CHAPTER III

Materials and Methods
3.0 Materials and Methods

3.1 Sampling

The objective of the sampling is to collect a portion of material small enough in volume to be conveniently transported to and handled in the laboratory while still accurately representing the material being sampled. This implies, firstly, that the relative portions of the concentrations of all pertinent components must be the same in the sample as in the material being sampled and secondly, that the sample must be handled in such a way that no significant changes in composition occurs before the test are performed. The analysis is generally intended to reveal the composition of the waters at the time or over the period of sampling. Consequently errors are introduced if changes take place between taking of the sample and analysis being carried out. There is in fact a strong likelihood that such changes will occur in most of the waters. The arrangements should be such that these are prevented or at least minimized.

3.1.1 Water sampling

Water is a dynamic system. During sampling the water is removed from its natural environment. Constituents of water sample may interact with the surface wall of the container and consequently their concentration may be altered. These considerations are therefore prerequisite of sampling program.

3.1.2 Site selection for lake water samples

The selection of sampling site was decided by the various uses of water and by their location, relative magnitude and importance. The chance of accidental pollution was also considered for sampling.

Water sampling stations were selected on the upstream Thanneermukkom bund towards the Alappuzha side of Vembanad Lake. Twenty representative stations were selected throughout the lake based on different stresses imposed on the system by different
activities (Figure 3.1). The factors which were considered during the site selection included the portion of the lake were it receives urban waste discharge, domestic waste discharge, agricultural waste discharge, pollution due tourism, barrage and fishing activities. The details of the stations are discussed in chapter 4.

3.1.3 Site selection for groundwater samples

The important parameters considered during the selection of groundwater sampling sites include parameters such as location, aquifer type, total yield, the population served, its value to industry and agriculture and the magnitude of threats to its water quality. The sampling stations were fixed in the basin of Vembanad Lake covering the whole southern part of the wetland system (Figure 3.1). Thirteen stations were fixed and the details are discussed in chapter 5.

3.1.4 Sample collection

The sampling was carried manually using a water sampler. Samples from various depth were collected using automatic depth sampler. The collected sample were transferred to transparent polyethylene bottles, which was thoroughly cleaned and rinsed three times with the water which was sampled. Care was exercised to clean the devices inside the bottle for any possible sediment or precipitate. Recorded the complete information regarding the source and the conditions under which the sample was collected. Attached a record tag to the sample container, by noting the sample number, source of sample and sampling location.

While collecting the water sample from a hand pump, care was taken to run at least fifteen minutes so that the sediment, precipitates already formed either on the surface of the well or in the pipelines due to drying of materials, were washed away and were prevented from contaminating the sample. In taking the sample from open well the bucket was lowered at least two to three meters below the surface and collected preferably from the centre of the well.
3.2 Parameters measured in the field

A number of parameters including pH, conductivity, ammonia, temperature and residual chlorine were measured in the sampling site immediately after the collection of water sample. For measuring the dissolved oxygen, the fixing was done in the site itself.

3.3 Preservation and handling of samples

Between the time that the sample is collected in the field and until it is actually analyzed in the laboratory, physical changes, chemical changes and biochemical reactions may take place in the sample container which will change the intrinsic quality of the
sample. It is necessary therefore to preserve the sample before shipping and to prevent or minimize their changes. This was done by various procedures such as keeping the sample in the dark, adding chemical preservatives, lowering the temperature to retard the reaction by freezing or by combination of all these methods.

For different parameters, different methods of fixing are required which vary widely in terms of physical conditions, nature and concentration of chemicals. Summary of the sampling protocols is shown in Table below (APHA 2006).

