CHAPTER-III

Materials and Methods

This study was carried out in Tinsukia district, Assam, which contains certain oil fields. Tinsukia is the eastern most district of Assam on the southern bank of river Brahmaputra with its head quarter at Tinsukia town. The district covers an area of 3790 km^2 It extends from 27^0 23' N to 27^0 48' N latitude and 95^0 22' E to 95^0 38' E longitude. The northern, eastern and southern boundary of the district is surrounded by Arunachal Pradesh. The southern border is shared with Changlang district, north by East Siang district and Lower Dibang Velley district and east by Lohit district of Arunachal Pradesh. To the north-west and south west it shares boundary with Dhemaji and Dibrugarh district of Assam respectively. The mighty river Brahmaputra flows through the northern margin of the district. Burhidihing, Nuadihing, Dibru, Dangori and Dhola are the major tributary of the Brahmaputra flowing through the district The district is located in Upper Brahmaputra Valley agroclimatic zone bordering Eastern Himalayan region. It experienced under subtropical monsoon climate with mild winter, warm and humid summer. The average annual rainfall ranges from 2300 mm to 3800 mm. It receives most of its rain during June to September due to south west monsoon. The maximum temperature of the district was recorded 39^0C during summer and minimum was 18^0C. Relative humidity recorded maximum 96% and minimum 65%. The soil of the district is basically alluvial, sandy to silty loams and slightly acidic in nature (Anonymous, 1999).

Based on the climatic characteristics such as ambient temperature, rainfall, rainy days, humidity, presence of fogs and thunderstorms, the climate of Tinsukia district could be classified into four distinct climatic seasons *viz.* hot and wet pre-monsoon (March to May), monsoon (June to September) with heavy rainfall, post-monsoon (October to November) with retreating monsoon and dry winter (December to February) incorporated with western disturbance rainfall, fog and cloudy weather.

Tinsukia district is mainly an industrial district in Assam. It is well known for its rich oil fields and coal mines. In fact, oil exploration in India commenced with the

