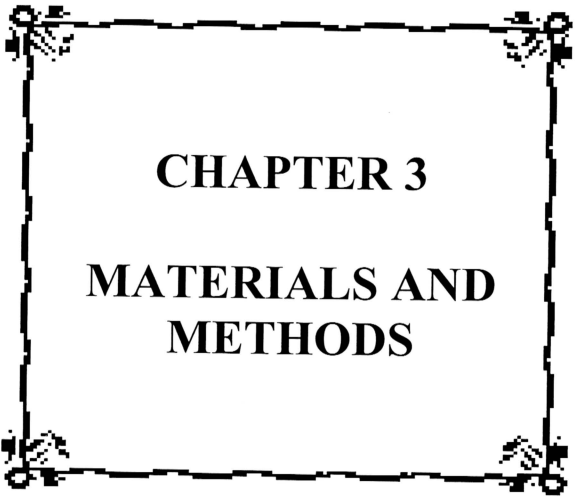

CHAPTER 3

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

3.1 The sampling sites

Twelve sampling stations were selected to cover as much of Tasek Bera as possible, as well as to coincide with the stations where water quality studies were conducted in 1997. Description of the sampling sites can be found in Appendix 1.

The locations for sampling stations are illustrated in Figure 9. The sampling stations were :

Station	Location	Station	Location
1	Tanjung Kuin (Figure 10)	7	Lubuk Kuang (Figure 16)
2	Sungai Tasik (Figure 11)	8	Kampung Baapa (Figure 17)
3	Lubuk Pathir (Figure 12)	9	Sungai Bera 1(Figure 18)
4	Tanjung Papan (Figure 13)	10	Sungai Bera 2 (Figure 19)
5	Kampung Benal (Figure 14)	11	Paya Kelantong (Figure 20)
6	Kuala Sungai Tembangan (Figure 15)	12	Sungai Tembangan (Figure 21)

3.2 Field measurements and sampling

Field sampling was conducted once a month between April to October 1998 and coincided with one dry season and one wet season. At each sampling station, three substations were established, with two substations near each side of the littoral zone and one in the middle of the stream. Three replicates were made for each measurement. Geographical Positioning System (GPS) readings of all stations were recorded using an Ensign Geographical Positioning System by Trimble Navigation.

