CHAPTER-2

MATERIALS AND METHODS

2.1 PHYSICO-CHEMICAL ANALYSIS OF WATER SAMPLES OF SOME RELIGIOUS PONDS OF KURUKSHETRA

2.1.1 SELECTION OF STUDY SITES

Kurukshetra district lies between latitude 29°-52' to 30°-12' and longitude 76°-26' to 77°-04' in the North Eastern part of Haryana State. According to Mahabharata the land between the Saraswati and Drishdvati was called Kurukshetra, which covered an area of five yojanas in radius. The territory between Tarantuka and Arantuka and between Machkruka and Ramhrada is called Kurukshetra, Samant Panchaka and the Northern Vedi of Brahma (Lal 1980, Trikha 2006). It was after the name of King Kuru, the ancestor of Kauravas and Pandavas this place was called Kurukshetra. Prior to the name of Kurukshetra this place of pilgrim was known as 'Brahmkshetra', 'Bhrigukshetera', 'Aryavarat' and 'Samant Panchak ' etc. All these names can be found in the holy pages of Mahabharata (Dandekar 1990, Buck 2004, Chapple 2009).

The plain area of Kurukshetra is remarkably flat and within it, are the narrow low-lying flood plains, known as either Betre Khadar of Naili. Saraswati, Markanda and Ghaggar are the important rivers of the district. The district covers a large number of holy places, temples and tanks connected with the ancient Indian traditions and the Mahabharata War. Some of the main aquatic ponds have been discussed in the previous chapter. Out of these religious ponds, the three big ones have been selected as the study sites for their exploration as follows:

(1) STUDY SITE-I (BRAHMA SAROWAR) - It is believed that this is the place where Lord Brahma, the creator of universe conceived the earth. A dip in the sarowar helps to gain sanctity as same as that of performing
'Ashvamedha Yajna’. It is of belief that bath on the days of the solar eclipse helps people to get away from all sins. Brahma Sarowar is 3600x1500 ft in size. It is also a place for hermits to meditate. 'Deep Daan, Pind Daan’ and 'Aarti’ are the ceremonies related with *Gita Jayanti* and *Solar Eclipse celebrations* conducted with much enthusiasm. During winter migratory birds from far off places visit this holy tank. The ‘Kurukshetra Festival’ is celebrated in the month of December in the Kurukshetra city of Haryana. The festival is celebrated as a commemoration of the **birth anniversary of the Srimad Bhagavat Gita.**

(2) **STUDY SITE-II (SANNIHIT SAROWAR)** – It is yet another sacred water tank at Kurukshetra. It is believed to be the meeting point of seven sacred Saraswati rivers. The Sarowar spreads 1500 ft in length and 550 ft in breadth. Bathing ghats and steps have been set up here for pilgrims. Temples dedicated to Dhruva Narayan, Goddess Durga and Lord Hanuman can be seen here.

(3) **STUDY SITE-III (JYOTISAR SAROWAR)** - It is also another sacred tank at village Jyotisar, approx. 5 km away from the Kurukshetra University Kurukshetra. It was here that the great battle of *Mahabharta* was fought and Lord Krishna preached his Philosophy of "Karma" as enshrined in the Holy Geeta to Arjuna at Jyotisar. It is the birth place of holy ‘Geeta’ where Lord Krishna preached the lesson ‘Geeta’ (the holy epic of Hindus) to ‘Arjuna’ during the war of *Mahabharata* and later became famous as the ‘Geeta Updesh Sthal’. It is believed that the same live ‘Akshya Vat Wriksha’ (the immortal *Ficus bengalensis* tree) is still present here since the time of Mahabharata. There are mainly two ponds here. Pond-1 is used mainly for religious purpose, while the 2<sup>nd</sup> one is used for either recreation purpose, lying idle or occasionally for *Nelumbo* cultivation by Govt. Agencies. Both ponds are interconnected via a tiny bridge. This non-religious adjacent pond is the breeding ground for some higher genera like *Eichhornia, Hydrilla and Nelumbo* etc.
Fig. 2.1 Some real images of STUDY SITE- I (*BRAHMA SAROWAR*)
Fig. 2.2 Some real images of STUDY SITE- II (SANNIHIT SAROWAR)
Fig. 2.3 Some real images of STUDY SITE-III (JYOTISAR SAROWAR)
2.1.2 **SAMPLE COLLECTION:**

The study period comprised three successive years (2009-2011). The periodic collection of water samples were made between 9:00 to 11:00 h from the selected water bodies. The water samples were collected from the selected water bodies in air tight containers and transported to the laboratory for analysis with a regular interval of fortnight.