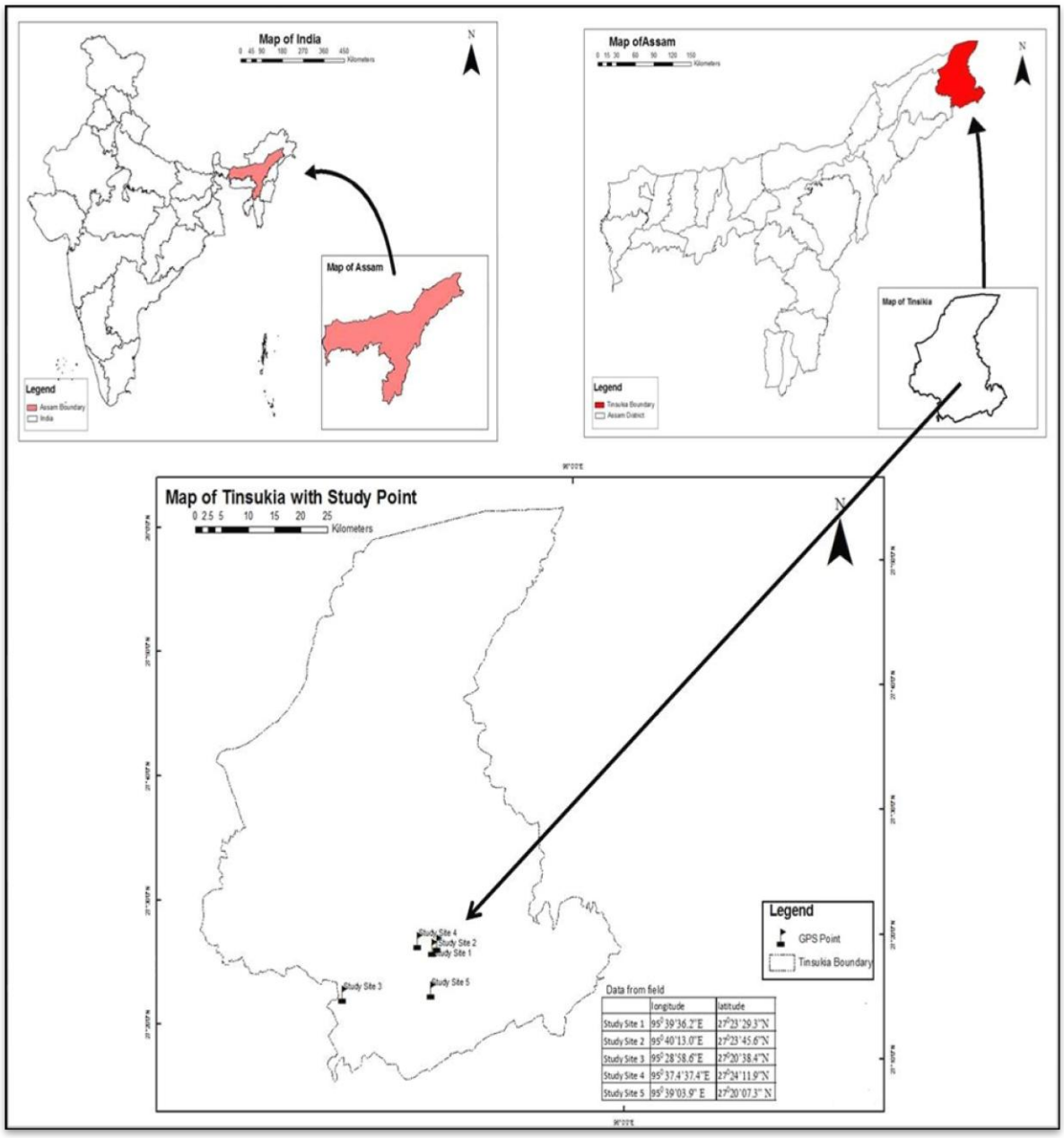

Fig.1: Map of the study sites in Tinsukia district, Assam, India

discovery of the Digboi oilfield in Tinsukia district more than 100 years ago. Presently oil India Limited carried out oil exploration from oil fields of Digboi, Jorajan, Makum, Barekuri, Baghjan and Dirok. The oldest oil refinery in Asia is situated at Digboi and places like Margherita, Ledo and Borgolai are famous for coal mines. The district is also famous for its tea gardens.

Study sites:

In the present study, five (5) water bodies of Tinsukia district were selected for observation and designated as S1, S2, S3, S4 and S5. The water bodies were selected based on extent of crude oil contamination. Out of these five water bodies, three (S1 to S3) were located within oil exploration and production areas and the other two (S4 and S5) were located away from oil exploration and production sites. Description of the study sites is given in the Table- 1.

Table-1: Description of study sites.

Site	Latitude	Longitude	Altitude (m)	Area (m^2)
S1	$27^0$23’29.3” N	95^0 39’36.2”E	154	297.60
S2	$27^0$23’45.6” N	95^0 40’13.0”E	129	363.20
S3	$27^0$20’38.4” N	95^0 28’58.6” E	112	276.94
S4	$27^0$24’11.9” N	95^0 37.4’37.4”E	133	173.62
S5	$27^0$20’07.3” N	95^0 39’03.9”E	119	103.48

S1 is a sludge pit inside Digboi oil field which contain free floating oil above the surface and oily sludge and wax at the base. S2 is a natural wetland used as waste pit inside Digboi oil field. S3 is a “produced water holding evaporation pond” of Jorajan oil collection station containing large quantity of free floating oil and wax. S4 is a man made pond within paddy fields far away from oil from oil fields located at Pawoi. S5 is a historic pond locally known as “Gupto Pukhuri” located in a residential area of Digboi Town used by the neighboring residents for their routine household purposes like bathing and washing of clothes and utensils.

