CHAPTER III
MATERIALS AND METHODS

3.1 ORGANISMS AND THEIR MAINTENANCE

The microalgae selected for the present study were two unicellular algae and a mixture of microalgae. The two unicellular algae were:

1. Tetraselmis gracilis
2. Dicrateria inornata

Both the strains are from CMFRI Algology Laboratory, and are used as live food in aquaculture practices. Taxonomic position of the selected species are as follows:

Tetraselmis gracilis

Class - Prasinophyceae
Order - Prasinocladales
Family - Prasinocladaceae

Dicrateria inornata

Class - Haptophyceae
Order - Isochrysidales
Family - Gephyrocapsaceae

3. Mixture of Microalgae

Microalgal samples were collected from different paddy fields, and they were enriched with nutrients and allowed to grow in the laboratory, under culture conditions. This was maintained as mixed culture of freshwater
microalgae in the laboratory and it was dominated by Chlorophycean members. Important strains in this culture were:

1. Chlorella ovalis - Chlorophyceae
2. Scenedesmus indicus - Chlorophyceae
3. Selenastrum gracile - Chlorophyceae
4. Nitzchia longissima - Bacillarophyceae

3.1.1. Culture condition:

Sea water medium: Prior to preparation of the culture medium the sea water collected from offshore was allowed to age in carbus. Further sea water was filtered through Sartorius filter paper, and then boiled. After boiling upto 100° C, it was allowed to cool down one overnight. The cool sterilized sea water was then enriched with the proper nutrient medium, and this medium was transferred to sterilized culture flasks. However, the cultures were not bacteria free.

Fresh water: For culturing mixed culture of fresh water microalgae, the freshwater was collected from non-contaminated ponds, filtered and sterilized. As in the case of sea water, the cool sterilized fresh water was then enriched with the nutrients and transferred to culture flasks.

3.1.2. Culture media:

The important culture media (Gopinathan, 1982) used for nanoplankton flagellate culture in the laboratory were:
1. Schreibers medium
2. Miquels medium
3. Pantastico's medium
4. Walnes or Conways medium

Among the above media, Walnes medium (Walne, 1974) was found to be the best for the growth of microalgae. In the present study, Walne's medium was selected for further experiments.

The composition of Walne's medium is as follows:

Solution A:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>100gm</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>20gm</td>
</tr>
<tr>
<td>EDTA</td>
<td>45 gm</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>33.4gm</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>1.3gm</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>0.36gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 litre</td>
</tr>
</tbody>
</table>

Solution B:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnCl₂</td>
<td>4.2gm</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>4.0gm</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>4.0 gm</td>
</tr>
<tr>
<td>Ammonium molybdate</td>
<td>1.8gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 litre</td>
</tr>
</tbody>
</table>
Solution C:

Vitamin B1 (Thiamine) : 200mg
Vitamin B12 (Cyanocobalamine) 100mg

Each of the chemicals was dissolved separately in 100 ml distilled water.

Solutions A, B & C were prepared in different reagent bottles. Added 1 ml of A, 0.5 ml of B and 0.1 ml of C to 1 litre of filtered and sterilized sea water.

The salinity of the sea water used to prepare the medium was 30-35 ppt, for culture of _Dicrateria inornata_, whereas _Tetraselmis gracilis_ was grown at 20-25 ppt.

**Freshwater culture media:**

Modified medium of Ward and Parrish (1982) was used for culturing the fresh water microalgae.

1. **Macronutrients:**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>6.37g</td>
</tr>
<tr>
<td>MgCl₂.6H₂O</td>
<td>3.05g</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>3.675g</td>
</tr>
<tr>
<td>CaCl₂.7H₂O</td>
<td>1.1g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.261g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 litre</td>
</tr>
</tbody>
</table>
2. Micronutrients

1. CoCl₂ : 0.078g/100 ml
   CuCl₂ : 0.9g/100ml
   Dilute 1 ml of each to 1 litre for working stock solution.

2. H₃BO₃ : 0.185g
   MnCl₂ : 0.264g
   FeCl₃ : 0.096g
   Na₃ EDTA : 0.3g
   Distilled water : 1 litre
   To this add 1 ml of each micronutrients - stock solution

3. Na₂SiO₃ : 1.2g
   Distilled water : 100ml

The maintenance and test medium was prepared by adding 4 ml of macronutrient solution and 1 ml of micronutrient stock solution and 1 ml of silicate solution to 1 litre of sterilized fresh water.

The stock culture and experiment cultures were maintained in 'corning' conical flasks plugged with sterilized cotton. They were maintained in the exponential phase. The stock cultures were used to reculture every 14 days in the case of marine species, and every 7 days in the case of fresh water mixed culture. All stock and test cultures were illuminated with day light fluorescent tubes. The light : dark period within the chamber was 10 : 14 hours with a mean light intensity of 34.61 x 10⁻¹⁵ quanta x cm⁻² x sec⁻¹. The ambient temperature ranged from 27°C to 32°C.
Aeration was not provided to the cultures. Instead the cultures were shaken manually to give three to four rotations every now and then to keep them in uniform suspension. Settling was not noticed for a month in the case of marine species. But freshwater species showed a tendency to settle down after one week. Experimental set up was as shown in the photograph (Plate 1).