The seasonal mean values of different parameters considered are hereby presented as different seasons, i.e. summer (March-June), rainy (July-October) and winter (November-February) seasons respectively.

![Fig. 2.4 Collection of Water Sample from Study Site-III](image)
2.1.3 **PHOTOGRAPHY:**

Photography was done with the help of a digital camera (Make: Canon, Model No. A3400, Japan) and printed with the help of a laser printer (Make: Canon, Model No. LBP-1210, Japan) connected to a computer.

The **microscopic photography** was carried out using a microscope (Make: Suswox Optik, India), an electronic camera (Make: Nikon, Model No. Coolpix S4000, Japan).

2.1.4 **STATISTICAL ANALYSIS:**

The statistical analysis of the data obtained was performed for their significance at various probability levels using the books for statistics (Snedecor and Cochran 1989, Parker 1991, Trudy *et al.*, 1997).

**STANDARD ERROR (SE):**

The Standard Error (SE) of the data was calculated as:

\[ SE = \frac{SD}{\sqrt{n}} \]

Where, SD = Standard Deviation, and n = number of variants.

**ANALYSIS OF VARIANCE (ANOVA):**

The data for various metal treatments and exposure time were verified for their significance at a particular probability level, and the variance ratio (F) (Parker 1991, Trudy *et al.*, 1997) was calculated as:

\[ F = \frac{\text{Treatment mean square}}{\text{Residual mean square}} \]

In the results section, \( F_{\text{Hg}^{2+}, 6, 48} \) means that the F value was calculated for six treatments of \( \text{Hg}^{2+} \) concentrations (including \( \text{Hg}^{2+} \)-less control also) and it included 48 observations.

**CORRELATION COEFFICIENT (r):**

The correlation coefficient (r) was calculated to account for the extent of correlation between the two variables (X) and (Y) using the following equation:

\[ r = \frac{\sum X \cdot Y - \sum X \cdot \sum Y / n}{\sqrt{[\sum X^2 - (\sum X)^2 / n] \cdot [\sum Y^2 - (\sum Y)^2 / n]}} \]

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2.1.5 **PHYSICO-CHEMICAL ANALYSIS OF WATER SAMPLES:**

The physicochemical parameters were determined in each case by applying the methods as described in ‘Standard Methods for Examination of Water and Waste Waters’ (APHA, 1995). The temperature, Free CO$_2$ and transparency were measured at the site. For DOC, the samples were fixed at the site and then transported to the laboratory for final analysis (Willard *et al.*, 1974, Ghosh *et al.*, 1983, Singh *et al.*, 2007).

2.1.5.1 **Temperature:** This was measured with the help of a Celsius thermometer.

2.1.5.2 **Transparency:** Transparency was measured with the help of Secchi disc (20 cm in diameter) pointed alternating black and white.

2.1.5.3 **Turbidity:** Turbidity was measured by using electronic turbidimeter (Electronics, India).

2.1.5.4 **Electrical Conductance:** Electronic conductivity meter (Electronics, India) was used to measure electrical conductance.

2.1.5.5 **pH:** Electronic pH meter (Electronics, India) was used to measure pH.

2.1.5.6 **Dissolved oxygen content (DOC):** Winkler’s modified iodide-azide method was used for the estimation of dissolved oxygen (Singh *et al.*, 2007). The samples were fixed at the site with the help of manganous sulphate and alkaline iodide azide. In the laboratory the precipitates were dissolved with the help of conc. H$_2$SO$_4$ and then titrated with sodium thio-sulphate using starch as indicator.

2.1.5.7 **Free CO$_2$ Content:** water sample was titrated at the site of sodium carbonate using phenolphthalein as an indicator (Willard *et al.*, 1974). The following formula was used to calculate free CO$_2$.

\[
\text{Free CO}_2 = \frac{V \times N \times 44,000}{\text{mg/l CaCO}_3} \times \frac{\text{ml sample}}{}
\]
where V and N are volume and normality of titrant, respectively.