Sampling:

The sampling was done at an interval of two month during April 2011 to March 2013 for a period of two years. The collections of samples were done between 6 am to

11 am. Five locations were identified in each selected fresh water body for collection of samples. Water samples were collected from each location at three vertical points- upper, middle and bottom using Nansen sampler and mixed to get composite sample.

Algal Samples:

Microalgal or phytoplankton samples were collected at each location by filtering techniques using 25 μ mesh size plankton net. About 10 liters of water was passed through the plankton net and the residual cellular materials were transferred to acid washed clean plastic tubes. Visible algal samples were collected by hand picking method with forceps from side of the ponds, on the floating wax, on the leaves and stems of aquatic plants. The tubes containing algal samples were properly labeled and brought to the laboratory of Department of Botany, Gauhati University for further investigation.

The collected samples were preferably examined in fresh. The rest portions of algal samples were preserved using Leugol's solution. Leugol solution was added in a ratio of 1 ml of solution to 1000 ml of sample. Leugol's solution was prepared by dissolving 10 mg of neutral potassium iodide in 20 ml of distilled water and to it 5 gm sublimed iodine was added. After that 50 ml of distilled water and 5 gm of sodium acetate or 10% of acetic acid were added.

Water samples:

For physico chemical parameters water samples collected in cleaned plastic jerry cans of 5 liters capacity previously rinsed with pond water, and brought to the laboratory of the Department of Botany, Gauhati University where total dissolved solids(TDS), total suspended solids (TSS), turbidity, free CO_2, total alkalinity, total hardness, biological oxygen demand (BOD), chemical oxygen demand (COD), calcium, magnesium, chloride, sodium, potassium, phosphate, nitrate, total oil content (TOC) were measured following APHA, (2012). The surface water temperature, pH, conductivity, turbidity were measured on the spot using Systronics digital water analyzer 371. Dissolved oxygen (DO) and Primary productivity of water bodies were also estimated on the spot by Winkler's method (Trivedi and Goel, 1986) and Winkler's dark and light bottle method (Vollenweider, 1969) respectively.

Microscopic studies:

Fresh algal samples were observed by mounting a drop of algal sample on a microscopic slide and carefully placed a cover slip upon it. The slide was then observed under compound microscope initially at lower magnification (10 x) and move sequentially up to higher magnification (40 x). The algal samples preserved in Leugol's solution were examined by preparing permanent slides with Glycerin solutions with an addition of little amount of suitable stain or dye. Frequently used stains were- Safranin, India Ink or Congo Red.

Identification:

The prepared algal materials were observed under compound microscope (Magnus-MLXz) in 40X to 100X magnification and photographs were taken. Measurements were taken using ocular scale and micrometer. Identification of algal samples were done by consulting literature and monographs of Fritsch (1935), Smith (1950), Desikachary (1959), Ramanathan (1964), Prescott (1938, 1939, 1962, 1969, 1973), Prasad and Srivastava (1992), Prasad and Mishra (1992), Gandhi (1999), Perumal and Anand (2009) and Yamagishi (2010).

Quantitative study:

Sadgwick-Rafter (S-R) cell was used for quantification of algal samples. The S-R cell is about 50 mm long x 20 mm wide x 1 mm deep. Its total bottom area is about 1000mm^3 and total volume is about 1000 mm^3 (1 ml). For quantitative estimation, first place the cover glass diagonnaly across the cell and pipetted out 1 ml of well mixed sample into the cell, carefully covered the cell by cover slip. Allowed the S-R cell stand for at least 15 minutes to settle algal samples and counted the algae on the bottom of the S-R cell. All the algae present in the cell were counted by moving the slide both horizontally and vertically. At least three replicates were done and then average is calculated out. For, the samples having very high density of algal specimens, sample water was diluted 10 to 50 times and the same procedure was followed. Algal number was then calculated by following formula:

$$\text{Number of algal unit/ml} = \frac{\text{number of organisms counted x dilution factor}}{\text{number of replicates taken}}$$

Water quality analysis

Surface water temperature

Surface water temperature was measured at spot soon after the collecting water sample in a 500 ml capacity beaker. It was measured using Systronics digital water analyzer 371. The instrument was calibrated prior to use with a thermometer of known accuracy to minimize the errors. It is expressed in degree Celsius (^{o}C).

pH

The pH of water samples were determined just after collection. The measurement was carried out using Systronics digital water analyzer 371. Standard buffer solutions were used for calibration of the electrode of the instrument.