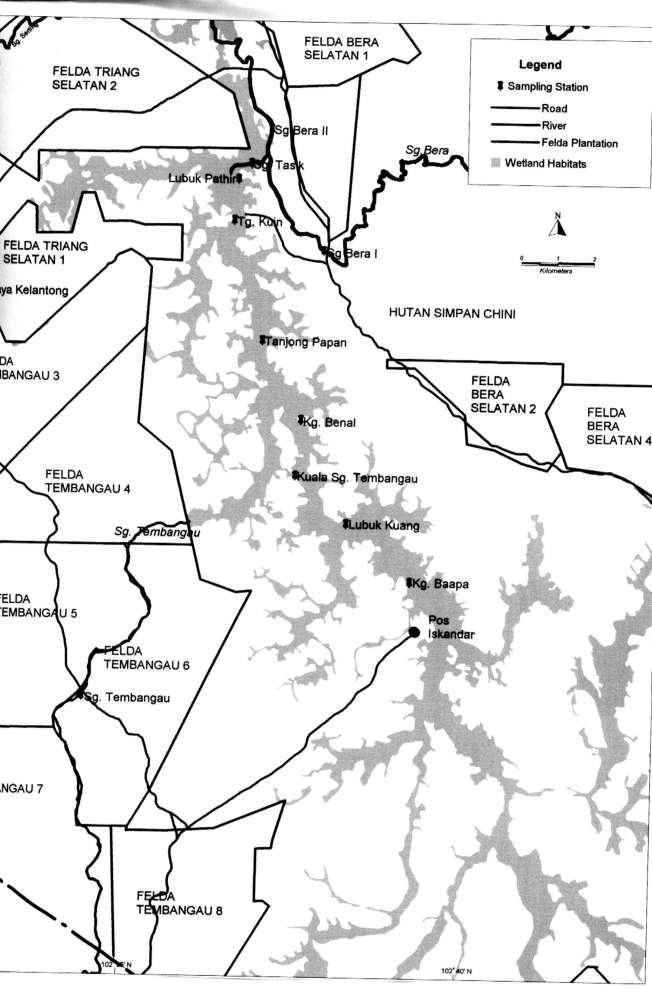

Figure 9. Location of Sampling Stations and Felda Plantations at Tasek Bera

Figure 10. Sampling Station 1 : Tanjung Kuin

Figure 11. Sampling Station 2 : Sungai Tasik

Figure 12. Sampling Station 3 : Lubuk Pathir

Figure 13. Sampling Station 4 : Tanjung Papan

Figure 14. Sampling Station 5 : Kampung Benal

Figure 15. Sampling Station 6 : Kuala Sungai Tembangan

Figure 16. Sampling Station 7 : Lubuk Kuang

Figure 17. Sampling Station 8 : Kampung Baapa

Figure 18. Sampling Station 9 : Sungai Bera 1

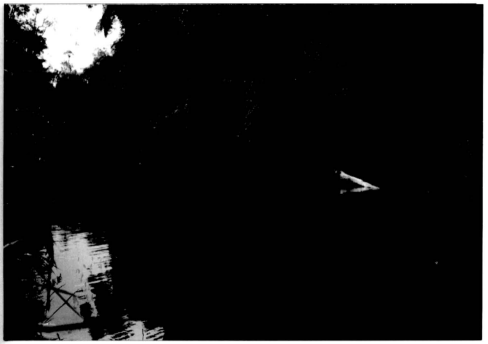

Figure 19. Sampling Station 10 : Sungai Bera 2

Figure 20. Sampling Station 11 : Paya Kelantong

Figure 21. Sampling Station 12 : Sungai Tembangan

3.2.1 Physical and chemical parameters

Temperature and dissolved oxygen were measured using the YSI dissolved oxygen meter Model 57 while pH was measured with a Hanna pHep 2 pocket pH meter. Conductivity was measured using Hanna instrument Model HI 8733 and Secchi disk was used to determine transparency of the water. Water samples were collected in 500 ml acid-washed plastic sample bottles and stored in the dark before analysis in the laboratory for determination of nitrate (NO_3), orthophosphate ($O\text{-}PO_4$) and ammoniacal-nitrogen concentration ($NH_3\text{-}N$).

3.2.2 Productivity measurements

The oxygen production capacity (OPL) as an expression of chlorophyll activity was measured. The basic reactions in algal photosynthesis involve uptake of inorganic carbon and release of oxygen, summarised by the relationship:

$$CO_2 + H_2O \text{ ---> } (CH_2O)_6 \quad + \quad O_2$$

The light and dark bottle method was used to estimate gross and net productivity and respiration in terms of oxygen used or produced per unit time. Pairs of 250 ml reagent bottles with ground glass stoppers were required. The dark bottles were darkened by covering with aluminium foil. Initial concentration of O_2 in both bottles was measured. A rope was tied to the bottles, anchored by a stone and suspended using a polystyrene float, or tied to a floating or anchored object. The pairs of bottles were suspended at depths of 0,1,and 2 or 3 m and incubated for 3 to 6 hours, the water having been taken from the same depth at which the bottles were incubated. After incubation,

the bottles were retrieved to measure the concentration of dissolved oxygen in ppm using a YSI dissolved oxygen meter Model 57.

Units of production measurements :

Three measurements were obtained for each depth at each site.

C_1 = Initial O_2 concentration (ppm)

C_2 = Final O_2 concentration (ppm) for dark bottles

C_3 = Final O2 concentration (ppm) for light bottles

Three qualities were calculated from these measurements :

Gross photosynthesis, $P_G = C_3 - C_2$

Net photosynthesis, $P_N = C_3 - C_1$

Respiratory activity, $R = C_1 - C_2$

Since the time of incubation was accounted for, the values at each depth were in units of $mg/O_2/l/hour$. For sites where the depth was more than 50m greater than the deepest production measurements made, the calculation was carried out only for the upper portion of the water column.

3.2.3 Phytoplankton sampling

Phytoplankton samples were obtained using a 3 L Van Dorn plankton sampler. 15 L of sample was collected and concentrated by filtering through a plankton net with a mesh size of 10μ, at each substation. Samples collected from the three substations

were kept in 500ml plastic sample bottles and preserved with formalin. $CuSO_4$ crystals were added to maintain the colour of the green and blue-green species.

