MATERIAL AND METHODS
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During present studies only aquatic algae have been studied. These algae were distinguished as macrophytic, attached and microphytic algae. Microphytic algae were designated as Phytoplanktons. It is difficult to put a border line between the macrophytic and microphytic algae. Filaments of algae like Spirogyra may form mats and can be collected easily through hand picking while floating filaments of Anabaena or Oscillatoria may sometimes be very long but cannot be collected through hand picking. Generally the algae were observed in living condition. However, for later observation and for storage they were preserved with appropriate preservatives.

Collection, preservation and observation of macrophytic and attached algae:

These algae were collected through hand picking and were preserved in 4% formalin.

Collection, preservation and observation of planktonic algae:

Collection:

Water samples for phytoplanktons were collected generally from the surface up to a depth of about 1 meter. Samples were collected occasionally with the help of plankton net, of fine bolting silk, to obtain a concentrated sample of planktons. However, nanno and pico planktons are generally lost by this method of collection. Samples were collected from different places of a pond or a river. As the sample has to reach the laboratory, as early as possible, only a single time sampling, in a day, was done for each site.
Phytoplanktons were concentrated either by keeping the sample standing for sufficient time (4 to 8 hrs.) or by centrifugation of the sample at a low speed, because high speed centrifugation may result in disruption or distortion of the plankton cell.

Reed stems or the twigs of submerged weeds, were collected and placed in polythene bags. Just enough water was added to the container to ensure a saturated atmosphere when it is closed. On return to the laboratory, the bottles were opened and the samples were examined as soon as possible.

To allow algae to live for a longer time their concentration was maintained low. During the collection of algae the natural water in which they were occurring was added in sufficient quantity to keep the alive. Another precaution is to refrain from placing them indiscriminately in any kind of water, especially tap water, which is usually highly chlorinated or sometimes contains toxic amounts of zinc or copper.

Macrophytic algae were collected in plastic bottles. Samples requiring transport more than 12 hours were preserved with 4% formalin.

Preservation

1. Algae were stored for subsequent morphological studies, in a 4% solution of formalin (prepared by adding 4 ml of .40% formalin to 96 ml of distilled water (40% commercial formalin is regarded as 100% for purposes of calculation). For preserving aquatic algae, an appropriate quantity or 40% formalin may be added directly to the sample so as to obtain a final concentration of about 4%.

2. Added 0.3 ml Lugol’s solution to 100 ml of sample and stored in dark. For long term storage added 0.7 ml of Lugol’s solution to 100 ml sample and buffered formaldehyde to minimum of 2.5% final concentration after 1 hr.

Lugol’s solution:
Potassium iodide 10 g
Iodine crystal 20 g
Distilled water containing 20 ml glacial
Glacial acetic acid 200 ml
3. For maintaining the algae in their natural (green) colour, following solution was employed. The algae were immersed in the preservative for a few days and then were transferred to a formalin acetic alcohol (FAA) solution.

<table>
<thead>
<tr>
<th></th>
<th>Amount</th>
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</thead>
<tbody>
<tr>
<td>Cupric sulphate (CuSO₄ 5H₂O)</td>
<td>0.25 gm</td>
</tr>
<tr>
<td>Water</td>
<td>38 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>4 ml</td>
</tr>
<tr>
<td>40% formalin</td>
<td>8 ml</td>
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<tr>
<td>95% ethyl alcohol</td>
<td>50 ml</td>
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Temporary Fixation

Temporary fixation was done by adding a drop of dilute Iodine Potassium Iodine (IKI) solution to a 1-2 ml sample of algae; this acts also as a temporary preservative. (The IKI solution was prepared by dissolving 2 gm of KI in 20-30 ml of water and then dissolving 1 gm of Iodine. The solution was then made up to 100ml with distilled water.)