Conductivity

It was measured using Systronics digital water analyzer 371. Conductivity was reported as micro siemens per centimeter (µS/cm).

Turbidity

Turbidity was measured following Nephelometric Method (APHA 2012). A nepheloturbidimeter (Model 131, Systronics, India) was used for the measurement of turbidity of water samples. The volumes were calibrated with respect to a suspension of known turbidity and are given in nepheloturbidity units (NTU).

Solids

Solids include "total suspended solids", the portion of total solids retained by a filter, and "total dissolved solids", the portion that passes through the filter.

Total Dissolved Solids

A well mixed water sample was filtered through a standard glass fiber filter, and the filtrate was evaporated to dryness in a weighted dish and dried to constant weight at 180^{0} C. the increase in dish weight represents the total dissolved solids and calculated following (APHA, 2012).

Calculation:

$$\text{mg total dissolved solids/l} = \frac{[(A-B)\ X\ 1000]}{\text{ml of Sample}}$$

Where,

A= weight of dried residue + dish in mg; *B*= weight of the dish in mg

Total suspended solids

A well mixed water sample is filtered through a weighted standard glass fiber filter and the residue retained on the filter is dried to a constant weight at 103 to 105 ^{0}C. The increase in the weight of the filter represents the total suspended solids.

Dissolved oxygen (DO)

DO in water was measured with the help of Winkler's method (APHA, 2012). Water samples were collected in specialized BOD bottles (200ml) and care was taken so as to avoid any agitation during collection. 1 ml each of MnSO4 and alkaline KI solution were added to the BOD bottle through the surface of the wall. After the appearance of precipitate, 2ml of H_2SO_4 was added by the side of bottle and shake for proper mixing until precipitate dissolved completely. At last 100 ml of this mixture was taken in a separate conical flask and was titrated against standard sodium thiosulphate solution using starch as indicator until the solution become colourless. The amount of DO was calculated by using the formula:

$$\text{DO in mg/l} = \frac{(\text{volume of sodium thiosulphate x N) of titrate x 8 x 1000}}{\text{V1}-\text{V}}$$

Where,

N = normality of sodium thiosulphate

V_1 = volume of the sample bottle after placing the stopper

V = volume of $MnSO_4$ and KI added

Or one another way, the calculation of DO can be obtained as:

DO = 2C mg/l

Where,

C = volume of thiosulphate used for titration

Free Carbon dioxide

Free CO_2 was determined by titrating the sample using strong alkali like sodium carbonate or sodium hydroxide (APHA, 2012). Free CO_2 reacted with these alkalis to from sodium bicarbonate. Completion of the reaction is indicated by the development of the pink colour characteristic of phenolphthalein indicator at the equivalent pH of 8.3

Calculation:

$$\text{mg } CO_2\text{/l} = \frac{[Ax\ N\ x\ 44000]}{\text{ml of Sample}}$$

Where:

A= ml titrant; *N*= normality of NaOH

Biochemical oxygen demand (BOD)

The biochemical oxygen demand (BOD) was determined following APHA, 2012. The method consists of filling with diluted and seeded sample, to overflowing, an airtight bottle of specified size and incubated at the specified temperature (20 ± 1^0 C) for 5 days. Dissolved oxygen was measured initially and after incubation, and the BOD was computed from the difference between initial and final DO. Because the initial DO was determined shortly after the dilution was made, the entire oxygen uptake occurring after this measurement was included in the BOD measurement.