3.3 Laboratory measurements

3.3.1 Chlorophyll-a measurements

Chlorophyll-a was measured to indicate standing crop. At each substation, 15 L of water sample was collected using a 3 L Van Dorn plankton sampler, concentrated to at least 100 ml by filtering through a plankton net with a mesh of 10μ and kept in the dark until analysis in the laboratory. Chlorophyll-a analysis was conducted by extraction in acetone according to Strickland and Parsons (1968). Algal sample that was strained on filter paper was homogenised with a known volume of 100% acetone in a plastic centrifuge tube and left overnight at 4°C in the refrigerator to facilitate pigment extraction. The tubes were centrifuged at 3,000 rpm for 10 minutes and the optical density (O. D.) of the clear supernatant obtained at wave lengths 630 nm (OD_{630}), 645 nm (OD_{645}) and 665 nm (OD_{665}), with a Shimadzu UV 160 Spectrophotometer. The calculation of chlorophyll-a concentration was based on the following equation (Strickland and Parsons, 1968):

$$\text{Chll-a } (mgm^{-3}) = \frac{(C_a)\,(V_a)}{(V_c)}$$

where

$C_a = 11.6\ OD_{665nm} - 1.31\ OD_{645nm} - 0.14\ OD_{630nm}$

V_a = Volume of acetone in mL

V_c = Volume of sample in L

3.3.2 Chemical analysis

3.3.2.1 Dissolved orthophosphate

The ascorbic acid method based on Murphy and Riley (1962) was used in the analysis. The reaction between ammonium molybdate and potassium antimonyl tartrate in acid medium forms phosphomolybdic acid that has an intense-coloured molybdenum blue after being reduced by ascorbic acid (APHA, 1989).

Prior to analysis, glassware were cleaned thoroughly with concentrated H_2SO_4 and rinsed well with distilled water. Apparatus not in use was kept filled with 0.1% v/v H_2SO_4.

15 g of A.R. ammonium paramolybdate, $(NH_4)_6Mo_7O_{24}.4H_2O$ was dissolved in 500 ml of distilled water to prepare the molybdate solution and was kept in a plastic bottle out of direct sunlight. The solution is stable only for a few weeks. 140 ml A.R. concentrated H_2SO_4 was added to 900 ml of distilled water for the sulphuric acid solution. 5.4 g of high quality ascorbic acid, $C_6H_8O_6$, was dissolved in 100 ml of distilled water. This solution was prepared only on the day it was to be used. As for the potassium antimonyl tartrate solution, 0.35 g of high grade potassium antimonyl tartrate, $KSbO.C_4H_4O_6$, was dissolved in 250 ml of water and stored in a plastic or glass bottle. This solution is stable for many months. In order to prepare the mixed reagent, 100 ml ammonium molybdate, 250 ml sulphuric acid, 100 ml ascorbic acid and 50 ml potassium antimonyl tartrate solutions were mixed together. The mixed reagent should be prepared shortly before use and must not be kept for more than 6 hours.

Standard phosphate solution was prepared by dissolving 0.439 g of dessicated potassium dihydrogen phosphate, KH_2PO_4 in distilled water and made up to 1000 ml. A

few drops of chloroform were added and the solution stored in a dark bottle. This solution contained 100,000 μg PO_4^{3-}-P L^{-1}. One litre of PO_4^{3-} solution containing 1000 μg PO_4^{3-}-PL^{-1} was prepared by diluting 10.0 ml of the above stock. The dilute solution could last for only 3 months.

Reference solutions that contained 0,2,5 and 7 μg PO_4^{3-}-P were made by diluting 0,2,5 and 7 ml of the 1000 μg PO_4^{3-}-PL^{-1} standard solution to 100 ml with distilled water. 10 ± 0.5 ml of mixed reagent from a 25 ml measuring cylinder was added to 100 ml of each sample and to the reference solutions and mixed at once. After 10 minutes and within two to three hours, the absorbance of the solution was measured with a 160 UV Shimadzu Spectrophotometer in a 10 cm curvette against distilled water at a wavelength of 885 nm.

3.3.2.2 Ammoniacal-nitrogen

A modified phenate method based on Solorzano (1969) was used. 10 g of reagent grade phenol, C_6H_5OH, was dissolved in 100 ml of 95% v/v ethyl alcohol, C_2H_5OH for the phenol-alcohol solution. The sodium nitroprusside solution (5% w/v) was prepared by dissolving 1 g of sodium nitroprusside, $Na_2Fe(CN)_5NO.2H_2O$, in 200 ml of water and stored in an amber bottle. The solution must not be kept for more than a month. 100 g of A.R. trisodium citrate, $C_3H_4OH(COONa)_3.2H_2O$, A.R. and 5 g of A.R. sodium hydroxide, NaOH, were dissolved in 500 ml of distilled water to make the alkaline citrate solution.

A solution of commercial hypochlorite solution, NaOCl with 12.5% available chlorine was used as sodium hypochlorite solution. The oxidizing solution was prepared by mixing 4 volumes of alkaline citrate solution and 1 volume of hypochlorite.