3.2. **BIOCIDES**

Biocides used for the present study come under three categories:

1. Insecticides : 3 Nos
2. Herbicides : 1 No
3. Fungicides : 1 No.

In the addition to these three groups two mixtures of biocides were also used. They are:

1. Mixture of all the five biocides
2. Mixture of one insecticide, one herbicide and one fungicide.

1. **Insecticides:**

There are three important group of insecticides. They are:

1. Organochlorines
2. Organophosphates
3. Carbamates
a) Experimental set up.

b) Stock culture

A. *Tetraselmis gracilis*

B. *Dicerotria inornata*

C. Mixed culture of freshwater algae.
One insecticide from each group was used for the present study. 'BHC' as organochlorine, 'Nuvacron' as organophosphate and 'Carbaryl sevin' as carbamate insecticide. The Herbicide used was 'Gramaxone', and the fungicide was 'Cuman L' (R). These five biocides were selected for the present study were purchased from the agrochemical shops. All of them were commercial grade. The detailed description about the biocides are as follows:

1. **'BHC':** Benzene hexa chloride— Gamma isomer - (Lindane) - commercial grade BHC 50% chemical name - Hexachlorocyclohexane.

2. **'Nuvacron' (Organophosphate) -** Monocrotophos 36% SL - Commercial grade.

   Insecticide with systemic and contact action based on Monocrotophos. Chemical name - Dimethyl Phosphate of 3-hydroxy-N-methyl-cis-crotonamide.

   A water soluble organophosphorous concentrate containing 360 gms monocrotophos as active ingredient in a kg. of product.

3. **'Carbaryl sevin':** Carbamate insecticide - 50% W.D.P. Commercial grade.

   **Composition:**
   
   Carbaryl (A.I.) : 50%
   Adjevants & carriers : 50%
   Chemical Name - 1-Naphthyl N-methyl carbamate.
2. Herbicide:

4. 'Gramaxone': Paraquat dichloride 24% WSC commercial grade.

**Composition:**

<table>
<thead>
<tr>
<th></th>
<th>W/W</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraquat dichloride</td>
<td>24</td>
</tr>
<tr>
<td>Other ingredients</td>
<td>76</td>
</tr>
</tbody>
</table>

Chemical name - 1-1-Dimethyl-4-4-bipyridilium dichloride

5. Fungicide: 'Cuman L'(R). Ziram 27% SC Commercial grade

An organic colloidal liquid fungicide containing 270gms ziram (zinc dimethyl dithiocarbonate) as active ingredient in a kg of product.

Chemical name - Zinc dimethyl dithiocarbonate

3.3. GROWTH MEASUREMENTS

3.3.1. Measurements of cell concentration:

The cells were fixed in Lugol's iodine solution and counted with a calibrated haemocytometer (improved, Neabaur, 0.1 mm deep, fein optic, made in GDR). Four counts were made from each sample to ensure counting accuracy and then mean value was taken.

3.3.2. Productivity measurements:

Productivity measurements were made using light and dark bottle oxygen method (Gaarder and Gran, 1927). From each experimental flask 3 samples - initial, light and dark - were analysed for dissolved O₂ content.
using Winklers method. The oxygen values were then converted into their carbon equivalents.

Productivity as determined by using the formula

\[ \text{mgc/l/hr} = \frac{O_2 \text{ml} \times 0.536}{\text{PQ} \times T} \]

The PQ (Photo synthetic quotient) is 1.25

Gross production, net production and Respiration were calculated using this method (Strickland and Parsons, 1968).

3.3.3. Determination of quantitative variation of algal pigments by Spectrophotometry

The quantity of pigments was also used as an index of physiological activity. The concentrations of chlorophylls and carotenoids were estimated by Spectrophotometric (Spectronic 1001) analysis of pigment extract. For marine species the pigment values were estimated as described by Parsons et al., (1984). For the estimation of fresh water pigments the method followed by Sartory and Grobbelaar (1984) was used.

A known volume of the culture was filtered through Millipore HA filters of pore size 0.4 µ. One or two drops of MgCo₃ were added to the sample, while filtering, to prevent acidification. The pigments were extracted by adding 10 ml of 90% acetone to each filter paper. The extraction was carried out at low temperature for 20 hours. The extracts were centrifuged and extinction of the clear solution was measured by Spectronic photometer.
For the estimation of fresh water phytoplankton pigments hot methanol was used instead of acetone (Sartory and Grobbelaar 1984).

The absorbance of the clear pigment extract was measured against the blank at different wavelengths such as 750, 664, 647, 630, 510 and 480 n.m. Concentration of various pigments (chlorophylls and carotenoids) were then calculated using the equation given by Parson's et al., (1984).

