CHAPTER III
MATERIALS AND METHODS

Algal cultures of *Spirulina platensis* and *Anacystis nidulans* were obtained from Indian Agricultural Research Institute, New Delhi. *Anacystis* and *Spirulina* were grown in the same aqueous medium (composition of which is given below) in flat bottomed boiling flasks maintained at 27 ± 2°C under an artificial light hood with an approximate light intensity of ~2500 lux. 16 hrs light and 8 hrs dark cycle was followed during the whole study period. The maintenance of the lag phase of growth of the algal cultures were acquired by replacing a fixed quantity by pure culture approximately every week. The algal cultures were shaken at least twice during the forenoon and afternoon period to optimise the algal density and avoid sticking. The composition of the culture medium used is given below (Menon 1981). The medium was devoid of EDTA in order to reduce the chelation of metal added, during the study (Table 2).

Most of the experiments except that for uptake and proteins contents were conducted in 15 ml screw cap tubes, with 10.0 ml of culture media. Protein and uptake studies were conducted in 50 ml or 100 ml round bottomed flasks. All the chemicals used for the culture media and also for other experiments were Analar grade. The metal used for the study were Cu as CuSO₄·5H₂O; Cd as 3CdSO₄·8H₂O; Ni as NiSO₄·5H₂O; and Cr as K₂Cr₂O₇. Necessary
Table - 2: Chemical Composition of culture medium

<p>| | | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>NaHCO₃</td>
<td>= 18.0 gm/l</td>
<td>As solution</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>= 0.5 ml</td>
<td>H₃BO₃ = 2.9 gm/l</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>= 2.5</td>
<td>MnCl₂ = 1.8</td>
</tr>
<tr>
<td>K₂PO₄</td>
<td>= 1.0</td>
<td>WnCl₂ = 0.11</td>
</tr>
<tr>
<td>NaCl</td>
<td>= 1.0</td>
<td>CuSO₄ = 0.08</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>= 0.2</td>
<td>(NH₄)MoO₄ = 0.18</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>= 0.04</td>
<td></td>
</tr>
<tr>
<td>FeSO₄</td>
<td>= 0.01</td>
<td></td>
</tr>
<tr>
<td>As solution</td>
<td>= 1.0 ml</td>
<td></td>
</tr>
</tbody>
</table>

Table - 3: Characteristics of GBC-902 Atomic Absorption Spectrophotometer for estimation of Cu, Cd, Ni and Cr.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Lamp Current (mA)</th>
<th>Slit Width (nm)</th>
<th>Wave Length (nm)</th>
<th>Optimum range (ppm)</th>
<th>Sensitivity (ppm)</th>
<th>Fuel and Flame type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>3.0</td>
<td>0.5</td>
<td>324.7</td>
<td>1-5</td>
<td>0.025</td>
<td>Air-Acetylene, oxidizing</td>
</tr>
<tr>
<td>Cd</td>
<td>3.0</td>
<td>0.5</td>
<td>228.8</td>
<td>0.2-1.8</td>
<td>0.009</td>
<td>Air-Acetylene, oxidizing</td>
</tr>
<tr>
<td>Ni</td>
<td>3.5</td>
<td>0.2</td>
<td>232.0</td>
<td>1.8-8.0</td>
<td>0.05</td>
<td>Air-Acetylene, oxidizing</td>
</tr>
<tr>
<td>Cr</td>
<td>6.0</td>
<td>0.2</td>
<td>357.9</td>
<td>2 - 15</td>
<td>0.05</td>
<td>Air-Acetylene, High-reducing</td>
</tr>
</tbody>
</table>
quantity of the metals were weighed and stock solution were prepared which contained 1000 ppm of the metal (Cu; Cd; Ni or Cr). In case of those experiments where higher volume of culture were used higher concentrated stock solutions were used. The stock solutions were added to the experimental cultures in necessary amounts so as to have the required concentration, in the medium. Alteration in the biomass, chlorophyll content, carbon assimilation, protein content and metal uptake by the algae were studied.

Statistical analysis like regression analysis were performed with the help of computer programmes following Hielborn (1981) using micro computer Eiko-II.

Measurement of Biomass:

For all the experiments to study the change in biomass with different concentration of metals and time, initial weight of the algae were made approximately to 100 mg/l dwt. The results are reported after correcting it to 100 mg dwt/l. Metals (Cu, Cd, Ni and Cr) were added to the medium to have concentration of 0.1, 0.5, 1.0, 2.0, 4.0 and 8.0 ppm of the metals. This concentration range was selected after a preliminary assay with higher range of concentrations. In case of Cr eventhough the reduction in biomass with the concentrations finally selected were quite low, the use of higher concentrations were limited by the higher absorbance that may result from Cr solution. The biomass were
monitored spectrometrically (Stein 1973) using a Spectronic 20 spectrophotometer at 490 nm, at 24 hr intervals upto varying time duration (minimum 72 hrs and maximum 168 hrs). The optical density of the culture to dry wt biomass of the species were performed using the relation:

For *Anacystis nidulans*

Biomass mg dwt/ml

\[
= (-2.4201 \times 10^{-6}) + (1.4398 \times 10^{-3}) \times \text{optical density.}
\]

For *Spirulina platensis*

Biomass mg dwt/l

\[
= 7.57 + (800.77 \times \text{optical density})
\]

Growth rate were estimated by the relation

\[
\ln \left( \frac{N_{t1}}{N_{to}} \right) = \frac{r}{t1 - to} \quad \text{(Stein 1973; Odum, 1971)}.
\]

where \( N_{t1} \) and \( N_{to} \) = Biomass at time \( to \) and \( t1 \); \( t1 \) and \( to \) = time in hrs; \( r \) = the instantaneous growth coefficient. The survival ratio were determined as

\[
\text{S.R} = \frac{B_{\text{exp}} \times 100}{B_{\text{cont}}}
\]

where \( B_{\text{exp}} \) = Biomass at time \( t \); with different treatments, and \( B_{\text{cont}} \) = Biomass of the control culture at time \( t1 \).