Calculation:

$BOD = (D_0 - D_5) \times$ dilution Factor

Chemical oxygen demand (COD)

COD was measured by open reflux method (APHA, 2012). A sample was refluxed in strongly acid solution with a known excess of potassium dichromate ($K_2Cr_2O_7$). After two hour digestion the remaining unreduced $K_2Cr_2O_7$ was titrated with ferrous ammonium sulfate to determine the amount of $K_2Cr_2O_7$ consumed and the oxidizable matter is calculated in terms of oxygen equivalent.

Calculation:

$$\text{COD as mgO}_2\text{/l} = \frac{[(A-B)\ x\ M\ x\ 8000]}{\text{ml of Sample}}$$

Where:

A= ml FAS used for blank; *B*= ml FAS used for sample; *M*=molarity of FAS and 8000 = milliequivalent weight of oxygen x 1000 ml/l.

Alkalinity

Alkalinity of surface waters is primarily a function of carbonate, bicarbonate and hydroxide content. Alkalinity was determined by titrating with 0.1N hydrochloric acid solution (standardized with 0.1N Na_2CO_3) using phenolphthalein and Murexide indicator from the following table (APHA 2012):

Result of titration	OH alkalinity	CO_3 alkalinity	HCO_3 alkalinity
PA = 0	0	0	TA
PA < ½ TA	0	2 PA	TA – 2PA
PA = ½ TA	0	2 PA	0
PA > ½ TA	2 PA – TA	2 (TA – PA)	0
PA > ½ TA	TA	0	0

Where, PA= phenolphthalein alkalinity, TA= Total alkalinity. *All values are in mg $CaCO_3$/l.*

According to this scheme:

(a) Carbonate (CO_3^-) alkalinity is present when phenolphthalein alkalinity is not zero but is less than total alkalinity.

(b) Hydroxide (OH^-) alkalinity is present if phenolphthalein alkalinity is more than half the total alkalinity.

(c) Bicarbonate (HCO_3^-) alkalinity is present if phenolphthalein alkalinity is less than half the total alkalinity.

PA and TA are determined using the following expressions:

(a) PA as mg CaCO3 /l = $\frac{[\text{A x N of HCl x 1000 x 50}]}{\text{ml water sample}}$

(b) TA as mg CaCO3 /l = = $\frac{[\text{B x N of HCl x 1000 x 50}]}{\text{ml water sample}}$

where, A = ml of HCl used only with phenolphthalein

B = ml of HCl used with phenolphthalein and methyl orange i.e. total HCl used with both the indicators.

Hardness

Total hardness of the water samples was determined by EDTA complexometric titration using Eriochrome Black T indicator (APHA, 2012). For calcium hardness, the same procedure was followed but the indicator used is murexide. Magnesium hardness was calculated by subtracting the value of calcium hardness from total hardness.

Total hardness (as mg/l $CaCO_3$) = $\frac{[\text{ml of EDTA used X 1000}]}{\text{ml water sample}}$

Calcium, (mg/l) = $\frac{[\text{ml of EDTA used X 400.8}]}{\text{ml water sample}}$

Magnesium, (mg/l) = $\frac{[(B - A) \text{ x } 400.8]}{\text{ml of Sample x } 1.645}$

Where, A = EDTA used for calcium determination for the volume of sample

B = EDTA used for total hardness (both Ca and Mg) determination

Chloride

It was determined by titrating water with $AgNO_3$ solution (APHA, 2012). 50 ml of the sample water was taken in a conical flask and 2 ml of K_2CrO_4 solution was added to it. After that the solution was titrated against 0.02 N $AgNO_3$ solutions until a persistent red tinge appeared.

Calculation:

$$\mathrm{Cl} = \frac{\text{Volume of AgNO3 x Normality of AgNO3 x 1000 x 35.5}}{\text{Volume of the sample}}$$

Potassium

Flame Photometric method was used in determination of potassium (APHA, 2012). Trace amounts of potassium can be determined in flame photometer at a wavelength of 766.5 nm. Potassium levels of approximately 0.1 mg/l can be determined.

