was kept for 10 minutes and filtered. After filtration 50 ml of filtrate was taken in a Nessler tube and the phosphate was determined.

Calculation:

ppm of phosphate in solution × 20 = mg P/100 g sediment.

4.3.6. Texture (Method- Hydrometric method, Strokes law):

Soil texture is an indicator of the proportionate of mineral fractions in soil and grouped into sand, silt, clay depending on the particle size. 100g of air dry soil was placed in a 500ml conical flask, in which 15ml 0.5 N sodium oxalate and 200ml distilled water were added and shook for one hour. Then the contents were transferred to a 1000 ml tall cylinder and make up the volume. The hydrometer was dipped in the liquid after 2 minutes of sedimentation and the percentage of clay +silt was noted.

The percentage of clay was similarly determined by noting the hydrometer reading after two hour. The reading gave directly the percentage of suspended matter. Percentage of sand was obtained by deducting clay + silt fraction from 100 percentage of silt was calculated by deducting clay component from clay + silt fraction.

4.4. PHYSICO-CHEMICAL CHARACTERISTICS OF WATER:

4.4.1 Temperature:

Procedure:

Temperature of the sample was recorded immediately after the collection using a sensitive (1/10) thermometer (0-110 °C).

4.4.2. pH (Colorimetric method):

Procedure:

Measurement of pH was carried out using a pH meter (Systronics digital pH meter -335) after standardization with buffer solutions of pH 4.0 and 7.0.

4.4.3. Dissolved Oxygen(APHA, 1989):

Procedure:

- The sample filled in a glass stopper bottle (BOD bottle), carefully avoiding any bind of bubbling and trapping of the air buckles in the bottle after placing the stopper.
- 1 ml of each ManganousSulphate ($MnSO_4$) and Alkaline Iodine (KI) solution were added to (200ml) of water sample, the reagents can also be poured at the bottom of the bottle with the help of special pipette syringed to ensure better mixing of the reagents with the sample. A precipitate will appear.

- The stopper was placed and shaken the contents well by inverting the bottle repeatedly. The bottle is kept for some time to settle down the ppt. If the sample at this stage with the ppt.
- 1-2 ml of con. H_2SO_4 is added and shaken well to dissolve the ppt.
- Either the whole contents or a part of them is taken in a conical flask for titration. To avoid further mixing of oxygen bubbling should be avoided.
- The content is titrated within 1 hour of dissolution of the ppt. against Sodium Thiosulphate ($Na_2S_2O_3$) solution using starch as an indicator. At the end point, initial dark blue colour changes to colour less.

Calculation:

$$DO = \frac{[ml * N] \text{ of titrant } *8*1000 \text{ mg/l}}{\frac{V_2 (V_1 - V)}{V_1}}$$

Where, V_1 = Vol. of sample bottle after placing the stopper

V_2 = Volume of part of the contents titrated

V = Volume of $MnSO_4$ and KI added.

4.4.4. Biochemical Oxygen Demand (APHA, 1989):

Procedure:

- The collected samples were diluted before incubation to bring the oxygen demand and supply into an appropriate balance. One liter of distilled water was mixed with nutrients (1 ml of each of buffer, calcium chloride, magnesium sulphate and ferric chloride) and used as the diluents.
- Samples were neutralized to pH 6.5 – 7.5 with H_2SO_4 or NaOH.
- The DO of the sample was determined initially and after 5 days incubation in a BOD incubator at 20^0C.
- A blank using distilled water alone was also run simultaneously.
- The BOD 5 was then worked out by the following formula:

BOD5 at 20^0C in mg/l = $(D_0 - D_5) - (C_0 - C_5) \times$ Dilution factor.

$$\text{Dilution factor} = \frac{1000}{\text{Vol. of Sample}}$$

Where,

D_0 = DO content of the sample on the 1st day

D_5 = DO content of the sample on the 5th day

C_0 = DO content of the blank on the 1st day

C_5 = DO content of the blank on the 5th day.

4.4.5. Chemical Oxygen Demand (APHA, 1989):

Procedure:

Chemical Oxygen Demand (COD) was determined following the methods suggested by APHA (1989).

