CHAPTER 3

METHODOLOGY

3.1. Field Methods

3.1.1. Sampling Stations

After a preliminary survey, six sampling stations were identified along the course of river Periyar which included an upstream region least disturbed by human activity (Edamalayar), industrial area and a down stream site (Figure 3). The location of the stations are given in Table 4.

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Sampling Stations</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Approximate distance from Cochin harbour mouth(km)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Edamalayar</td>
<td>10°15'N</td>
<td>76°43'E</td>
<td>64</td>
</tr>
<tr>
<td>2.</td>
<td>Alwaye</td>
<td>10°8'N</td>
<td>76°21'E</td>
<td>27</td>
</tr>
<tr>
<td>3.</td>
<td>Pathalam</td>
<td>10°4'N</td>
<td>76°18'E</td>
<td>16</td>
</tr>
<tr>
<td>4.</td>
<td>Edayar</td>
<td>10°4'N</td>
<td>76°17'E</td>
<td>15</td>
</tr>
<tr>
<td>5.</td>
<td>Eloor</td>
<td>10°4'N</td>
<td>76°17'E</td>
<td>10</td>
</tr>
<tr>
<td>6.</td>
<td>Ernakulam</td>
<td>9°57'N</td>
<td>76°15'E</td>
<td>2</td>
</tr>
</tbody>
</table>

3.1.2. Collection of water samples

Water samples of 6000 mL were collected from surface and bottom at each station using a 'Ruttner' water sampler made of perspex. The sampler was of 1L capacity. Water samples were
Fig. 3. Location of sampling stations
collected by lowering the sampler from a country boat at 3 points at each station, one at the midstream and others from one third distance from either bank. Sampling was done every fortnight for a period of one year starting from January 1986. The fortnightly values were averaged to find the monthly means at each station. Water temperature, pH, stream depth and Secchi disc transparency were recorded at each station during sampling. Temperature was read with a mercury thermometer calibrated 1/10°C. pH value of the samples were measured using a portable pH meter (L.G. Nester, phase IV). Stream depth was determined by lowering a weighted graduated string into the river. The depth of the light penetration was measured using Secchi disc (Welch, 1948).

The samples for analysing dissolved oxygen were siphoned into 150 mL glass bottles and fixed in manganous sulphate followed by alkali-iodide-azide reagent. The samples for salinity estimation were stored in special salinity bottles. 25 mL sample was fixed in Lugol's iodine to examine the phytoplankton composition. The remaining samples collected were stored in polyethylene bottles and taken to the laboratory under cool dark conditions within 4 hr of collection. The samples for analysing biochemical oxygen demand (BOD) were incubated immediately after reaching the laboratory. The water samples for estimation of chlorophyll and nutrients were filtered and stored in a refrigerator until analysed.
3.1.3. **Analytical Methods**

The water samples were analysed for the following parameters:

Salinity, Dissolved oxygen (DO), Biochemical oxygen demand (BOD), Nitrite (NO$_2^-$-N), Nitrate (NO$_3^-$-N), Ammonia (NH$_3$ -N), Phosphate (PO$_4^{3-}$-P), Chlorophylls and Pheopigments.

Salinity was determined by titration with silver nitrate solution. The value for chlorinity was obtained from hydrographical tables (Knudsen, 1901).

Dissolved oxygen was determined by titration against standard sodium thiosulphate (APHA, 1985).

Biochemical oxygen demand of the undiluted samples were determined according to the method described by APHA (1985).

The procedure given by APHA (1985) was used to estimate nitrite. The method is based on the formation of a pinkish azo-dye on addition of sulphanilamide and N-(1-naphthyl)-ethylene diamine dihydrogen chloride. The optical density was measured at 543 nm in a Hitachi spectrophotometer (model 200-20).

Nitrate was reduced to nitrite by passing through a cadmium reduction column and determined as nitrite. The optical density was measured at 543 nm in spectrophotometer. The nitrate concentration was read from a standard graph (APHA, 1985).
The phenolhypochlorite method of Solorzano (1969) was used to estimate ammonia. The optical density was measured at 640 nm and the concentration was read from standard graph.

Phosphate was determined by the ascorbic acid method (APHA, 1985). The absorbance was measured at 880 nm and concentration obtained from standard graph.