3.3.4. Biochemical compounds:

1. Protein (Dorsey et al., 1978).

Algal cells were collected after centrifugation of the culture and the algal pellet was washed with an isotonic solution of Ammonium molybdate. The protein was extracted with appropriate reagents at 100°C for 100 minutes. The concentrations and final volumes of the reagents were as published by Winkfors et al., (1984). After the heated biuret-folin colour development was complete, this colour was measured in Spectronic 1001 Spectrophotometer at 660 n.m. Protein nitrogen values were determined by interpolation from a standard curve obtained with prepared solutions of bovine serum albumin. Total protein was then calculated using a conversion factor of 6.25 generally accepted for most algal species (Dorsey et al., 1978).

ii) Carbohydrate (Kochert 1978)

Carbohydrate determinations were made using phenolsulphuric acid method for analysis of algae as reported by Kochert (1978), based on procedures developed by Dubois et al., (1956).
Algal culture samples were collected and washed with isotonic solution by repeated centrifugation. Cells to be analysed were then homogenized in 1 ml 80% sulphuric acid as in Myklestad and Haug (1972), and the total amount of carbohydrate in the solution was measured by the phenol sulphuric acid method using glucose as standard.

3.4. ALGAL BIOASSAY PROCEDURE

Each species of microalgae was exposed to the pesticides in various concentration to study the effect of pesticides on each microalgal species. Short term bioassay test was almost always of the static type (Reish and Oshida, 1986).

Initially a wide range of pesticide concentrations were used to determine the effect on the microalgae by using the method - range finding test, as outlined by Ward and Parrish (1982) and Reish and Oshida (1986). Using the results of range finding test, pesticide concentrations of the narrow range were prepared and its effect on each microalgal species was determined using - Definitive test (APHA, 1980).

All the tests were conducted as follows: clean, sterilized 500 ml conical flasks were filled with 200 ml of culture medium. Then the required strength of pesticide was added to the medium by using micro-pipett. While transferring the pesticide solution, the pipetts were rinsed with the pesticide solution of particular concentration prior to delivery in order to obtain good reproducible aliquots. After the pesticide concentration was added, known number of microalgal cells were inoculated from
a stock culture of exponential growth phase. The contents were hand shaken and incubated. Each concentration was established in triplicate. Control was also maintained of the same volume used for the test. All the cultures were periodically hand shaken to keep the cells in suspension.

3.5. BIOACCUMULATION STUDIES:

Only one insecticide - organochlorine, 'BHC' was selected for the study of bioaccumulation by the microalgae, - Tetraselmis gracilis. The method followed by Schauberger and Wildman (1977) was adopted to find out the bioaccumulation of the organochlorine insecticide.

The alga was harvested by centrifugation, lyophilized to ensure uniformity among samples and extracted 3 times with 2.5ml acetonitrile. An equal volume of 2% aqueous Na2SO4 was added to the collected supernatant. The mixture was extracted 3 times with 2 ml hexane. The extracts were evaporated to 1 ml under nitrogen.

Clean up procedure:

A glass column of size 200 mm x 14 mm was packed with florisil and anhydrous Na2SO4 (1 : 1). After elution with 200 ml 6% ethyl ether/petroleum ether (v/v), and then with 200 ml 15% ethyl ether/petroleum ether (v/v), the elute was evaporated to 1 ml on a steam bath.

Chromatographic analysis was performed on a Chemito-8510 Gas chromatograph equipped with 1 bar stainless steel column packed with 5% SE 30, and Ni 63 as electron capture detector and nitrogen as carrier gas.
The operating temperatures for the various components of the gas chromatogram were as follows:

- Oven temperature: 200°C
- Injector temperature: 230°C
- Detector temperature: 250°C

The bioaccumulation of the organochlorine pesticide was found out using the formula given by AOAC (1984).

Each residue (ppm, µg/ml) = \( \text{Conc. of Peak size of sample} \times \frac{\mu L \text{ Std. Peak size of standard}}{\mu L \text{ sample Weight of sample}} \times \text{Dilution volume} \)

3.6. **PHOTOMICROGRAPHY**

In order to understand the morphological deformities of microalgal cells as a result of biocide treatment, photomicrographs were taken. The photomicrographs of the algal cells which were fixed in 50% glycerine, were taken on Olympus Universal Research Microscope (Vonvox model P.M. 10 A.D.) equipped with an automatic exposure system. Kodak colour film was used for taking photographs (Kodak colour 35 mm 100 ASA).

3.7. **STATISTICAL ANALYSIS**

The effective concentration of biocide that would inhibit the growth of test algae by 50% as compared to control growth as EC\(_{50}\) and was calculated by probit procedure using computer (SAS Institute, 1985). The advantage of probit method is that it provides a more reliable statistical
treatment of data (Reish and Oshida, 1986).

In order to understand the effect of various concentrations of different biocides and their mixture on three microalgal culture, two-way analysis of variance (ANOVA) technique was employed (Snedecor and Cochran, 1967) and the F value was taken at 5% and 1% significant level. In Anova Tables, different concentrations of toxicant were considered as treatments, and between days as replicates.