Sodium

Sodium was determined by flame emission photometry at 589 nm (APHA, 2012). Sample was nebulized in to a gas flame under carefully controlled, reproducible excitation condition. The sodium resonant spectral line at 589 nm was isolated by interference filters or by light-dispersing device such as prism or gratings. Emission light intensity is measured by a phototube, phtomultiplier, or photodiode. The light intensity at 589 nm is approximately proportional to the sodium concentration.

Nitrate

Nitrate was determined by the phenol disulphonic Acid Method (APHA, 2012). Nitrate reacts with phenol disulphonic acid forming a nitro-derivative, which in alkaline solution developed a yellow colour. The colour derived was directly proportional to the concentration of nitrate present in water sample. The concentration of nitrate was determined spectophotometrically using Systronics UV-Vis spectrophotometer119.

Phosphate

Phosphate content in the water was estimated by stannous chloride method (APHA, 2012). In this method, 50 ml of the clear and colourless water sample was taken in a conical flask, followed by addition of 2 ml of ammonium molybdate solution (25g of $(NH_4)_6Mo_7O_{24}.4H_2O$ in 175 ml distilled water, mixed with 280 ml concentrated H_2SO_4 and diluted to 1 liter) and 5 drops of stannous chloride reagent (2.5 g $SnCl_2.2H_2O$ in 100 ml glycerol, dissolved by heating in a water bath with constant stirring). A blue colour develops and the optical density is measured at 690 nm (using Systronics UV-Vis spectrophotometer 119) after 5 minutes but before 12 minutes of the addition of stannous chloride reagent using the blank which was treated exactly as the samples. The concentration of phosphate was determined with the help of a standard

curve obtained with standard phosphate solutions (KH_2PO_4) of at least five different concentrations in the equivalent range.

Total oil content (TOC)

TOC in the water samples were estimated by soxhlet extraction with dichloro methane, followed by evaporation at 70^{o}C (APHA, 2012).

Calculation:

$$\text{TOC (mg/l)} = \frac{[(A - B) \times 1000]}{\text{ml of Sample}}$$

Where, A = Final weight of the beaker and residue in mg.

B = Initial weight of the beaker in mg.

Primary Productivity

Primary productivity in water bodies was estimated by Winkler's dark and light bottle method (Vollenweider, 1969). First of all, the initial DO of the water sample was taken. Two similar glass bottles were taken one dark coloured and other is light coloured. Both the bottles were filled with water and caped properly right inside the water of the aquatic body itself and kept it under water from dawn to dark in the day, DO of both the bottles were determined and productivity of the water body during the study period was calculated as follows:

Calculation:

$$\text{Gross primary productivity, } O_2 \text{ mg/l/hr} = \frac{Dl - Dd}{hr}$$

$$\text{Net primary productivity, } O_2 \text{ mg/l/hr} = \frac{Dl - Di}{hr}$$

$$\text{Community respiration, } O_2 \text{ mg/l/hr} = \frac{Di - Dd}{hr}$$

where,

Di= initial DO in mg/l

Dl= DO in the light bottle in mg/l

Dd= DO in the dark bottle in mg/l

h= duration of exposure in hours

Statistical Analysis

The data generated during field as well laboratory observations were tabulated in spread sheets. The mean average, minimum and maximum values of the studied parameters were calculated using Microsoft office excel 2013 version 15.0.4779.1002.

Pearson's Correlation Analysis

The correlation between studied physico chemical parameters of water and algal population were analyzed by Pearson's correlation coefficient ('r' at significant level $p<0.05$). The Pearson's correlation coefficient (r) is calculated as-

$$r = \frac{\sum XY - \frac{\sum X \sum Y}{N}}{\sqrt{\left(\sum X^2 - \frac{(\sum X)^2}{N}\right)\left(\sum Y^2 - \frac{(\sum Y)^2}{N}\right)}}$$

The significance of 'r' was established by using the table values of 'r' corresponding to a range of probability levels for different degree of freedom.

