CHAPTER 3

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS

3.1 Location of study area

The study area is located within the Federal Territory of Putrajaya. The location of five water sampling stations is as shown in Figure 2. A handheld Geographical Positioning System Model Garmin 12CX was used to determine position of each of the sampling station and data summarised in Table 2.

Water samples were collected from the Lower Bisa Lake which is part of the Phase IA Lake. The sub-catchment of Lower Bisa Lake is about 5.9 km^2 (Khor, 1988). This sub-catchment receives surface runoff mainly from the surrounding development area, mainly Precincts 1, 14, 15, 16 and 17 of the Federal Territory of Putrajaya.

Table 2. Description of water sampling stations

Station	Location	Latitude	Longitude
I	Upper Bisa wetlands, near the weir (Figure 3)	N 02°56.058'	E 101°42.111'
II	Upper Bisa Lake, near Precinct 16 pumping station (Figure 4)	N 02°56.013'	E 101°42.086'
III	Upper Bisa Lake, near Bridge 1 (Figure 5)	N 02°56.012'	E 101°41.932'
IV	Upper Bisa Lake, opposite Deputy Prime Minister's Residence (Figure 6)	N 02°55.969'	E 101°41.651'
V	Near Putra Bridge (Figure 7)	N 02°55.985'	E 101°41.442'

3.2 Sampling occasions and period

The frequency of water sampling was carried out at approximately two week intervals between each occasion from 9 December 2000 to 24 March 2001. A total of eight occasions of water sampling was conducted for this study. During each sampling day, water samples were collected from the five water sampling stations between 1000h and 1500h.