2.1.5.8 **Nitrate** (NO₃⁻) **Content**: The phenol-disulphonic acid method was applied for the analysis of nitrate nitrogen (Willard et al., 1974, Ghosh et al., 1983). The water sample was dissolved in phenol-disulphonic acid. The alkaline medium was made by adding NH₄OH. The development of yellow colour denoted the presence of nitrate. The colour intensity was proportional to the amount of nitrate nitrogen and was measured with the help of spectrophotometer (Electronics, India) at a wavelength of 410 nm in terms of optical density. The final calculations were made with the help of standard graph using KNO₃.

2.1.5.9 **Sulphate** (SO₄²⁻) **Content**: Barium chloride colorimetric method was adopted to analyse the sulphate content of water (Chesnin and Yein 1950, Singh et al., 2007). BaCl₂ was added to the sample water to remove turbidity and the optical density was taken at a wavelength of 420 nm in a spectrophotometer. The final calculations were be made with the help of standard graph of K₂HPO₄.

2.1.5.10 **Chloride** (Cl⁻) **Content**: Mohr’s method was applied for the determination of the amount of chloride present in the water sample (Ghosh et al., 1983). The water was titrated with silver nitrate (AgNO₃) using potassium chromate as an indicator. The chloride content was calculated using the following formula:

\[
\text{Cl}^{- \text{ Content}} = \frac{(a-b) \times N \times 35.45}{\text{ml sample}} \times 1000 \text{ mg/l}
\]

where, 
- a= volume of AgNO₃ used for the sample
- b= volume of AgNO₃ used for the blank
- N= normality of AgNO₃ (0.0141)

2.1.5.11 **Ammonium** (NH₄⁺) **Content**: The ammonium content of the sample was estimated using Nessler’s reagent (Burris and Wilson 1957). **Preparation of Nessler’s Reagent**: 11.3 g of resublimated iodine
was mixed with 10.0 ml of distilled water, 10.0 g potassium iodide (KI) and 15.0 g mercury in a 100 ml capacity volumetric flask by constant shaking and simultaneous cooling in a cold water bath until the yellowish colour disappeared. The volume of the solution was raised to 100 ml by adding distilled water, and poured subsequently in 465 ml of 2.5 N NaOH solution. The solution was filtered thereafter, and stored in a dark coloured glass bottle.

**Procedure:**

A 1.5 ml of Nessler’s Reagent was added to 5.0 ml of water sample and incubated for 10 min at room temperature. The intensity of the yellow colour so developed, was recorded at 420 nm against reagent Blank run simultaneously. The amount of ammonium ions was quantified with the help of a standard curve obtained with NH₄Cl.

**2.2 CYANOBACTERIAL AND ALGAL BIODIVERSITY OF DIFFERENT STUDY SITES:**

**2.2.1 SAMPLING PROCEDURE:**

In order to evaluate seasonal variation in species diversity and distribution, monthly collection of water samples from different study sites was done. The planktonic algal samples were collected from the epilimnion at the different fresh water ponds of Kurukshetra, using plastic plankton nets (0.1mm pore size), forceps and knifes.

Water samples were collected using air tight bottles containers and transported to laboratory.

**2.2.2 ISOLATION AND CULTIVATION OF SAMPLES:**

The traditional methods of isolation and cultivation of algal samples were followed (Venkataraman 1969, 1981, Kaushik 1987). The collected cyanobacterial samples were isolated and transferred to conical flasks with Allen and Arnon’s nitrogen free (AA-) medium (Allen and Arnon 1955) to grow them in laboratory conditions for further ecophysiological studies.
For isolation and culturing of cyanobacteria the samples were inoculated in the enrichment medium (Allen and Arnon 1955). Germanium dioxide was used in the nutrient medium to inhibit the growth of diatoms. The cultures were incubated at 25°C with an illumination of 2000 lux for 30 days. The cultures were observed under the light microscope (400 x magnifications) and morphological characteristics of the species were carefully studied for their identification (Desikachary 1959, Round 1981).

The AA- medium was found to be most suitable to isolate and maintain the strains like *Anabaena*, *Nostoc* and *Merismopedia* etc. However, *Chlorella*, *Vaucheria* and *Cladophora* were maintained in Allen and Arnon’s nitrogen supplemented (AA+) medium.