General Staining

Different algae vary in their affinity for stains. More frequently employed stains were Methylene Blue, Gentian Violet and Acid Fuchsin (up to 1% aqueous solutions). The following simple procedure was applied:

Staining was done by mounting algae in a drop of water on a slide and then applied a coverglass. A drop of the stain was placed to one edge of the coverglass and let it diffuse to the opposite edge by absorbing the water from the other side with a piece of dry blotting paper. In this way, a spectrum of staining was obtained, the algae near one side of the coverglass are intensely stained and on the opposite side weakly stained.
Arresting the Movements

In the living state, certain algae are actively motile or exhibit gliding movements. Others may exhibit a passive Brownian movement. To slow down such movements any one of the following was applied:

(i) added a tiny drop of IKI solution to a drop of algal suspension;
(ii) added a pinch of powdered gum arabic or dextran to a drop of algal suspension and applied a coverglass;
(iii) mixed a drop of chloroform water (i.e., a drop of chloroform in 5 ml of distilled water) with a drop of algal suspension and applied a coverglass;
(iv) placed a drop of algal suspension directly in the center of a slide or petri dish containing a thin sheet of 2.5% aqueous agar, applied a coverglass and observed under a microscope. This method also arrest the Brownian movement.
(v) Isabgol (*Plumbago ovata*) seeds were placed in the sample, after a few minutes the seeds swell restricting the movement of algae. However, the seed coat releases some dye, which can stain some organisms.

Flagella:

Most algal flagella cannot be seen under an ordinary light microscope without special staining. To render flagella visible fixed the algae in dilute IKI solution and examined. If overstrained, decolorized to the desired degree by adding dilute sodium thiosulphate solution.

Cleaning diatom frustules:

Diatom frustules were cleaned, as far as possible, free from the other accompanying material, through repeated washing and decantation. The diatom material was then dried and boiled in conc. HNO₃. Washed the acid thoroughly. Then was boiled with Conc. H₂SO₄ and washed. The process cleaned the frustules.
Analysis for physico-chemical characters:

Temperature (°C), pH, conductivity (mmhos, cm⁻¹) and dissolved solids (mg.l⁻¹) of water were determined with the help of a Century, Portable, Water Analyser Kit.

Analysis of water for other physico-chemical characters was done following Standard Methods (APHA-AWWA-WPCF, 1979), as follows:

1. Alkalinity:

Alkalinity (mg⁻¹ as CaCO₃) was determined in three forms as phenolphthalein, methyl orange and total alkalinity by titrimetric method.

100 ml, or a suitable volume of, sample was taken. Two drops of phenolphthalein solution was added to this sample. If purple colour was developed, it was titrated with 0.01N HCl to the disappearance of the colour (end point pH 8.3) and was recorded as phenolphthalein alkalinity (PA). Then methyl orange solution was added to this sample and titration was continued with 0.01 N HCl till the development of red colour. This was recorded as methyl orange alkalinity (MA). Total alkalinity was obtained by adding the values for PA & MA. Calculations were made as follows:

\[
PA (\text{mg} l^{-1} \text{ as CaCO}_3) = \frac{\text{ml titrant} \times \text{Normality of titrant} \times 50,000}{\text{ml sample}}
\]

\[
MA (\text{mg} l^{-1} \text{ as CaCO}_3) = \frac{\text{ml titrant} \times \text{Normality of titrant} \times 50,000}{\text{ml sample}}
\]

Total alkalinity (TA) = PA + MA
2. Free Carbon Dioxide (F. CO₂):

F. CO₂ was determined by titrimetric method. 100 ml or a suitable size of sample was taken. Phenolphthalein was added to this sample. If no colour developed the sample was titrated with 0.01N NaOH till the development of pink colour of the phenolphthalein. F. CO₂ was estimated using the formula:

\[
\text{ml titrant} \times \text{Normality of titrant} \times 44,000
\]

\[
\frac{\text{F. CO}_2 (\text{mg l}^{-1})}{\text{ml sample}}
\]

3. Dissolved Oxygen (DO):

DO content in water sample was determined by azide modification method:

Reagents:

Manganese Sulfate Solution: Dissolve MnSO₄.4H₂O (480g) or MnSO₄.2H₂O (400g) or MnSO₄·H₂O (364g) in 1l water and filter. It should not give colour with starch when added to an acidified solution of KI.