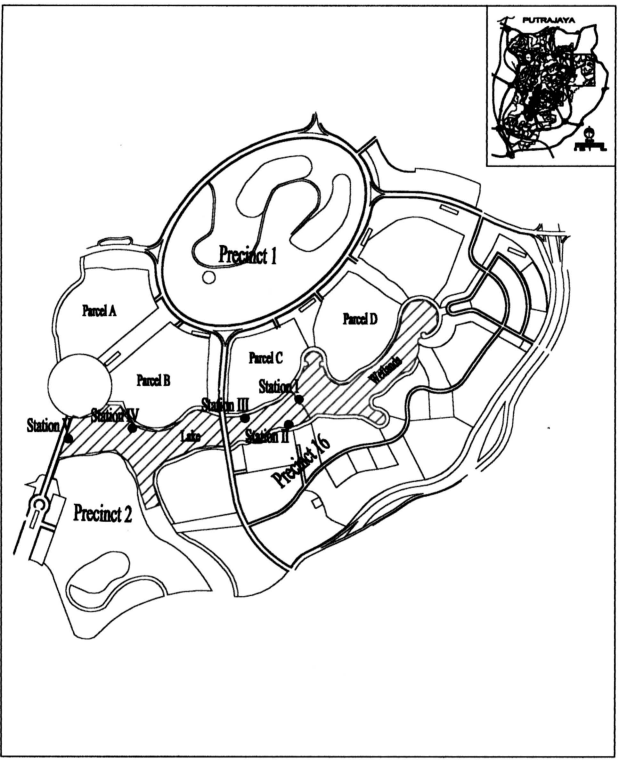

Figure 2. Location of sampling stations

Figure 3. Water sampling site at station I

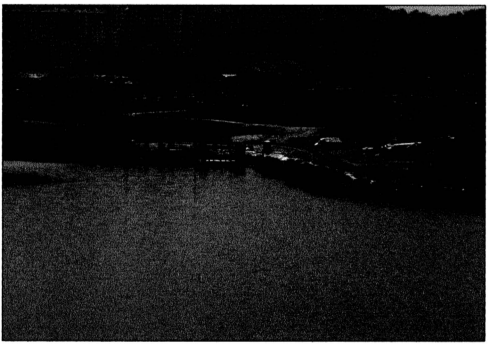

Figure 4. Water sampling site at station II

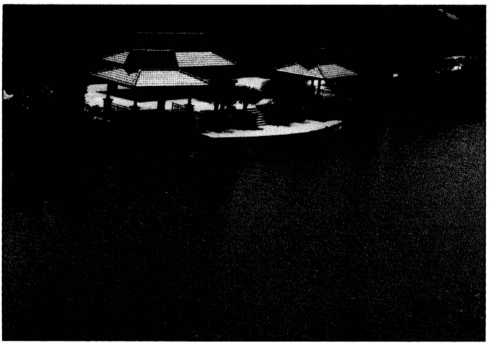

Figure 5. Water sampling site at station III

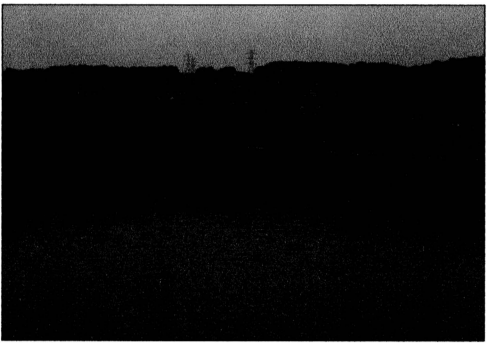

Figure 6. Water sampling site at station IV

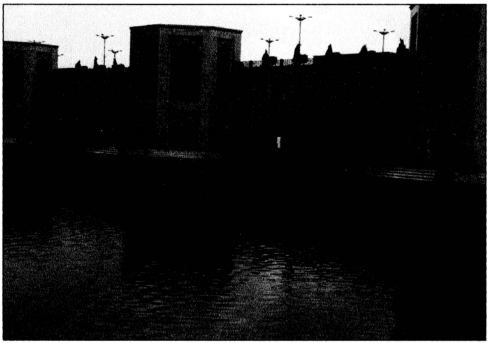

Figure 7. Water sampling site at station V

3.2.1 Sampling procedure

At each sampling station, duplicate water samples were taken from each station which were located within 5 m from each other.

3.3 Physical and chemical parameters

Physical parameters for each sub-station were measured *in-situ* using the following instruments:

Yellow Springs Instruments Model 57 : temperature and dissolved oxygen

Hanna Instruments HI 8733 : conductivity

Hanna Instruments HI 8424 : pH

All the instruments were pre-calibrated ahead before being brought to the field. Secchi depth was measured using a Secchi disc to determine the transparency of water at each station, the measurement was done in the littoral zone from the bank and not in the pelagic zone from a boat.

Water samples were collected in 500 ml pre-washed polyethylene sample bottles with plastic stoppers and screw-top caps to ensure no air was left in the bottle according to the standard methods (APHA, 1989). Only surface water was collected for the chemical parameter analysis. The water samples for orthophosphate ($O\text{-}PO_4$), ammoniacal nitrogen ($NH_3\text{-}N$) and silica (Si) analysis were stored in the dark in ice container during the sampling period before brought back to the laboratory.

3.4 Phytoplankton sampling

Water samples for phytoplankton were collected using a 3 L Van Dorn sampler. 12 L of water sample collected and concentrated by filtering through by a plankton net (mesh size of 10 μm) to a final volume of about 300 ml at each station, yielding a concentration factor of 40.

The concentrated water samples were kept in 500 ml sample bottles with 10 % formaldehyde. $CuSO_4$ crystals were added to maintain the colour of the green and blue-green algal species (after Phang & Murugadas, 1997).

For chlorophyll-a analysis, 6 L of water samples were collected using the same sampler and concentrated by the plankton net. No preservative was used.

3.5 Laboratory analysis

Upon return to the laboratory, the water quality samples for nutrients were refrigerated.

3.5.1 Chlorophyll-a analysis

Chlorophyll-a analysis was conducted by extraction in acetone according to Strickland and Parsons (1968). Water samples were filtered with glass fibre filters (0.45 μm) and rinsed with deionised distilled water and then mashed with a glass rod in 10 ml acetone. The extracts were left overnight at 4°C in the refrigerator to facilitate pigment extraction. The tubes were centrifuged at 3,000 rpm for 10 minutes. The optical density of the clear supernatant was measured with a Shimadzu UV 160 Spectrophotometer at 630 nm (OD_{630}), 645 nm (OD_{645}) and 665 nm (OD_{665}). The calculation of chlorophyll-a concentration was based on the following equation (Strickland & Parsons, 1968).

$$\text{Chl-a concentration} = \frac{(Ca)\ (Va)}{Vc \times 1000}$$

where

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

Va = Volume of acetone in mL

Vc = Volume of sample in mL

3.5.2 Chemical analysis

3.5.2.1 Dissolved orthophosphate

Ascorbic acid method based on Murphy and Riley (1962) was used. Prior to analysis, glassware was 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 in distilled water solution.