The cultures were observed under the light microscope (400x magnifications) and morphological characteristics of the species were carefully studied. The traditional taxonomic criteria based on the morphological characteristics were used for their identification (Desikachary 1959, Round 1981, Kaushik 1987).

### 2.2.3 PREPARATION OF CULTURE MEDIA:

The collected water samples were used for isolation and cultivation of different cyanobacterial strains and the blue green algal cultures were developed in the laboratory using Allen and Arnon’s (Allen and Arnon 1955) algal growth medium (both solid as well as liquid medium).

**Preparation of Stock Solutions:** The macronutrient solutions were prepared separately in different dark coloured stock bottles. A single solution was prepared for all the micronutrients (except Fe-EDTA) as shown in table no. 2.1.

**Preparation of Fe-EDTA:** The chelating solution of Fe-EDTA was prepared as per the methodology of Waris 1953. A 5.2 g of EDTA was dissolved in distilled water. A 5.4 g of KHCO₃ was dissolved in a separate beaker. The EDTA solution was added to it and boiled for one hour. After
removing CO$_2$, 5.0 g of FeSO$_4$ was added to this solution and boiled for one hour. After cooling, the red coloured solution was filtered and final volume was raised to 1000 ml. The 1.0 ml of this solution was added to 1.0 L of the Allen and Arnon’s medium.

Table No. 2.1 The composition of basal growth medium i.e. Allen and Arnon’s medium (Allen and Arnon 1955):

<table>
<thead>
<tr>
<th>NUTRIENT (SALT)</th>
<th>CONCENTRATION (mM)</th>
<th>CONCENTRATION (mg/L)</th>
<th>STOCK (ml)</th>
<th>ALIQUOT VOLUME (ml/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MACRONUTRIENTS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>1.0</td>
<td>9.858</td>
<td>400.0</td>
<td>10.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>4.0</td>
<td>9.350</td>
<td>400.0</td>
<td>10.0</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>0.5</td>
<td>2.940</td>
<td>400.0</td>
<td>10.0</td>
</tr>
<tr>
<td>K$_2$HPO$_4$*</td>
<td>2.0</td>
<td>13.934</td>
<td>400.0</td>
<td>10.0</td>
</tr>
<tr>
<td>KNO$_3$(+) or ( - )</td>
<td>20.0</td>
<td>8.088</td>
<td>400.0</td>
<td>10.0</td>
</tr>
<tr>
<td><strong>MICRONUTRIENTS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn (MnSO$_4$.4H$_2$O)</td>
<td>0.5</td>
<td>400.0</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Mo (MoO$_3$)</td>
<td>0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zn (ZnSO$_4$.4H$_2$O)</td>
<td>0.05</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cu (CuSO$_4$.5H$_2$O)</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B (H$_3$BO$_3$)</td>
<td>0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co [Co(NO$_3$)2.6H$_2$O]</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CHELATING AGENT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe-EDTA</td>
<td>4.0</td>
<td>1000.0</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

*K$_2$HPO$_4$ and Fe-EDTA were added after sterilization for liquid culture media & simultaneously for agar-based culture media.
The Allen and Arnon’s (1955) growth medium, free from any combined nitrogen source, was invariably used as the basal liquid medium (pH= 8.5) for the general photoautotrophic growth of the cyanobacterial strains. The solid medium was prepared by adding 1.0% Bacto-agar (5.0 g for 500 ml and 10.0 g for 1000 ml of culture medium) to the nutrient medium before sterilization. The basal culture medium and glass wares were sterilized in a vertical autoclave (Model: NSW-227, Make: NSW, India) at 1.0546 Kg/cm² pressure and 121°C for 15 minutes. All the manipulations, involving transfer of the organism to fresh liquid/solid cultures were aseptically performed under a laminar flow hood (Suswox Optic, India). The cyanobacterial strains were grown axenically in 200 ml basal medium contained in cotton stoppered Erlenmeyer flasks (capacity, 500 ml, Borosil) and incubated phototrophically in a culture room (24 ± 1°C).

2.2.4 Organism and growth conditions:

The N₂-fixing cyanobacterium *Anabaena variabilis* Breb., isolated from a local ancient sacred pond (Brahma Sarowar) of the holy city Kurukshetra, India, was axenically cultured and routinely grown in 500 ml Erlenmeyer flasks having 200 ml Allen and Arnon’s nitrogen-deficient growth medium (Allen and Arnon 1955), containing A6 trace elements at 24 ± 1°C under cool fluorescent light on the surface of the culture vessels with a 18/6 h light/dark cycle.