- 20 ml of the sample was taken in a 250 ml round bottom flask and added with 0.4 g of $HgSO_4$ was added to it.
- The contents were further added with 30 ml of con. H_2SO_4 containing 0.4 g of Ag_2SO_4.
- Later 20 ml of $K_2Cr_2O_7$ (0.25 N) was added.
- The contents in the flask was refluxed for 2 hour and then cooled and the solution was made upto 140 ml.
- 3-4 drops of feroin indicator were added.
- Then the contents were titrated against ferrous ammonium sulphate (0.25N).
- The end point of the titration was the first sharp colour change from blue-green to reddish brown.
- A blank was also run simultaneously in the same manner using distilled water.
- COD was calculated using the formula

$$\text{COD mg/l} = \frac{(A - B) \times N.Fe(NH_4)_2SO_4 \times 8 \times 1000}{\text{Volume of Sample}}$$

Where,

A = Vol. of Fe $(NH_4)_2SO_4$ consumed for blank (ml).

B = Vol. of Fe $(NH_4)_2SO_4$ consumed for Sample (ml).

4.4.6.Alkalinity (APHA, 1989):

Procedure:

- 100 ml of sample is taken in a conical flask and add 2 drops of phenolphthalein indicator.
- If the solution remain colour less, PA=0 and Total Alkalinity is determined.
- If the colour changes to pink, other addition of phenolphthalein, titrated it with 0.1 (N) HCL until the colour disappears at end point. This is Phenolphthalein Alkalinity (TA).

Calculation: TA as **$CaCO_3$** mg/lit = $\frac{[A*(N)] \text{ of HCL} * 1000*50}{\text{ml of water sample}}$

4.4.7. Salinity (APHA, 2000):

Salinity was measured by the (Electrometric method, multiline P4 WTW) by following the method of (APHA, 2000).

4.4.8. Nitrate(Jenkin and Medsken, Brucine method, 1964):

Procedure:

Sample (10 ml) was taken in a boiling tube and placed in a cool water bath. 2 ml of sodium chloride solution and 10 ml of sulphuric acid were mixed with the samples. The mixture was allowed to cool, and 0.5 ml of Brucine sufanilic acid was added. The reaction mixture was stirred and mixed and placed in a water bath at 95^0C for 20 minutes. The mixture was cooled in a water bath and the yellow colour developed was read at 410 nm in a Spectrophotometer.

4.4.9. Phosphate (APHA, 1989):

Sample (10 ml) was taken in a boiling tube and placed in a cool water bath. 2 ml of sodium chloride solution and 10 ml of sulphuric acid were mixed with the samples. The mixture was allowed to cool, and 0.5 ml of Brucine sufanilic acid was added. The reaction mixture was stirred and mixed and placed in a water bath at 95^0C for 20 minutes. The mixture was cooled in a water bath and the yellow colour developed was read at 410 nm in a Spectrophotometer.

4.5. HEAVY METAL ANALYSIS (WATER AND SEDIMENT):

The heavy metal contents were analysed in water and sediment samples using Atomic Absorption Spectrophotometer (Varian Techtron, AA-6D).

4.5.1. Digestion of sediment samples:

Sediment samples was taken in a Petri dish and dried overnight at 50^0C, in a hot air oven. 100 g of dried sediment was taken, then ground and sieved through a 250-mesh screen, to produce fine

grains. For digestion 0.5g of the dried sediment was taken and 15 ml of 65% HNO_3 was added and gently heated in a water bath, to ensure proper digestion. When the mixture formed a slurry, with a volume of approximately 7 ml, 10 ml more of 65% HNO_3 was addedand the slurry heated again to reduce the volume to 5 ml . At this point, 15 ml of de-ionized distilled water was added and the mixture was brought to a boil, once again by gradual heating in a water bath, for a short period of a time, to produce a clear solution. The solution was removed from the water bath, cooled and filtered using a Whatman 40-41 filter paper. This filtrate was poured into a 50 ml volumetric flask and the volume brought to the mark by adding requisite amount of de-ionized-distilled water. Heavy metal analysis was carried out using the Atomic Absorption Spectrophotometer (Clesceri et.al., 1998). Water sample were filtered with Whatman 40-41 filter paper and analyzed directly.

4.5.2. Atomic Absorption Spectrophotometer (AAS):

Atomic Absorption Spectrophotometer (AAS) determines the presence of metals in liquid samples. Metals include Fe, Cu, Al, Pb, Ca, Zn, Cd and many more. In order to analyze a sample for its atomic constituents, it has to be atomized. The atomizers most commonly used now a days are flames and electro thermal (graphite tube) atomizers. The atoms should then be irradiated by optical radiation, and the radiation source could be an element-specific line radiation source or a continuum radiation source. The radiation then passes through a monochromatic grid in order to separate the element-specific radiation from any other radiation emitted by the radiation source, which is finally measured by a detector. Proper cathode lamp for each metal was chosen and allowed to warm up for a minimum of 5 minutes. Then a series of standards for each metal (5, 10, 15 and 20 ppm) were run and a calibration curve was plotted on linear graph paper (absorbance of standards versus their concentration). Afterwards the heavy metal analysis of the

sample was conducted done one by one and a blank was analyzed between samples, to ensure the precision of the reading that were noted and documented.