Chlorophyll and pheopigments were estimated by filtering 1 L each of the samples through Whatman GF/C filter papers (pore size 0.45 μm). 1 mL of 1% magnesium carbonate suspension was added to the samples while filtering. The filters were extracted in 90% acetone under cool dark conditions (refrigerator) for 20 hr. The acetone extracts were centrifuged at 4000 rpm for 15 minutes and the absorbance measured at 750, 664, 647 and 630 nm in a spectrophotometer. The extracts were then acidified with 1 N HCl and the absorbance read at 750 and 665 nm according to the procedure of Lorenzen (1967). The amount of pigments were computed from the equations of Jeffrey and Humphrey (1975) and Lorenzen (1967).

The water samples that were fixed in Lugol's iodine (APHA, 1985) were examined microscopically (Zeiss Telaval 2 Inverted Microscope) to assess the phytoplankton composition.

3.1.4. Analyses of Data

The monthly distribution of hydrographic features is represented graphically. The sampling year has been divided
into three seasons: monsoon (June to September), postmonsoon (October to January) and premonsoon (February to May). The data were analysed using Student's t-test to find whether there was any significant difference between surface and bottom samples. The spatial variation of the variables was assessed by Page's L (trend) test (Ray Meddis, 1975). A multiple regression relationship was set up with chlorophyll as the dependent variable and the hydrological features such as pH, temperature, dissolved oxygen, nitrite, nitrate, ammonia and phosphate as independent variables (Steel and Torrie, 1960). All calculations were done in a WIPRO PC/XT computer.

3.2. Laboratory Methods

3.2.1. Test Algae

Axenic cultures of two freshwater algae: *Nitzschia palea* (Kütz) W.Sm. and *Oocystis pusilla* Hansgirg var. *major* Skuja were isolated from the upstream of river Periyar. Cultures were developed according to the standard procedure (Stein, 1973). The taxonomy of the species is given below.

Division: Chrysophyta

Class: Bacillariophyceae

Order: Bacillariales

Family: Nitzschiaaceae

Genus: *Nitzschia* (Hassall, 1845; W. Smith) Grunow Ch. em., 1880.
Species : *palea* (Kütz) W.Sm. (S.B.D., ii., p. 89; H.V.M. Atl., pl. 69, f. 22b and 22c; in Types Nos. 165, 196, 343 and 479; different varieties in Types Nos. 411 and 413), plate 17, fig. 554.

Valves linear lanceolate with apices shortly rostrate. Length 25-65 μm; breadth about 5 μm; 33-36 striae per 10 μm; freshwater in distribution (Heurck, 1896).

Division : Chlorophyta
Class : Chlorophyceae
Order : Chlorococcales
Family : Oocystaceae
Genus : *Oocystis* Naegeli in A. Braun, 1855, p 94.
Species : *pusilla* Hansgirg

A. Hansgirg, 1890, p 9; H. Printz, 1913, p 181, pl 4, f 31-32; J. Brunnthaler, 1915, p 124; G.W. Prescott, 1951, p 246, pl 51, f 15, pl 54, f 4-5 = *Oocystis naegelii* A. Br. var. *minutissima* Bernard, 1908, p 172.

Variety : major Skuja


Cells solitary, elongate-ellipsoid with rounded ends measuring 6-11 μm in length. Chromatophores 2-3, almost filling
the cell, cell division by formation of 2-4 autospores. Cell membrane thin without polar thickenings; distributed in freshwater (Philipose, 1967).

3.2.2. Maintenance medium

The algae were maintained as axenic cultures and tested for various parameters in freshwater medium, the composition of which is given below (Ward and Parrish, 1982). All nutrient solutions were prepared in glass distilled water.