For general maintenance of the cyanobacterial strains, exponentially growing (8 d old) cyanobacterial cells were harvested by centrifugation (3000 x g, 3 min), (Model: R-8C, Make: Remi, India) and washed twice with sterile distilled water before inoculating into the fresh growth medium.

2.3 MERCURIC ION (Hg²⁺) CONTENT IN DIFFERENT COMPONENTS OF STUDY SITES:

The mercury ion content of different water samples, soil samples, crude algal biomass samples and the macrophyte plant samples was done as per the methodology dealt in APHA (1995). Mercury ion (Hg²⁺) content was
determined in a cold vapour atomic absorption spectrophotometer, Mercury Analyzer (Model, MA 5840, Electronics Corporation of India Limited, India; detection limit, 0.1 μg Hg²⁺/l). The various samples (except water samples) were first digested in HNO₃ as per the method described in Standard methods for Examination of Water and Waste Water by APHA (19th Edition, 1995). The Hg²⁺ content of the acid digested samples was estimated as per the methodology described in the manual of Mercury Analyzer (ECIL, India). The standard curve was obtained with HgCl₂.

Requirements:
1. 1% w/v KMnO₄ in 10% H₂SO₄
2. 20% NaOH
3. 20% SnCl₂ in 10% HCl
4. 10% HNO₃
5. Standard solution of HgCl₂

Procedure:
Sample Digestion: The crude algal biomass samples, angiospermic macrophyte plant samples and the soil samples were first digested in HNO₃ as per the method described in Standard methods for Examination of Water and Waste Water by APHA (19th Edition, 1995). The acid digested sample solutions (supernatant) and the water samples were proceeded for estimation of mercury content.

Estimation of Mercury Content:

All the control knobs present in the instrument were adjusted. The stopper was removed. A suitable aliquot of the blank, standard or sample solution were taken in the reaction vessel. 10% Nitric acid was added to maintain a volume of 10 ml. 2ml of stannous chloride was also added and the stopper was replaced immediately. Magnetic stirrer was switched on and stirred vigorously for about 5 min and then adjusted to ‘0’ and 100% Transmittance. The filter rod was left in the position. ‘HOLD’ mode of operation was switched on. The pump was started and air was allowed to purge through the reaction vessel. The
absorbance was noted at 253.7 nm as early as possible within a min and switched back to ‘NORMAL MODE’. The meter indication should be back to 100% Transmittance. The pump and the magnetic stirrer were switched off. Before each measurement it was adjusted with 0% and 100%. Measurements were repeated for standard also. The amount of mercury content was later on calibrated through the standard graph.

2.4 **IMPACTS OF INTRACELLULAR ACCUMULATION OF MERCURY ON PHYSIOLOGY OF *ANABAENA VARIABILIS***

2.4.1 **MERCURY UPTAKE STUDIES**

**Sample Collection** - Hg$^{2+}$ uptake by *A. variabilis* was determined in a cold vapour atomic absorption spectrophotometer, Mercury Analyzer (Model: MA 5840, Electronics Corporation of India Limited, India; detection limit, 0.1 μg Hg$^{2+}$/l), in terms of quantifying the total intracellular built-up of Hg$^{2+}$. For this, 10.0 ml of the exponentially growing cyanobacterial cells were centrifuged as above, and inoculated in a fresh sterile medium, having 0.05-1.0 μM HgCl$_2$, as above. The 5.0 ml aliquots from these experimental suspensions were withdrawn at desired intervals for Hg$^{2+}$ content estimation in the solution.

**Sample Digestion** - The cyanobacterial samples (pellets) and the supernatant spent medium were digested in HNO$_3$ as per the method described in Standard methods for Examination of Water and Waste Water by APHA (1995).

**Hg$^{2+}$ uptake** –

The Hg$^{2+}$ content of the acid digested samples was estimated as per the methodology described in the manual of Mercury Analyzer. The Hg$^{2+}$ content was quantified as μmol Hg$^{2+}$/mg protein by reference to a standard obtained with HgCl$_2$ in the methodology as above.