45.8 mg of ammonium chloride, NH_4Cl, was dissolved in distilled water and made up to 1 litre in a volumetric flask. The solution is quite stable. A ten times dilution of this stock gave a working solution containing 1.2 μg of ammonia nitrogen per ml. The standard ammonia solution was prepared using 0 ml, 0.25 ml, 0.50 ml and 1.00 ml of the stock with ten times dilution by further diluting to 50 ml with distilled water. This made up reference solutions of 0, 6, 12 and 24 μg NH_3-N L^{-1}.

Two ml of phenol alcohol solution, two ml of sodium nitrotrisside solution and five ml of oxidizing solution were added to 50 ml of each sample or reference solution and mixed thoroughly after each addition. The blue colour of indophenol was allowed to develop at room temperature for at least 1 hour. The blue colour is stable for at least 18 hours. Absorbance of standards and sample were measured in a 10 cm cuvette, with distilled water in the reference cell at 640 nm using a 160 UV Shimadzu Spectrophotometer.

3.3.2.3 Nitrate

A Waters Quanta 4000 Capillary Electrophoresis (CE) system was used for the determination of nitrate. Analysis was based on ion mobility of NO_3^- in an electrolyte of sodium chloride, NaCl and OFM (osmotic flow modifier) solution in a capillary made from silica. To prepare electrolyte, 0.1648g of sodium chloride solution was added to 100 ml of deionised water and 2.5 ml of OFM-BT solution (20mM) into a 150ml

volumetric flask and the mixture was shaken until evenly mixed. The electrolyte was filtered and then degased for 15 minutes using a sonicator. Standard solution was prepared using ammonium nitrate, NH_4NO_3 and made up into reference solutions of 0, 0.1,0.5,1.0 and 2.0 ppm. A capillary tube with a 50μm diameter and a dimension of 37.5 cm (total), 30.0 cm (to window) was used for hydrostatic injection of samples at a rate of 10 cm for 30 seconds into the electrophoresis with a separation voltage of 15 kV. Carrier electrolyte consisted of 28 mM NaCl, 0.45 mM Waters OFM™ modifier. Detection was through indirect UV absorbance at 214 nm. Prior to analysis, the capillary electrophoresis was purged with deionised water and then with 0.5 M potassium hydroxide, KOH, for five minutes. The capillary was purged again with deionised water for 15 minutes and electrolyte for another five minutes. 500μl of samples was transferred using a micropipette into centrifuge tubes and placed in the carousel before the analysis was run. Output of calibration data and results were measured with computer software that was linked to the capillary electrophoresis and measurements were based on peak area and peak height value plotted by the graphs in the computer.

3.3.3 Microscopic identification and quantification of phytoplankton

3.3.3.1 Microscope calibration

A standard compound microscope, equipped with a set of 10x oculars and 10x, 20x, 40x and 100x objectives, was used for algal identification and enumeration. A Sedgewick-Rafter counting chamber was used for phytoplankton identification and enumeration under standard light microscope at the magnification of 200X. The

Sedgewick-Rafter cell measured approximately 50mm long by 20 mm wide by 1 mm deep. The total area of the bottom of the counting chamber was approximately 1000 mm^2 and the total volume approximately 1000 mm^3 or 1 ml. Therefore, the grid on the counting chamber consisted of 50 x 20 squares of $1mm^2$.

The microscope was calibrated using an eyepiece graticule and a stage micrometer. The eyepiece graticule consisted of ten units of accurately ruled grid. The stage micrometer consisted of standardised, accurately ruled scale on a glass slide, totalling 0.01mm. The two scales for eyepiece graticule and stage micrometer were overlapped. The line at the left of the eyepiece graticule was matched with the zero mark on the stage micrometer scale. The width of the eyepiece graticule was determined to the nearest 0.01mm from the stage micrometer scale. If the width of the grid on the eyepiece graticule was exactly 0.01mm, the smallest graduation for the eyepiece was 1/0.01mm (10 μm). Therefore, direct measurement was done using the eyepiece graduation and then converted based on the conversion with stage micrometer.