Molybdate solution - 15 g of A.R. ammonium paramolybdate, $(NH_4)_6Mo_7O_{24}.4H_2O$ was dissolved in 500 mL of distilled water and kept in a plastic bottle out of direct sunlight.

Sulphuric acid solution - 140 mL A.R. concentrated H_2SO_4 was added to 900 mL distilled water and mixed.

Ascorbic acid solution - 5.4 g high quality ascorbic acid, $C_6H_8O_6$ was dissolved in 100 mL distilled water. It was prepared on the day of analysis.

Pottassium antimonyl tartrate solution - 0.34 g of high grade potassium antimonyl tartrate, $KSbO.C_4O_6$, was dissolved in 250 mL of water. The solution is stable for many months either in a plastic or glass bottle.

50 mL ammonium molybdate, 125 mL sulphuric acid, 50 mL ascorbic acid, and 25 mL potassium antimonyl tartrate solution were mixed together to prepare mixed reagent solution. It was prepared shortly before use and kept for not more than 6 hours.

Standard phosphate solution - 0.439 g of A. R. dessicated potassium dihydrogen phosphate, KH_2PO_4 (A.R.), was dissolved in distilled water and made up to 1000 mL. A few drops of chloroform were added and stored in a dark bottle. The solution contains 100,000 ug PO_4^{3-} -P $litre^{-1}$. One litre of PO^{3-}_4 solution containing 1000 ug PO_4^{3-}-P $litre^{-1}$ was prepared by diluting 10.0 mL of the above stock. The diluted solution was kept not longer than three months.

Reference solutions contained 0, 2, 5, 7 and 10 μg PO_4^{3-} were made by taking 0, 2, 5, 7 and 10 mL of the 1000 μg PO_4^{3-}P $litre^{-1}$ standard solution and diluted to 100 mL with distilled water. 10 mL of mixed reagent was added to 100 mL of each sample and to the reference solutions and mixed at once. After 10 minutes and within 2-3 hours, the absorbance of the solutions in a 10 cm cuvette was measured with a 160 UV Shimadzu Spectrophotomer against distilled water at 885 nm.

3.5.2.2 Ammonia

Phenol-alcohol method based on Solorzano (1969) was used.

Phenol-alcohol solution - 10g of reagent grade phenol, C_6H_5OH, was dissolved in 100 mL of 95% v/v ethyl alcohol, C_2H_5OH.

Sodium nitroprusside solution (0.5% w/v) - 1 g of sodium nitroprusside $Na_2Fe(CN)_5.NO.2H_2O$ was dissolved in 200 mL of water. The solution was stored in an amber bottle for not more than a month.

Alkaline citrate solution - 100g of A.R. trisodium citrate, $C_3H_4OH(COONa)_3.2H_2O$, and 5g of A.R. sodium hydroxide, NaOH, was dissolved in 500 mL of distilled water.

Oxidizing solution - 4 volumes of alkaline citrate solution were mixed with 1 volume of commercial hypocholorite, NaOCl (12.5% available chlorine). The unstable mixture was used at the same day.

Standard ammonia solution - 4.8 mg of ammonium chloride, NH_4Cl was dissolved in distilled water and made up to 1 litre in a volumetric flask. A ten times dilution of this stock gave a working solution containing 1.2 ug of ammoniacal nitrogen per mL.

The reference solution was prepared by taking 0 mL, 0.25 mL, 0.5 mL, 1.0 mL and 2.0 mL of the standard ammonia solution (= 0, 6, 12, 24 and 48 ug NH_4-N $litre^{-1}$) and diluted to 50 mL with distilled water.

2 mL of phenol alcohol solution, 2 mL of sodium nitroprusside solution and 5 mL of oxidising solution were added to each 50 mL of sample or reference ammonia solution. The solutions were mixed thoroughly after each addition. The blue colour indophenol was allowed to develop at room temperature for at least 1 hour. The blue colour is stable for at least 18 hours. The absorbance was measured at 640 nm in a 10 cm cuvette, with distilled water in reference cell using 160 UV Shimadzu Spectrophotometer.

3.5.2.3 Silica as SiO_2

Molybdosilicate method based on Kahler (1941) was used.

Ammonium molybdate reagent - 10 g of ammonium paramolybdate, $(NH_4)_6Mo_7O_{24}.4H_2O$ was dissolved in 100 mL of distilled water. The solution was adjusted to pH 7 to 8 and kept in a polyethylene bottle.

Oxalic acid solution - 7.5 g of $H_2C_2H_4.H_2O$ was dissolved in distilled water.