2.4.2 **MEASUREMENTS OF GROWTH YIELD**

Blue green algae behave as simple microorganisms undergoing a simple asexual life cycle and multiplying only by cell division. Hence, common growth techniques and analyses used in bacteriological studies can also be applied to blue green algal cultures. The growth of algal cultures is expressed
usually as an increase in biomass, number of cells, and amount of protein; pigments etc., over a given period of time. There are various methods and procedures that are commonly used for such determinations.

As cyanobacteria form homogenous suspension in liquid medium, direct optical density readings (or absorbance at $\lambda = 650$ nm) gave a reliable measurement of growth yield. Such turbidometric determinations were made in a microprocessor spectrophotometer (Electronics, India).

The specific growth rate constant ($k$) was calculated for plotting Lineweaver Burk Plots (double reciprocal plots), by the formula given by Kratz and Myers (1955):

$$
 k = \frac{2.303 \left( \log N_2 - \log N_1 \right)}{(T_2 - T_1)}
$$

Where, $N_1$ is initial cell density at time $T_1$ and $N_2$ is the final cell density at time $T_2$.

The initial cell density was adjusted to 35 µg protein/ml culture (absorbance, 0.02; unless otherwise mentioned for particular experiments) to study the long-term (2-14 days) experiments.

2.4.3 **ESTIMATION OF PROTEIN**

Proteins form a major part of all living systems. The protein content of the cyanobacterial cultures was estimated by the method adopted by Lowry *et al.*, (1951), modified by Herbert *et al.*, (1971). In this method, proteins react with Folin-Ciocalteau reagent to give a blue coloured complex. The colour is produced because of the reduction of phosphomolybdate by tyrosine and tryptophan liberated from the protein by the action of alkaline copper. The intensity of the colour depends upon the amount of these aromatic amino acids present and thus will vary for different proteins.

**Requirements:**

1) **Reagent A** – 1.0 N NaOH

2) **Reagent B** – (1) 5.0 % Na$_2$CO$_3$
(2) 0.5 % CuSO$_4$.5H$_2$O in 1.0 % sodium potassium tartarate

# Reagent B was prepared by adding (1) and (2) in the ratio of 25:1 (v/v).

3) **Reagent C** – 1.0 N Folin-phenol reagent (BDH, India)

*All the chemicals were products of BDH, India.

**Procedure:**

A 5.0 ml of the cyanobacterial suspension was centrifuged (3000 rpm, 3 min, R-8C, Remi, India). To the pellets (≈ 0.5 ml), distilled water was added to raise the volume up to 5.0 ml. To this, a 0.5 ml of 1.0 N NaOH was added and incubated for 10 min in a boiling water bath. A ‘blank’ was also run simultaneously with 5.0 ml of distilled water. After sufficient cooling, 2.5 ml of reagent B was added and the reaction mixture was incubated for 15 min at room temperature. This was followed by the addition of 0.5 ml Folin-phenol reagent and incubation for another 15 min. The intensity of the resulting blue colour was optically determined at $\lambda = 650$ nm and the amount of algal cell protein was calculated in µg protein/ml culture by reference to a standard calibrated curve, obtained with Lysozyme (Sigma, USA).

**Sensitivity:** 10-100 µg protein /ml culture

**Precautions:**

1) Protein solution should be alkaline.
2) Folin-Ciocalteau reagent should be kept in brown bottle and the reagent colour should not be green.
3) Vigorous mixing should be done immediately after adding Folin’s reagent. This reagent is degraded soon after coming to neutral or alkaline pH. The degraded reagent is incapable of reducing and hence will not give colour.
4) Since the method is very sensitive, great care should be taken to prepare the reagents, cleaning glassware and in performing the analysis.

2.4.4 **ESTIMATION OF CARBOHYDRATES**

Blue green algae contain a great variety of carbohydrates ranging from mono to polysaccharides, containing both aldehyde and keto groups. They are
important structural and nutritional components of the cell. Obviously, no single reagent will determine all these substances and the so called ‘Total carbohydrate’ estimations in fact, determine only the simple sugars and their polymers. All colorimetric methods of total carbohydrate determination are based on the well known Molisch test, which involves heating of the material with strong (20 N or more) \( \text{H}_2\text{SO}_4 \) and a colour developer, which is either an aromatic amine or phenol. The whole process involves: (i) hydrolysis of polysaccharides to monosaccharides, (ii) dehydration and rearrangements of monosaccharides to form furfurals (in case of pentoses) or hydroxyl-methyl furfurals (in case of hexoses) and (iii) reaction of furfural with colour developer to form a coloured compound. e.g. Furfurals produce an orange yellow colour with phenols. The total carbohydrate content of the cyanobacterial cells was estimated by the method described by Dubois et al., 1956.