**Macronutrient stock solution**

1. Dissolve 25.5 g NaNO$_3$ in 1 L water
2. Dissolve 12.2 g MgCl$_2$·6 H$_2$O in 1 L water
3. Dissolve 14.7 g MgSO$_4$·7 H$_2$O in 1 L water
4. Dissolve 4.41 g CaCl$_2$·2 H$_2$O in 1 L water
5. Dissolve 15.0 g NaHCO$_3$ in 1 L water
6. Dissolve 1.044 g K$_2$HPO$_4$ in 1 L water

**Micronutrient stock solution**

1. Dissolve 0.78 g CoCl$_2$ in 1 L water
2. Dissolve 0.90 g CuCl$_2$ in 100 mL water
   Dilute 1 mL of this solution to 1 L for working stock solution
3. To 1 L water add 0.1855 g H$_3$BO$_3$, 0.2643 g MnCl$_2$, 0.0327 g ZnCl$_2$, 0.0073 g Na$_2$MoO$_4$·H$_2$O, 0.0960 g FeCl$_3$, 0.300 g Na$_3$EDTA and 1 mL of micronutrient solutions (1) and (2).
The maintenance medium was prepared by adding 1 mL each of macronutrient solution and 1 mL of the micronutrient stock solution number (3) to 1 L of glass distilled water. The medium was autoclaved for 30 minutes at 121°C and 1.1 kg cm⁻². The cool sterilised medium was equilibrated with filtered air for 24 hr. The pH of the medium was between 7.6 and 8.2. The maintenance medium was transferred to sterilised 150 mL conical flasks (Borosil glass) plugged with non-absorbent cotton. The cultures were inoculated under aseptic conditions and exposed to illumination from day-light fluorescent lamp assembly = 2000 µ W.cm⁻² on a 12:12 light-dark cycle at 27 ± 1°C. The cultures were shaken at 6 hr interval on a rotary shaker at 100 rpm.

3.2.3. Test Procedure

The standard procedure for algal toxicity test (Ward and Parrish, 1982) was followed throughout the study. Both the species were maintained and tested in the same medium. Axenic cultures of Nitzschia palea and Oocystis pusilla var. major at exponential phase of growth were inoculated into 75 mL each of test media in 150 mL culture flasks so as to yield 1 x 10⁴ cells mL⁻¹. All tests were performed in triplicate. Cultures were incubated on a uniformly illuminated rotary shaking platform under identical conditions as in the case of maintenance cultures. Test duration was 96 hr.
After incubation, aliquots of cultures were fixed in Lugol's iodine and the cell number was counted using haemocytometer.

The photosynthetic pigments were estimated by spectrophotometric method (Jeffrey and Humphrey, 1975). Cultures of *N. palea* (50 mL) were filtered through Whatman GF/C (pore size 0.45 μm) and extracted in 90% acetone (Vollenweider, 1974). Cultures of *O. pusilla* var. major were filtered (50 mL) through Sartorius membrane filters (pore size 0.45 μm) and extracted in dimethyl sulfoxide (DMSO) because it did not give satisfactory extraction with acetone (Burnison, 1980). The absorbance was measured in a spectrophotometer at 664, 647, 630 and 480 nm.

3.2.4. Growth kinetics of test algae

The test species were inoculated into 750 mL medium in 1 L culture flasks in triplicate and incubated for 16 days. Aliquots of culture were removed every 24 hr to enumerate the cell count which were subsequently plotted on semi-logarithmic graph paper to obtain the growth curve.

The growth rate was calculated as doublings per day (k) according to the equation of Eppley and Strickland (1968).

\[
k (\text{division/day}) = \frac{3.32}{t-t_0} (\log_{10} n_t - \log_{10} n_{t_0})
\]

where time is in 24 hr day, 3.32 = \log_2 10, n_t = final cell number, n_{t_0} = initial cell number, t-t_0 = final-initial (days).
The doubling time or generation time ($t_g$) was calculated as follows

$$t_g = \frac{\ln 2}{k} = \frac{0.69}{k} \text{ (h}^{-1}) = \frac{16.5}{k} \text{ (days}^{-1})$$

On alternate days, 50 mL each of the cultures was filtered to estimate chlorophyll a, b, c and carotenoid. Chlorophylls were computed using equations of Jeffrey and Humphrey (1975) and carotenoid using that of Strickland and Parsons (1968). The mean value of the replicates were plotted on a graph paper to obtain the growth curves.