4.6. Diversity of Plankton in the mouth of Haldi River:

4.6.1. Sample Collection and preservation:

The plankton samples were collected from the three study location mouth of Haldi River by filtering 50 lit. of water from each location through standard plankton net (Heron-tranter net). It had a square frame nd the filtering cone of mesh size of 0.2 mm and the moth area of the net was 0.25 m^2. The samples were collected during the early hour of the day between 7 am to 10 am. The samples were fixed in 5 % formalin immediately after collection and stored in the dark. Then 2 drops of mild detergent solution was added to prevent any clumping of the zooplankton.

4.6.2. Enumeration of plankton:

Sub-sampling pipette method was followed for enumeration (Dodson and Thomas, 1964; Gannon, 1971: Alden *et.al.*, 1982;Clesceri*et. al.*,1998). The enumeration of small plankton (e.g., protozoa, rotifers etc.) was made in a 2 ml clear acrylic plastic counting cell fitted with a glass cover slip, and placed under a compound microscope with the magnification of 100X. for larger organisms and mature microcrustacea, a counting chamber holding 5 ml was used under the binocular dissecting microscope at desired (20X – 40X) magnifications. To reduce any movement of the organisms, one drop of the mild detergent solution was placed once more in the chamber before counting. Small planktons were recorded as number per lit., whilst the larger forms were recorded as number per cubic meter. Zooplankton identification was done with the help of standard manuals (Alfred *et. al.*, 1973; Adoni*et. al.*, 1985). The quantitative analysis of

planktonic organisms was carried out using Sedgwick Rafter plankton counting cell in accordance with the method outlined by Welch (1948).

4.6.3. Community structure analysis:

Four indices were used to obtain the estimation of species diversity, species richness and species evenness.

4.6.3.1. Margalef species richness index:

It is a measure of the number of species present for a given number of individuals.

The values range from 0 to 5 (in exceptional cases up to 12). The demerit of this index is that it is sensitive to sample size. The advantage of this index is that values can come more than 1unlike Simpson index which ranges from 0 to 1. That way comparing species richness between different samples is easy.

Species richness (R1) was obtained using the equation:

$R1 = (s-1)/ \log N$ (Margalef, 1951)

Where:

R= the index of species richness

S = total number of species

N = total number of individuals

4.6.3.2. Pielou's evenness index:

Evenness index is also an important component of the diversity indices. This expresses how evenly the individuals are distributed among the different species. It varies from 0 to 1. When the species are distributed evenly, the index is on the higher side. When unevenness (dominance) is there, this index is on the lower side. Species equitability or evenness was determined by using the expression of Pielou (1966) and Sheldon (1969).

$El = N_1/N_0$

Where:

N_0 = number of species on the sample

N_1 = number of abundant species in the sample

4.6.3.3. Shannon Wiener diversity index:

It is a benchmark measure of biological diversity and denoted as H'. it is a widely used measure of diversity index for comparing diversity between various habitats (Clarke and Warwick, 2001). Shannon and Wiener independently derived the function which has become known as Shannon index of diversity. The value of Shannon diversity is usually found to fall between 1.5 and 3.5 and only rarely it surpasses 4.5. Shannon and Weaver (1949) diversity index value was obtained using the following equation:

$D = \sum pi_2 (\log pi)$ (Shannon's index)

Where :

Pi = is the proportion of the first species. The proportions are given $Pi = ni/N$

4.6.3.4. Simpson dominance index:

Simpson gave the probability of any two individuals drawn at random from an infinitely large community belonging to different species. The Simpson index is therefore expressed as 1-D or 1/D. the merit of this index is that it is a widely used measure of richness. The demerit of this index is that it is heavily weighted towards the most abundant species. This index is denoted as D. When D increases, diversity decreases. When diversity increases, dominance decreases.

Simpson (1949) dominance index value was obtained using the following equation:

$D=\sum Pi_2$ (Simpson index)

Where:

Pi = is the proportion of the first species. The proportions are given Pi = ni/N

4.7.DATA ANALYSIS:

The statistical analysis like t- test and Pearson correlation were done through Ms- Excel and correlation test was done by IBM SPSS. The data were expressed as Mean ± SD.