3.3.3.2 Phytoplankton counting techniques

The Sedgewick-Rafter chamber, a counting cell that limits the volume and area for ready calculation of population densities, was used for counting phytoplankton. The Sedgewick-Rafter chamber was approximately 50 mm long by 20 mm wide by 1 mm deep and has grid lines marked on the glass slide. Prior to filling the Sedgewick-Rafter cell, the cover glass was placed diagonally across the cell while a large-bore pipette was used to transfer the sample into the cell. This was to prevent formation of air bubbles in

cell corners. The cover slip will usually rotate slowly to cover the inner portion of the Sedgewick-Rafter cell during filling. Overfilling should be avoided as this would yield a volume of more than one ml and produce an invalid count. A small drop of distilled water was placed on the edge of cover glass during examination to prevent formation of air spaces caused by evaporation. Before counting, the Sedgewick-Rafter chamber was let to stand for at least 15 minutes to settle the plankton. Phytoplankton on the bottom of the Sedgewick-Rafter chamber was counted. However, the disadvantage of using a Sedgewick-Rafter chamber is that magnification was limited to approximately 200X.

Some phytoplankton are unicellular while others are multicellular (colonial). The natural unit or clump, the most easily used system, was applied during enumeration. Dead cells or broken diatom frustules were not counted. Plankters were counted in random fields. The number of cells per mililiter were multiplied or divided by a correction factor to adjust for sample dilution or concentration. The number of plankton per mililiter were calculated as follows :

$$\text{No. / ml} = \frac{C \times 1000\ mm^3}{A \times D \times F}$$

where

C = number of organisms counted

A = area of a field , mm^3

D = depth of a field (Sedgewick-Rafter cell depth), mm

F = number of fields counted

Total cell count = $\frac{\text{Number of cells per mililiter}}{\text{Concentration factor}}$

Concentration factor = V / C

where

V = Volume of water filtered, mL

C = Concentrated volume,mL

Drawings of each species found in the samples were made while a Nikon HFS-IIA camera is also used to take photographs of the species to assist in identification. Taxonomic references by Smith (1950), Hasle (1964), West and West (1971), Prescott (1978), Whitford and Schumacher (1973), Shamsudin (1991) and Yamagishi (1992) were used in phytoplankton identification.

3.4 Data analysis

3.4.1 Frequency

For each species identified, its frequency was calculated as :

Frequency (%) = n/N x 100

where

n = Number of individual species

N = Total number of individual for all species

3.4.2 Diversity Studies

The Shannon-Weiner Diversity Index, H', was calculated for each station as follows :

$$H' = - \sum_{i-1}^{n} Pi \log_{10} Pi$$

where n = the number of individuals of the ith genus

P = the proportion of each genus

$\log_{10}$ = the logarithm of base 10

The Margalef's Species Index (d) was calculated as :

$d = (s - 1)/N$

where

s = total number of species

N = total number of individual

Equitability or species evenness (J) was calculated as :

$J = H' / \ln s$

where

H' = Shanon-Weiner's Diversity Index

s = total number of species

Sorensen's Coefficient of Similarity (%) $= 2a / (2a + f)$

where

a = number of species common to both quadrats

f = number of species present in one or other (but not both) of the quadrats

3.4.3 Statistical Analysis

Several statistical techniques were used in data analyses. This included Multiple Regression Analyses, Two-way analyses of variance (ANOVA), Newman-Keules Test as Post Hoc Test and Cluster Analyses (Table 10).

Prior to analyses, data were normalised using a $\log_{10}(x + 1)$ transformation as required for statistical analyses. The statistical software Statistica Version 5 was used for all statistical analyses.

A Two-way analysis of variance was used to assess difference in environmental parameters and biotic parameters : i) of all the nine stations; ii) between different sampling occasions (April to October 1998)

The Newman Keuls test was used to trace the significant difference resulting for the interaction of sampling occasions and stations for environmental parameters and for biological parameters such as chlorophyll-a, cell count, productivity and diversity indices of phytoplankton in Tasek Bera.

Multiple regression analysis was used to investigate which of the environmental parameters were more effective and contributed more to the chlorophyll-a concentration, cell count, productivity and diversity of phytoplankton in Tasek Bera.

Cluster analyses was used to measure similarity or distance among the sampling stations in terms of environmental parameters as well as biological parameters.

Table 10. Summary of the statistical analysis

Objective	Statistical test
Difference in environmental parameters of all the nine stations Difference in environmental parameters between different sampling occasions (April to October 1998) of the same station.	A Two-way Analysis of Variance (ANOVA) followed by Newman-Keules test as Post Hoc test
Contribution of the environmental parameters to the chlorophyll-a concentration, cell count, productivity and diversity of phytoplankton in Tasek Bera.	Multiple Regression Analysis
Measurement of similarity or distance among the sampling stations in terms of environmental parameters as well as biological parameters.	Cluster Analysis