EC50 at different intervals were computed by plotting the survival ratio against the log concentration (Vocke 1978).
Chlorophyll estimation

Experiment to study the alteration in Chla content under heavy metals stress were conducted in 15 ml Screw Cap tubes with 10 ml of algal cultures. Stock solution of the metal were added to the culture to have final concentrations of 0.01; 0.1; 1.0 and 10.0 ppm of the concerned metals. Half number of the tubes were covered with black paper and aluminium foil completely so as to prevent light entry and were kept along with the other half under light hood for 6 hrs. After 6hrs the tubes were collected and algae was filtered on Whatman GF/C filter paper of 2.5 cm. The samples were kept in refrigerator before extractions. Chlorophyll a content was extracted (Strickland and Parsons, 1965) with 80% acetone and the optical density was determined by Spectronic 1001 spectrophotometer at wavelengths 663 nm and 645 nm. The optical density to the Chla content was performed by the relation of Venkataraman (1983b).

Carbon assimilation

The experiments were conducted in 15 ml screw cap tubes with 10 ml of the algal cultures. The metals were added in the same concentration ranges as in case of chlorophyll a study. One tube among each concentration set (4 replicates) were wrapped with black paper and aluminium foil to give the background data. Labelled carbon was added to the experimental culture as Na$_2$CO$_3$ of 5 uCi/ml activity obtained from isotope division, BARC, Bombay. After 6 hrs exposure cultures were filtered on Whatman
GF/C filter paper. The filter paper with algae were exposed to conc. HCl fumes in a fuming chamber for 20 mls (APHA/AWWA/WPCF, 1976) to remove the possible extracellular sorbed carbon (C\textsuperscript{14}). After HCl fumigation, filter papers were taken out and dried in a desiccator. Dried filter papers were transformed to plastic scintillation vials and 5 ml of toluene based scintillation fluid (Scintillation cocktail) containing 5.0 gm PPO/l; and 0.330 gm POPOP/l were added. Counting was done with Beckman scintillation counter LS-700. Using the total carbon content determined by titration (APHA/AWWA/WPCF, 1976), counts per minute obtained and the algal weight, the net carbon uptake was estimated (Vollenweider 1974).

**Protein Estimation**

Experimental algal cultures grown in 50 ml round bottomed flask with 40 ml culture, were treated with metals in concentrations 0.1, 1.0 and 10.0 ppm. At every 48 hrs samples were collected, filtered on GF/C filter paper and extracted with 0.5 N NaOH. Protein estimation was performed by Lowry's method (Lowry et al. 1951). Folin-ciocalteu reagent necessary for the estimation was prepared in the laboratory following the method given by Oser (1979). To 0.5 ml of protein extract 5 ml Reagent A’ (1 ml 2.7% sodium potassium tartrate and 1.0 ml of 1% CuSO\textsubscript{4}.5H\textsubscript{2}O to 100 ml 2% Na\textsubscript{2}CO\textsubscript{3} solution) and 0.5 ml 1.0 N Folin-Ciocalteu reagent (Reagent B) were added. Standard calibration
curve was prepared using egg albumin dissolved in 0.5 N NaOH. Optical density was determined at 750 nm using Beckman Spectrophotometer No. 34.

Optical density to protein ug/ml was performed by the relation:

\[
\text{Protein ug/ml (SE: 3.773) } = -12.019 + 286.877 \times \text{O.D.}
\]

**Metal Uptake:**

To study metal uptake by the algal species the algae (80 ml) were taken in 100 ml round bottomed flask. Always it was taken care to keep the algal biomass approximately 200 ml dwt/l in the experimental culture. At every 12 hrs intervals samples were filtered on a GF/C filter paper (2.5 cm diameter). The filter papers were washed once with a little volume of distilled water to minimise the culture medium absorbed by the filter papers. After drying in desiccatort the filter papers were transferred to a mixture of 3 ml conc. HNO₃ and 1.0 ml conc. HClO₄ (Enk and Mathis, 1977) and kept for 24 hrs. Then the digested samples were made up to 10 ml with distilled water and stored in polyethylene vials in a cool place till the analysis was done.

Metal analysis was performed by Atomic Absorption Spectrophotometry with a GBC-902 Spectrophotometer. The lamp characteristics are given below (GBC scientific equipments) (Table 3). Metal concentration in the described samples were determined using the calibration curves shown in Fig. 1.
CALIBRATION CURVE FOR Cu, Cd, Ni AND Cr—ATOMIC ABSORPTION SPECTROPHOTOMETRY