**Table 3.1 Summary of sampling protocol**

<table>
<thead>
<tr>
<th>Determinant</th>
<th>Minimum sample size</th>
<th>Method of preservation</th>
<th>Maximum storage Preferable/Regulatory</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkalinity</td>
<td>200</td>
<td>Refrigerated to ~4°C</td>
<td>24hour/14days</td>
</tr>
<tr>
<td>BOD</td>
<td>1000</td>
<td>Refrigerated to ~4°C</td>
<td>6hour/14days</td>
</tr>
<tr>
<td>Color</td>
<td>500</td>
<td>Refrigerated to ~4°C</td>
<td>24hour/48hour</td>
</tr>
<tr>
<td>Order</td>
<td>100</td>
<td>Analyzed as soon as possible</td>
<td>24hours</td>
</tr>
<tr>
<td>Turbidity</td>
<td>300</td>
<td>Analyzed the same day, stored in dark up to 24h, refrigerated</td>
<td>28days</td>
</tr>
<tr>
<td>Fluoride</td>
<td>300</td>
<td>None</td>
<td>28days</td>
</tr>
<tr>
<td>Oil&amp;Grease</td>
<td>1000</td>
<td>Added H₂SO₄ to bring pH&lt;2 and refrigerated at ~ 4°C</td>
<td>28days/28days</td>
</tr>
<tr>
<td>Hardness</td>
<td>100</td>
<td>Added HNO₃ to pH&lt;2</td>
<td>6months/6months</td>
</tr>
<tr>
<td>Metals in general</td>
<td></td>
<td>For dissolved metals, filtered immediately through 0.45 micron filter, added HNO₃ to pH&lt;2</td>
<td>48hours/28days</td>
</tr>
<tr>
<td>Nitrate</td>
<td>100</td>
<td>Refrigerated to ~4°C</td>
<td>None/28days</td>
</tr>
<tr>
<td>Nitrite</td>
<td>100</td>
<td>Refrigerated to ~4°C</td>
<td>7days/7days until extraction</td>
</tr>
<tr>
<td>Organic compounds/pesticides</td>
<td>200</td>
<td>Refrigerated, added 100mg ascorbic acid/l</td>
<td>40day after extraction</td>
</tr>
<tr>
<td>Oxygen dissolved pH</td>
<td>300</td>
<td>Fixed from the field</td>
<td>24hours</td>
</tr>
<tr>
<td>Phosphate</td>
<td>100</td>
<td>Analyzed immediately</td>
<td>2hours/none</td>
</tr>
<tr>
<td>Sulphate</td>
<td>100</td>
<td>Refrigerated to ~4°C</td>
<td>48hours</td>
</tr>
<tr>
<td>Conductance</td>
<td>200</td>
<td>Analyzed immediately</td>
<td>7days</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24hours</td>
</tr>
</tbody>
</table>

(Source: APHA 2006)
Samples were analyzed as quickly as possible. In case immediate analysis was not possible, the sample was stored at 4°C in dark. Additional sampling precautions were taken depending upon the sample condition at particular location.

3.4 Field quality control

Quality control is an essential element of a field quality assurance program. It requires the submission of blank, ie, bottle blanks, filter blanks, sample blanks, field blanks and duplicate sample to test the purity of chemical preservatives: to check for contamination, sample containers, filter papers, filtering equipments that is used in the sample collection or handling and to detect systematic or random errors occurring from the time of sampling to the time of analysis. Replicate samples were taken to check the reproducibility of the sampling.

3.5 Physico-chemical analysis of water sample

Preservation of samples and estimation of various water quality parameters were done as per standard procedures reported in APHA (APHA 2006).

3.5.1 Temperature

Temperature of the water sample was measured insitu using mercury thermometer having a scale marked for every 0.1°C, checked against a precision thermometer.

3.5.2 pH

pH of the samples was measured using Systronics digital pH meter. The instrument was calibrated using pH 4, 7 and 9.2 buffer solutions.

3.5.3 Color and Turbidity

Color and turbidity were measured using Merck SQ 118 spectrophotometer
3.5.4 Electrical conductivity and Salinity

Electrical conductivity and salinity was measured using ELICO conductivity meter and salinometer. The instrument was standardized against standard conductivity solution of 1413µS/cm. The results are reported in micro- Seimens/cm.

3.5.5 Total dissolved solids

The TDS of the water sample was determined using gravimetric technique. The sample was filtered through a Whatmann No. 30 filter paper, evaporated the filtered sample, dried the residue and weighed. The increased weight gave the total dissolved solids.

3.5.6 Dissolved oxygen and Biochemical Oxygen Demand (DO&BOD)

Dissolved Oxygen was estimated volumetrically. Oxygen present in the sample was made to react with divalent manganese hydroxide, which gets oxidized to its higher state of valence, and precipitates as brown hydrated oxide after addition of NaOH and KI. Upon modification, manganese reverts to divalent state and liberates iodine from KI equivalent to the original DO content. The liberated iodine was titrated against sodium thiosulphate solution using starch as indicator. To determine the Biochemical Oxygen Demand (BOD) incubation of the sample at a constant temperature (at 20°C) for a time period of 5 days was done so that micro organisms can act upon the bio-degradable matter. After 5 days the DO content was determined. The difference between the initial and final DO gave BOD.

3.5.7 Alkalinity

Using the principle of acid-base titrations total alkalinity of the sample was determined. The water sample was titrated against std. H₂SO₄ (0.02N) using methyl orange as the indicator. The results were expressed as mg/l as CaCO₃.
3.5.8 Hardness

Complexometry was the principle used for the determination of total hardness and calcium hardness. Total hardness was estimated using std. EDTA (0.01M) and Eriochrome Black-T indicator in presence of NH₄Cl-NH₄OH buffer of pH 10. For the determination of calcium hardness Murexide indicator in presence of NaOH buffer was used. The difference between the two gave the magnesium hardness.

3.5.9 Calcium and Magnesium

Calcium and Magnesium concentrations in the water samples were estimated from their corresponding hardness using the equations.