3.2.5. Nitrate requirement of test species

Nitrogen starved cultures of Nitzschia palea and Oocystis pusilla var. major were used to determine the nitrate requirement of the species (Eppley and Thomas, 1969; Dortch, 1982). The inoculum was prepared by growing the test algae in nitrogen-free maintenance medium for 96 hr so that the cells were nitrogen starved. Nitrogen depletion was indicated by reduction of growth rate and change in colour of the culture. These were inoculated into media of nitrate concentrations 1, 3, 5, 10, 15, 20 and 25 $\mu$g-at NO$_3$-N L$^{-1}$ to give an initial cell density of $1 \times 10^4$ cells mL$^{-1}$. The test concentrations were prepared by adding the required amount of Analytical Reagent grade sodium nitrate to the maintenance medium devoid of nitrogen. The cultures were harvested after 96 hr incubation to measure the cell count and photosynthetic pigments. The growth rate was plotted as a function of nitrate on a graph.
paper. Half-saturation constant ($K_s$) and maximum growth rate ($k_{max}'$) were determined graphically (Thomas, 1970).

3.2.6. **Phosphate requirement of test species**

Test media of phosphate concentration 0.16, 0.32, 0.48, 0.64, 0.96, 1.28 and 1.60 µg-at PO$_4$-P L$^{-1}$ were prepared by adding the required amount of Analytical Reagent grade potassium dihydrogen phosphate to the maintenance medium devoid of phosphorus. Phosphate starved cultures of *Nitzschia palea* and *Oocystis pusilla* var. *major* were used to determine the phosphate requirement of the species (Thomas and Anne, 1968; Qasim and Joseph, 1975). The inoculum was prepared by growing the test algae in phosphorus-free maintenance medium for 96 hr so that the cells were phosphorus starved. Phosphorus depletion was indicated by reduction of growth rate. Phosphate depleted test algae were inoculated to a final concentration of $1 \times 10^4$ cells mL$^{-1}$ and incubated for 96 hr. The cell counts and photosynthetic pigments were determined and the data analysed as in 3.2.5.

3.2.7. **Salinity tolerance**

The test was conducted in maintenance media having 0, 5, 10, 15 and 20 x $10^{-3}$ salinity. The saline medium was prepared by adding Analytical Reagent grade sodium chloride to the maintenance medium. The test conducted in triplicate was of 96 hr duration. The cells were harvested after incubation
to determine the cell counts and photosynthetic pigments.

The exponential growth constant, \( k' \) was computed from the cell count using the formula

\[
    k' = \ln \left( \frac{N_{t_1}}{N_0} \right) / (t_1 - t_0)
\]

where \( N_{t_1} \) = final cell count, \( N_0 \) = initial cell count, \( t_1 - t_0 \) = period of exposure in days (Reynolds, 1984). The significance of \( k' \) was tested by Student's t-test (Snedecor and Cochran, 1967).

3.2.8. Toxicity test

Algal assays were conducted to study the response of the test species to liquid wastes collected from the fertilizer factory producing nitrogen and phosphorus fertilizers located on the banks of river Periyar. The effluent collected from the discharge point every 3 hr interval were mixed to get a homogeneous sample which was then stored and kept cool in polyethylene container. In the laboratory the sample was filtered first through absorbent cotton and then through Whatman glass microfiber filters (GF/C) of pore size 0.45 \( \mu \)m to remove all the suspended materials (Walsh et al., 1980). The filtrate was stored in clean polyethylene container, and kept in refrigerator at 4°C until use.

The effluent was analysed immediately after filtration to estimate the following parameters: pH, Chemical Oxygen Demand (COD), ammonia, phosphate and fluoride. COD was determined
by the Open Reflux Method described by APHA (1985). The effluent was refluxed with sulphuric acid in the presence of excess potassium dichromate and titrated with ferrous ammonium sulphate.

Fluoride content was measured by complexing the acid-distilled effluent (APHA, 1985) with lanthanum - alizarin reagent according to the procedure described by Martin (1968).

The effluent was allowed to attain room temperature and further filter sterilized using Whatman GF/C filter papers. Same quantity of macro and micro nutrients used to prepare maintenance medium were added to the effluent samples for enrichment.

The enriched effluent was diluted using maintenance medium to get different dilutions of the effluent, keeping the concentration of added nutrients unaffected. These diluted effluent grades were used for the assays.

A preliminary range finding test using 10, 25, 50, 75 and 100% effluent was conducted to choose the concentrations for definitive tests. The concentrations of 5, 10, 30, 50, 70 and 90% and 5, 10, 15, 20, 25 and 30% effluent were selected for Nitzschia palea and Oocystis pusilla var. major respectively. Tests were carried out in triplicate. The maintenance medium was used as control.