Alkali-Iodide-Azide: Dissolved 500g NaOH or 700g KOH and 130g NaI or 150g KI in water and then diluted to 1l. Added 10g NaN₃ dissolved in 40ml water. Acidified solution of this should not give colour with iodine.

Sodium thiosulfate 0.025N: Dissolve about 6.3g Na₂S₂O₃·5H₂O in 1l water. Preserved by adding 5ml chloroform or 1g NaOH. Standardised against dichromate solution.

Potassium dichromate (dried at 130°C for 2 hrs): Dissolved 12.259g K₂Cr₂O₇/l = 0.25 N. Prepared 0.025 N solution from this stock solution.
Starch indicator: Dissolved 2g soluble starch in hot water, added 0.2 g salicylic acid as preservative.

Sod. thiosulfate standardization: To 10 ml of 0.025 N K₂Cr₂O₇ soln. added 5 ml conc. H₂SO₄. Titrated with sod. thiosulfate till light straw colour. Added starch indicator and completed the titration.

Procedure: BOD bottle was filled with the sample. Added 2ml MnSO₄ solution followed by 2 ml of alkali-iodide-azide solution, well below the surface. Stoppered carefully to exclude air bubble. Mixed the contents by inverting the BOD bottle several times. Allowed the ppt to settle then added 2 ml conc. H₂SO₄, restoppered, mixed the contents gently. Titrated with 0.025N thiosulphate adding the starch indicator towards the end of titration (solution light straw coloured). 1 ml of 0.025N Sod. thiosulphate = 0.2 mg DO

4. Chloride (Cl⁻):

Chloride content was determined by argentometric method.

Reagents:

Potassium chromate indicator: 50 g of K₂CrO₄ was dissolved in little water, AgNO₃ solution was added till a definite red ppt was formed. Filtered after 12 hrs. volume was made up to 11.

Silver nitrate (0.0141N): Dissolved 2.395 g AgNO₃ in 11 distilled water, standardised against standard chloride. Stored in a brown bottle. 1ml of 0.0141 N AgNO₃ = 500 g Cl.

Standard chloride (0.0141N): 824.1 mg NaCl, dried at 1400°C, was dissolved in 11, chloride free water. 1ml = 500 g chloride.
Reagent to remove interference: 125 g AlK(SO₄)₂ or AlNH₄(SO₄)₂·12H₂O was dissolved in water. 55 ml of NH₄OH was added slowly. The mixture was transferred to a bottle. The precipitate was washed thoroughly until free from chloride. Volume was made up to about 1 l.

If the sample was coloured, 3 ml of Al(OH)₃ was added and filtered.

An appropriate volume of sample was taken. pH was adjusted to 7-10 but, without using H₂SO₄ or NaOH. It was titrated with AgNO₃ using K₂CrO₄ as indicator. End point was pinkish yellow.

\[
\text{Cl}^- \text{mg}^{-1} = \frac{\text{ml of titrant} \times \text{normality of titrant} \times 35,400}{\text{ml sample}}
\]

5. Hardness:

Hardness was determined only as calcium hardness by EDTA titrimetric method.

Ethylene diamine tetra acetic acid (EDTA) or its salts when added to water containing both calcium and magnesium, it combines with calcium first, forming a purple complex in the presence of suitable indicator as ammonium perpurate or murexide indicator at sufficient high pH of about 12 to 13.

Reagents:

i. NaOH (1.0N)

ii. Murexide indicator (Ammonium perpurate): 200 mg murexide powder was mixed with 100 g solid sodium chloride and was grinded to 40 to 50 mesh.
iii. Standard EDTA (0.01M): Dissolved 3.723 g sodium salt of EDTA/litre in distilled water was standardised against standard calcium solution.

iv. Standard calcium solution: 1.0 g anhydrous calcium carbonate powder was dissolved in 1+1 HCl. To this 200 ml distilled water was added and boiled to expel carbon dioxide. After cooling 2 drops of methyl red indicator was added and the intermediate orange colour was adjusted by adding 3N NH₄OH or 1+1 HCl as required. The volume was made to 1.0 liter giving a strength of 1.0 mg CaCO₃/l.