Canonical Correspondence Analysis (CCA)

CCA is multivariate method to explain the relationships between species and their environment. This method is designed to extract synthetic environmental gradients from ecological data sets. The gradients are the basis for concisely describing and visualizing the differential habitat preferences (niches) of taxa via an ordination diagram (Braak and Verdonschot, 1995). CCA was done using statistical software PAST version 3.10 to explain the relationship between algae, water quality variables and seasons.

Sorenson similarity coefficient

Sorenson similarity coefficient was used to measure the similarity between algal communities of two study sites. It is a simple similarity measure deals only with presence-absence data. It was developed by the botanist Thorvald Sorensen and published in 1948. It has the value always in 0 to 1range. Sorenson similarity coefficient is calculated by using the following formula:

$$S_s = \frac{2C}{A + B}$$

Where,

S_s= Sorenson similarity coefficient

A = Number of plant species in community A

B = Number of plant species in community B

C = Number of plant species common to both the species

Species diversity index

Species diversity index is a mathematical measure of species diversity in a community. Diversity indices provide more information about community composition

than simply species richness (i.e., the number of species present). Diversity index provide information about rarity and commonness of species in a community. Diversity index is widely used in assessment of water quality. Lower the value of diversity index, higher will be the pollution level of water (Trivedy, 1980). Species diversity was measured using Shannon and Weaver Diversity Index. It was calculated as follows:

$$D = -\Sigma \, pi \log_2 pi$$

Where,

D = Shannon and Weaver Diversity Index

Pi = ni /N (where, ni = number of individuals in species

N = total number of individuals in the sample

Trivedy (1980 and 1981), demonstrated a scale for the degree of pollution and diversity index. The scale was as follows:

Diversity index value	Water condition
> 4.0	Clean water
3.0 -4.0	Very light pollution
2.0 -3.0	Moderate pollution
< 2.0	Heavy pollution

Evenness index

Species evenness is an ecological index. It is a measure of biodiversity which quantifies how the community is numerically equal. The evenness of a community can be represented by Pielou (1966) evenness index. Evenness is constrained between 0 and 1. It is considered as the measure of equality of abundances in a community.

It was calculated from the Shannon- Weaver diversity index by following formula:

$$J = H / H_{max}$$

Where,

J=Pielou's (1966) evenness index

H = diversity index derived by Shannon-Weaver equation

$H_{max} = \log_2 S$, where, S = number of species

Palmer's pollution index

Water pollution indices are generally used to detection and evaluation of water pollution. The species composition, diversity of species, their distribution pattern, the presence or absence of indicator species or their group etc. may used as indicator to get information on pollution status. Palmer (1969) identified and prepared a list of 60 genera and 80 species of algae tolerant to organic pollution. Based on the algal data he developed Palmer's pollution index for evaluation of organic pollution in water bodies. This index table included 20 algal genera most tolerant to organic pollution and also their respective score (or number) depending on their relative tolerance. An algae is called present when 50 or more individuals of it are present in 1 ml of water. The numbers scored by each genera present in a sample are totaled to get the Palmer's pollution index of the water body.

The Palmer pollution indexes of algal genera are:

Sl. No.	Genera	Pollution Index	Sl. No.	Genera	Pollution Index
1.	*Anacystis* (*Microcystis*)	1	11.	*Micractinium*	1
2.	*Ankistrodesmus*	2	12.	*Navicula*	3
3.	*Chlamydomonas*	4	13.	*Nitzschia*	3
4.	*Chorella*	3	14.	*Oscillatoria*	4
5.	*Closterium*	1	15.	*Phormidium*	1
6.	*Cyclotella*	1	16.	*Pendorina*	1
7.	*Euglena*	5	17.	*Phacus*	2
8.	*Gomphonema*	1	18.	*Scenedesmus*	4
9.	*Lepocinelis*	1	19.	*Stigeoclonium*	2
10.	*Melosira*	1	20.	*Synedra*	2

Depending on the above table, Palmer (1969) formulated the following pollution index scale for assessment of organic pollution of water body.

Pollution index	Pollution Status
< 15	Very light organic pollution
15 – 20	Organic pollution
> 20	High organic pollution