**Requirements:**

1) **5.0 % (w/v) aqueous phenol** – Weigh 5.0 g phenol (BDH, India) in a clean 100 ml beaker. Liquefy this at 50° C. Dilute and make up the volume to 100 ml in a volumetric flask (capacity, 150 ml).

2) **Concentrated \( \text{H}_2\text{SO}_4 \)** (AR grade, specific gravity 1.84, Glaxo, India).

3) **Standard Glucose Solution** – Dissolve 10 mg glucose (BDH, India) in 100 ml distilled water in a volumetric flask (capacity, 150 ml) to prepare a working standard of 100 µg ml\(^{-1}\).

**Procedure:**

A 1.0 ml of sample suspension was taken in a test tube (16-20 mm diameter). A reagent ‘Blank’ containing 1.0 ml of distilled water was also run simultaneously. A set of glucose standard (10-100 µg ml\(^{-1}\)) was prepared by taking 0.1 – 1.0 ml of glucose solution and making the final volume upto 1.0 ml by sufficiently adding distilled water. Then, 1.0 ml of 5.0 % aqueous phenol was added to each test tube. After thorough mixing, a 5.0 ml of concentrated \( \text{H}_2\text{SO}_4 \) was added using a fast flow pipette for fast mixing. Incubate at room temperature for 10-15 min to complete the reaction. The intensity of the
characteristic straw colour thus developed, was determined optically at 488 nm using a microprocessor spectrophotometer (Electronics, India) and the carbohydrate content (µg carbohydrate/ml culture) was calculated from a standard curve as obtained from the glucose standard.

**Sensitivity:** 10-100 µg carbohydrate/ml culture

**Precautions:**
1. Absorbance should be determined precisely at 488 nm.
2. H₂SO₄ should be added rapidly with the pipette placed well above the liquid level.

### 2.4.5 **ESTIMATION OF PHOTOSYNTHETIC PIGMENTS:**

Blue green algae have chlorophyll a as the major light harvesting pigment along with carotenoids and phycobilins as accessory pigments. These pigments were extracted using specific organic and inorganic solvents and quantified in terms of absorbance at particular wavelengths.

#### a) Acetone soluble pigments

The pellets obtained from the known volume of cyanobacterial cultures (i.e. 10 ml) were suspended in 10 ml of 80% acetone (v/v) and incubated at 4°C for 12 hrs. In a test tube, 10 ml of 80% acetone is used as blank. After centrifugation, the supernatant were optically analyzed at 663 nm for chlorophyll a, and at 460 nm for carotenoids in a spectrophotometer. The relative amounts of the photo pigments was calculated (µg/mg protein) using the specific absorption coefficients 82.04 and 200.0, respectively (Myers and Kratz 1955; Allen 1968).

\[
\text{Chl.a (µg/ml)} = \frac{A_{663} \times 12.63 \times V_1}{V_c}
\]

Where

\[
A_{663} = \text{Absorbance at 663 nm}
\]

\[V_1 = \text{volume of acetone used for extraction i.e. 10 ml}\]

\[V_c = \text{volume of culture used i.e. 10 ml}\]
12.63 = absorption coefficient of pigments

\[
\text{Carotenoid (mg/ml)} = \frac{A_{450} \times V_{\text{extract}} \times f}{2500 \times 100}
\]

Where

\[A_{450}=\text{Absorbance at } 450\,\text{nm}\]
\[V_{\text{extract}}=\text{volume of extract i.e. } 10\,\text{ml}\]
\[f=\text{dilution factor i.e. } 1\]
\[2500 = \text{Average extraction coefficient of pigments}\]

b) Water soluble pigments

The dominant water soluble phycobiliprotein, c-phycocyanin [PC] was extracted from the residue (pellets) remaining after acetone – treatment of cells. Such residue were suspended in water and subjected to repeated freezing (4°C) and thawing (37°C). After complete extraction, the suspension was centrifuged and the intensity of the resultant clear supernatant (blue) was optically determined at 615 and 652 nm respectively. The phycobiliproteins were quantified (µg/ml culture), using the equations derived from the extinction coefficients of purified phycobiliproteins as follows (Brody and Brody 1961):

\[
\text{PC} = \frac{A_{615} - 0.474 \times A_{652}}{5.34} \, \mu\text{g/ml}
\]