The cell number and photosynthetic pigments were determined after 96 hr incubation. EC50 (interpolated or
calculated concentration of a toxicant that would inhibit population growth or any other biological process of algae by 50% compared to the controls in a specific period of time) was obtained graphically by plotting effluent concentration against percentage inhibition of growth (cell count) on a semi-logarithmic graph (Walsh, 1987). The exponential growth constant k' was computed and the significance tested by Student's t-test.

The values of EC\textsubscript{50} were used to compute the 7 day, 10 year low-flow volume (Q\textsubscript{r}) required for safety in the river receiving effluent, following the relation given by Walsh et al. (1982).

\[
0.01 \times EC_{50} = \frac{Q_w}{Q_r + Q_w} \times 100
\]

where \(Q_w\) = volume of discharge of effluent, \(Q_r\) = the 7 day, 10 year low-flow volume of the receiving water and 0.01 = a safety factor currently used by US EPA in instream waste concentration calculations for issuance of discharge permits.

In order to assess the recovery of the test species after 96 hr exposure to the effluent, in each case 1 mL of the culture was transferred aseptically to 75 mL of sterilised control medium contained in 150 mL flasks. These resuspension cultures were incubated for a period of 9 days and harvested to determine the cell number. The growth rates were compared by Student's t-test.
3.2.9. Toxicity vs. salinity

Tests were conducted to assess the variation of toxicity at EC$_{50}$ effluent concentration in different salinities using test species. Two sets of test media were prepared, controls and treatments. Controls were prepared by adding Analytical Reagent grade sodium chloride to the maintenance medium to obtain 5, 10, 15 and 20 x $10^{-3}$ salinities. These were inoculated with the test species, which served as controls. Treatment media were prepared in EC$_{50}$ concentration of effluent having the same salinities as in control. N. palea and O. pusilla var. major were acclimated for 96 hr in respective EC$_{50}$ effluent concentrations. The acclimated species were inoculated into the treatment media. Both control and treatment cultures were incubated for 96 hr. The cultures were harvested after incubation to determine cell counts and photosynthetic pigments. The effect of salinity on effluent toxicity was evaluated statistically.

3.2.10. Toxicity at low nitrate concentration vs. ammonia

Experiments were conducted using the test species to assess the variation of toxicity at EC$_{50}$ effluent concentration in different ammonia levels when the nitrate concentration was low. For this the maintenance medium was modified by keeping nitrate concentration at 25 $\mu$ g-at NO$_3$-N L$^{-1}$. After conducting range finding tests using Analytical Reagent grade ammonium chloride in modified maintenance medium the concentrations for definitive tests were selected. In both
test species definitive test concentrations selected were 0.04, 0.08, 0.16, 0.32, 0.64, 0.80 and 2.40 μ g-at NH₃-N L⁻¹. The modified maintenance media containing different ammonia concentrations were served as the controls. Treatment media containing selected concentrations of ammonia were prepared in the same manner by substituting the maintenance media with EC₅₀ effluent concentration to which nutrient solutions having 25 μ g-at NO₃-N L⁻¹ were added.

Test species acclimated for 96 hr in maintenance medium containing 25 μ g-at NO₃-N L⁻¹ were inoculated into control and treatment media. These cultures were harvested after an incubation period of 96 hr to determine the cell counts and photosynthetic pigments. The data were analysed by Student’s t-test.

3.2.11. Toxicity at high nitrate concentration vs. ammonia

The toxicity experiments (3.2.10) were repeated with 500 μ g-at NO₃-N L⁻¹, which is approximately the highest concentration found in the industrial area of river Periyar.

3.2.12. Toxicity at low nitrate concentration vs. phosphate

Identical toxicity experiments (3.2.10) were repeated using selected definitive test concentrations of phosphate, keeping nitrate at 25 μ g-at NO₃-N L⁻¹. Analar Reagent grade potassium dihydrogen phosphate was used to prepare concentrations
of 0.60, 1.80, 5.40, 16.20 and 48.60 μg-at PO₄-P L⁻¹.

3.2.13. **Toxicity at high nitrate concentration vs. phosphate**

The experiment (3.2.12) was repeated with 500 μg-at NO₃-N L⁻¹.