Procedure:

Pre-treatment of sample was done by acidifying 100 ml sample with 5 ml conc. nitric acid. After this a small amount of 30% hydrogen peroxide was added till the formation of white residue. This residue was dissolved in warm 1+1 HCl solution and was then neutralized with conc. NH₄OH. Final volume was adjusted to 100 ml. Sample containing 5-10 mg calcium of above pre-treated sample was taken. Sufficient NH₄OH was added to produce a pH of 12-13. 0.1-0.2 g of indicator mixture was added and titrated with EDTA.

\[
\text{ml titrant x mg CaCO}_3 \text{ equivalent to 1 ml EDTA x 400.8} \]

\[
\text{Ca mg}^{-1} = \frac{\text{ml titrant x mg CaCO}_3 \text{ equivalent to 1 ml EDTA x 400.8}}{\text{ml sample}}
\]

\[
\text{ml titrant x mg CaCO}_3 \text{ equivalent to 1 ml EDTA x 1000} \]

\[
\text{Hardness as CaCO}_3 \text{ mg}^{-1} = \frac{\text{ml titrant x mg CaCO}_3 \text{ equivalent to 1 ml EDTA x 1000}}{\text{ml sample}}
\]
6. **Nitrate-Nitrogen:**

Nitrate-nitrogen was determined by phenoldisulphonic acid method.

**Reagents:**

NO$_3$ free 6N NH$_4$OH, Ca(OH)$_2$, MgCO$_3$, activated charcoal, approximately 1N CuSO$_4$ (125 g. of CuSO$_4$.5H$_2$O/l), Ag$_2$SO$_4$ 0.6% solution.

Phenol 2-4disulphonic acid: 25 g. of crystal white pure phenol was dissolved in 150 ml. of conc. H$_2$SO$_4$. To this was added 75 ml. of fuming H$_2$SO$_4$. The flask was heated in boiling water for two hrs. and the solution was stored in a brown bottle.

**Standard nitrate:** 0.7221 g. KNO$_3$/l; 1 ml = 100 $\mu$g NO$_3$-N

Nitrate extraction solution: 20 ml. of CuSO$_4$ + 100 ml. of 0.6% Ag$_2$SO$_4$ were mixed and diluted to 1 l. If chloride is absent in sample Ag$_2$SO$_4$ may be omitted.

**Extraction of nitrate:** 100 ml. or a suitable volume of sample was taken. It was made alkaline by adding 0.25 g. CaCO$_3$ and was evaporated to dryness. To the residue 100 ml. of extraction solution, 0.2 g Ca(OH)$_2$ and 0.5 g. MgCO$_3$ were added, shaken, and filtered. First 10 ml. of filtrate was discarded and then about 40 ml. was collected. If the filtrate was coloured with organic matter, 1 g NO$_3$ free activated charcoal was added, shaken and filtered.

**Colour development:** 20 ml. of filtrate was evaporated to dryness, cooled and then 3 ml. of phenol disulphonic acid was added. Reaction was allowed for 10 minutes with rotation and then 15 ml. of cold water was added. After cooling 6N NH$_4$OH was added till the solution turned alkaline (indicated by yellow colour), then 3 ml. more was added, this was diluted to volume with water. Absorbance was read in a spectrophotometer at 420 nm.
7. Total Phosphorus:

Total phosphorus was determined as phosphate by Vanado- molybdophosphoric acid method.

Reagents:

Phenolphthalein, Conc. HCl, Activated carbon, Vanado-molybdate reagent.

a. 25g. of (NH₄)₆Mo₇O₂₄H₂O (ammonium molybdate) was dissolved in 300 ml. water.

b. 1.25 g. ammonium metavanadate NH₄VO₃ was dissolved in 300 ml. water with heating to boiling. It was then cooled and added 330 ml. conc. HCl, again cooled. Solution a was poured on to solution b and diluted to 1 l.