Stock silica solution - 4.73 g sodium metasilicate monahydrate, Na2SiO3.9H20, was dissolved in 1000 mL distilled water.

Standard silica solution - 10 mL stock silica solution was diluted in 1000 mL of distilled water.

Potassium chromate solution - 630 mg of K_2CrO4 was diluted in 1000 mL of distilled water.

Borax solution - 10 g of sodium borate decahydrate, $Na_2B_4O_7.10H_2O$ was diluted in 1000 mL of distilled water.

The standard silica solution was prepared by taking 0 mL, 5 mL, 10 mL, 15 mL, 20 mL and 30 mL of standard silica and diluted to 50 mL with distilled water.

1.0 mL (1 + 1) HCl and 2.0 mL ammonium molybdate were added to each 50 mL of sample or reference solution, mixed by inverting for 6 times. After 10 minutes, 2.0 mL oxalic acid solution was added and mixed thoroughly. After 2 minutes but before 15 minutes, the absorbance of the solution was measured with a 160 UV Shimadzu Spectrophotometer at a wavelength of 410 nm.

3.5.3 Identification and quantification of phytoplankton

3.5.3.1 Microscope calibration

A Sedgewick-Rafter counting chamber was used for identification and enumeration of phytoplankton under standard light microscope at the magnification of 200X. The chamber provides 50 x 20 squares of $1mm^2$ and depth 1 mm. Therefore, the total volume is approximately 1000 mm^3 or 1 ml.

3.5.3.2 Phytoplankton counting techniques

Cover glass was placed diagonally across the cell and sample transferred with a large-bore pipette before filling the Sedgewick-Rafter cell to prevent formation of air bubbles in cell corners. Before counting, the Sedgewick-Rafter chamber was let to stand for 15 minutes to settle the plankton. The phytoplankton was counted at magnification of 200X.

The phytoplankton was either unicellular or multicellular. The natural unit count was used as the enumeration method, where colonies and filaments were counted as 1 unit

(APHA, 1989). Dead cells or broken diatom frustules were not counted. Plankters were counted in random fields. The number of cells per mililiter was 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 field, mm^3

D = depth of a field, mm

F = number of fields counted

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

where:

V = volume of water filtered, mL

C = concentrated volume, mL

Phytoplankton was sketched and drawn; and photographs were taken with a Nikon HFS-IIA camera attached to the microscope, to assist in identification. Taxonomic identification was based on the keys published by Smith (1950), Whitford and Schumacher (1973) and Yamagishi (1992).

3.6 Data Analysis

3.6.1 Shannon-Weiner Diversity Index

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

$$H' = - \sum_{I-1}^{n} Pi \ln Pi$$

where :

n = the number of individuals of the ith genus
P = the proportion of each genus

3.6.2 Margalef's Species Index

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

$d = (s - 1)/\ln N$

where :

s = total number of species

N = total number of individual

3.6.3 Sorensen's Coefficient

Sorensen's Coefficient = $2a / (2a + b + c)$

where :

a = number of species in both stations A and B

b = number of species in station A but not in station B

c = number of species in station B but not in station A

3.6.4 Frequency

Frequency (%) = $n / N \times 100$

where :

n = number of stations or occasions

N = total number of stations or occasions

3.6.5 Statistical Analysis

Several statistical techniques were used in data analyses. These included one-way and two-way analysis of variance (ANOVA), multiple regression analyses and Cluster Analysis.

One-way ANOVA was used to examine each variable based on the mean values for each station. Tukey honest significant difference test was conducted to make comparisons among means of all stations.

Prior to analyses, data were normalised using a log (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 for the five sampling stations and between different sampling occasions.

Multiple regression analysis was used to investigate which of the environmental parameters have more influence and relationship to the chlorophyll-a concentration, cell counts and diversity of phytoplankton.

Cluster analysis was used to measure similarity or distance among the sampling stations in terms of environmental parameters as well as biological parameters. Cluster analysis was conducted to classify the five sampling station based on the water quality variables, the biotic variables and the species composition. For all the cluster analyses, hierarchical agglomerative clustering was performed using Unweighted Pair Group Method Average (UPGMA) (Legendre & Legendre, 1988). For species composition, Sorensen's coefficient was used in the cluster analysis.

For water quality variables, temperature, pH, dissolved oxygen, conductivity, ammonical nitrogen, silicon and dissolved orthophosphate were used in the cluster analysis excluded secchi depth due to unavailable data at two stations.