Where

\[A_{615}=\text{Absorbance at } 615\,\text{nm}\]
\[A_{652}=\text{Absorbance at } 652\,\text{nm}\]

2.4.6 STUDIES ON CELL MEMBRANE DAMAGE

Estimation of Cellular Pigment Loss: This was estimated by measuring the amount of photopigments (phycocyanin) extruded out in the ambient /spent medium as per the methods dealt above.

2.4.7 NITRATE UPTAKE

It was measured by the colorimetric method of Nicholas and Nason 1957.
Requirements:

4% Brucine Solution: A 4.0 g Brucine (BDH, India) was dissolved in 100 ml of Chloroform. After shaking, that was kept in dark in refrigerator for 2 h. Then the brucine solution was filtered into a dark coloured bottle for further use.

Procedure:

A 5.0 ml of cyanobacterial suspension was centrifuged. To the supernatant, 2.0 ml of brucine solution was added. Then a 5.0 ml of conc. H₂SO₄ was also added slowly. The solution was boiled with continuous shaking on a water bath. The absorbance of the yellow colour so obtained was recorded at 420 nm against the reagent Blank run simultaneously. The amount of nitrate taken up by the algae was quantified using the standard graph prepared with the help of KNO₃.

Sensitivity Range: 10-100 µg NO₃⁻/ml culture.

Precautions:

1) Add H₂SO₄ slowly, otherwise boiling will occur; and if too slowly, then chloroform remains in the reaction mixture
2) If residual chloroform remains, boil in water bath at 90⁰C.

2.4.8 AMMONIUM UPTAKE – It was measured by adopting the colorimetric method of Burris and Wilson 1957.

Requirements:

Nessler’s Reagent (Qualigens, India)

Procedure:

A 5.0 ml of cyanobacterial sample (taken out at a regular interval of 30 min) was centrifuged. To the supernatant, 1.5 ml of Nessler’s Reagent was added and incubated for 10 min at room temperature. The intensity of the yellow colour so developed, was recorded at 420 nm against reagent Blank run simultaneously. The amount of ammonium ions taken up by the algae was quantified with the help of a standard curve obtained with NH₄Cl.

Sensitivity Range: 0.4-5.0 µg NH₄⁺ /ml culture.
Precautions:

1) Do not shake the Nessler’s reagent bottle before use. Use the clear supernatant solution.

2) Close the reagent bottle immediately after use.

2.4.9 NITRATE REDUCTASE (NR) ACTIVITY:

The NR activity was estimated by calculating the amount of nitrite (NO$_2^-$) ions formed in the nitrate supplemented cyanobacterial cultures (Snell and Snell 1949).

Requirements:

1) Toluene (Himedia, India)

2) 1% Sulphanilamide: 1.0 g sulphanilamide (Himedia, India) was dissolved in a 100 ml mixture of 80 ml distilled water and 20 ml HCl (1:4, v/v).

3) 0.2% N-(1-naphthyl)-ethylene diamine dihydrochloride reagent (NEDD): 200 mg of NEDD (Qualigens, India) was dissolved in 100 ml of distilled water.

Procedure:

A 1.0 ml of cyanobacterial suspension was centrifuged and to the pellets, 0.5 ml of toluene was added. After vigorous shaking, incubate at 4$^\circ$C for 10 min. The toluene layer was removed by centrifugation. A 2.0 ml of sulphanilamide reagent was added and incubated for 10 min at room temperature. Then a 2.0 ml of NEDD reagent was also added and further incubated for 15 min at room temperature. The intensity of the pink colour so developed, was measured in terms of optical density at 540 nm against reagent Blank run simultaneously. The amount of nitrite formed was estimated using a standard graph as obtained with NaNO$_3$.

Precautions:

1) Prepare the reagents very carefully.

2) Take distilled water in a 100 ml measuring cylinder. Add to it 20 ml of HCl slowly and with continuous shaking.