Standard phosphate solution: 219.5 mg. anhydrous KH₂PO₄ was dissolved in water and diluted to 1 l. 1 ml. = 50 µg PO₄-P

Procedure:

Digestion: Measured a desired sample volume. Acidify to methyl orange (red) with conc. HNO₃, added another 5 ml. of conc. HNO₃. Evaporated to 15-20 ml. Cooled, added another 5 ml. of conc. HNO₃, cooled and then added 10 ml. of 70-72% HClO₄ and a few boiling chips. Heated on a hot plate till white fumes of HClO₄ appeared. Cooled and added one drop of aqueous phenolphthalein, neutralized with 6N NaOH and volume was made up to 100 ml. with distilled water.

Colour removal: If the sample was coloured, 200 mg. Activated carbon was shaken with 50 ml. sample and filtered. Sample pH and P concentration: Sample pH if between 4 and 10 no adjustment required. If less than 4, diluted 50 ml sample to
100 ml. with water. If more than 10, added 1 drop phenolphthalein to 50 ml. sample and discharged red colour with conc. HCl, before diluting to 100 ml. (but never required). Dilution was required also when PO4-P concentration was more than 15 mg/l.

Colour development: 35 ml. or less sample with 50 to 1000 μg P was taken in 50 ml. volumetric flask, 10 ml. Vanadate-molybdate reagent was added and diluted to the mark with water. Absorbance was measured at 400-490 nm (470 nm usually used). Colour is stable for days.

Chemical Oxygen Demand (COD):
It was determined by dichromate reflux method.

Reagents:
i. \( \text{K}_2\text{Cr}_2\text{O}_7; 0.25N \)

ii. Sulfuric acid reagent: Added 22g of\( \text{Ag}_2\text{SO}_4 \) to 4 kg of\( \text{H}_2\text{SO}_4 \). Kept for 1 to 2 days for dissolution.

iii. Ferrous ammonium sulfate 0.1N: Dissolved 36 g \( \text{Fe(NH}_4\text{)}\text{SO}_4\text{6H}_2\text{O} \) in water, Added 20 ml conc. \( \text{H}_2\text{SO}_4 \), cooled and diluted to 1l. For standardization, to 10 ml of this solution, 30 ml of \( \text{H}_2\text{SO}_4 \) was added and was titrated against \( \text{K}_2\text{Cr}_2\text{O}_7 \) using ferroin indicator.

iv. \( \text{HgSO}_4 \) crystals

v. Sulfamic acid: required only for nitrite removal

50 ml or a smaller sample was diluted to 50 ml in a 500 ml refluxing flask. Added 1 g \( \text{HgSO}_4 \) (for up to 2000 mg Cl/l), several boiling chips and 5 ml of acid. Cooled while mixing. Added 25 ml of 0.25N \( \text{K}_2\text{Cr}_2\text{O}_7 \). Attached the flask to the condenser and started the cooling water, added the remaining 20 ml of acid reagent through the open end of the condenser with swirling and mixing. Covered the open end of the condenser with a small beaker. Refluxed the mixture for about 2 hrs. Cooled and diluted the mixture to about double the volume with water. Titrated the excess dichromate with standard ferrous ammonium sulfate using 2-3 drops of ferroin indicator. End point from blue-
green to reddish brown. Simultaneously a blank was also run. Calculation for COD was done using the following formula:

\[
\text{COD mg l}^{-1} = \frac{\text{ml of FAS for blank} - \text{ml of FAS for sample} \times \text{molarity of FAS} \times 8000}{\text{ml sample}}
\]

**Algal indices**

Indices for different Phytoplankton groups were calculated following Nygaard (1949 in Hutchinson 1967).

1. **Myxophycean Index** = \( \frac{\text{Number of species of Myxophyceae}}{\text{Number of species of Desmideae}} \)

2. **Chlorophycean Index** = \( \frac{\text{Number of species of Chlorococcales}}{\text{Number of species of Desmideae}} \)

3. **Euglenophycean Index** = \( \frac{\text{Number of species of Euglenophyta}}{\text{Number of species of Myxophyceae and Chlorophyceae}} \)

4. **Compound Index** = \( \frac{\text{Number of species of Myxophyceae, Chlorococcales, centric diatoms and Euglenophyta}}{\text{Number of species of Desmideae}} \)