Calcium in mg/l = Calcium hardness x 0.4

Magnesium in mg/l = Magnesium hardness x 0.243

Magnesium hardness = Total hardness-Calculator hardness

3.5.10 Sodium and Potassium

Alkali metals like Sodium and Potassium were estimated using Flame Emission Photometry. Followed the instructions of flame photometer manufacture for selecting proper photocell, wavelength, slit width adjustments, fuel gas and air pressure, steps for warm up, correcting for interference and flame background, rinsing of burner, sample ignition and emission intensity measurements. Prepared a blank solution and sodium calibration standards of 25, 50, 75 and 100 mg sodium per liter. The instrument was set at zero containing no sodium (blank solution). Measured emission at 589 nm and prepared the calibration curve. Determined the sodium concentration of the sample, or diluted sample, from the curve. For potassium the calibration graph was plotted using the standards 2, 4, 8 and 10 mg potassium per liter and measured the emission at 766.5 nm.
3.5.11 Chloride

Argentometric titration was adopted for the estimation of chloride in the water sample. The sample after the addition of potassium chromate indicator was titrated against std. AgNO₃ solution (0.0282N).

3.5.12 Sulphate

Sulphate concentrations of the samples were determined using Nephelometric technique. Sulphate ion was precipitated in an acid medium with barium chloride in such a manner as to form barium sulphate crystals of uniform size. Nephelo turbidity meter measured the absorbance of barium sulphate suspension and the sulphate ion concentration was determined by comparison of the reading with a standard curve.

3.5.13 Phosphate-phosphorous

Ammonium molybdate–spectrophotometry was the method used for the estimation of Phosphate-P. Ammonium molybdate was made to react with Phosphate-P in the water sample to form molybdophosphoric acid, which was then reduced to a blue colored complex ‘Molybdenum blue’ by the addition of stannous chloride. The estimation of the blue colored complex was made at 690nm. A standard curve was plotted using known standards of 0.2, 0.4, 0.8 and 1.0 mg per liter of phosphorous. The instrument directly calculates the concentration in the sample and reported as mg/l of phosphate-P.

3.5.14 Nitrate-nitrogen

Cadmium reduction technique-Spectrophotometry was used for the estimation of nitrate-N. By passing the water sample through a column containing amalgamated cadmium fillings nitrate was reduced to nitrite. The nitrite–N thus produced was determined by diazotizing with sulphanilamide and coupling with N (1-Naphthy1) ethyl diamine to form a highly colored azo dye which was measured colorimetrically. The amounts of azo dye formed will proportional to the initial concentration of Nitrate-N over
a wide range of concentration. The estimation of dye was made at 543nm using UV-Visible spectrophotometer (Hitachi, U-2800).

3.5.15 Fluoride

Colorimetric technique using SPADNS reagent was used for the estimation of fluoride. Under acid condition fluoride react with zirconium SPADNS solution and the ‘lake’ (color of SPADNS) gets bleached due to the formation of ZrF$_6$. The concentration was estimated using the UV-Visible spectrophotometer.

3.6 Microbial analysis—Total coliform and Faecal coliform

The major microbial estimation conducted was the coliform estimations. The method used for their estimation was the Multiple Tube Dilution (MTD) technique. In this technique the results were reported in terms of Most Probable Number (MPN) of bacteria in 100ml of the sample. MPN value for a given sample was obtained by the use of MPN table. Mac Conkey broth, Brilliant Green Lactose Broth (BGLB) and peptone water were used as the media for the determination of total coliforms, faecal coliforms and E coli respectively. For total coliform estimation, 10, 1 and 0.1ml of the samples were inoculated in to the Mac Conkey media and incubated at 37.5$^\circ$C for 48 hours. Tubes having both gas and growth were taken as the positive tubes and counted the number of positive tubes. The positive tubes were gently shaken re-suspend the growth and with a sterile loop, transferred three loopful to a fermentation tube containing BGLB. This was then incubated at 44.5$^\circ$C for 24 hours. In a similar manner the number of positive tubes were noted. In both the above cases the count was determined using MPN index. The previous positive tubes were gently shaken to re-suspend the growth and with a sterile loop, transferred three loopful to a fermentation tube containing peptone water. The inoculated tubes were incubated at 44.5$^\circ$C for 24 hours. After the incubation added 2-3 drops of Kovac’s reagent. Formation of a violet ring indicated the presence of Escherichia coli.
3.7 Biological analysis-Chlorophyll

The Chlorophyll pigments, \( a, b \) and \( c \) were extracted from the plankton, concentrated using aqueous acetone 90% (v/v) and the estimation was done spectrophotometrically. The extract was kept overnight at 4°C in the dark. The tube was centrifuged for 20 minutes at 2000rpm. Decanted the extract, measured the volume and read the absorbance at 663, 645 and 630nm. Using the respective equations the concentrations of the three pigments were calculated. The algal biomass was estimated by multiplying the chlorophyll \( a \) content by a factor of 67 (here the assumption is that chlorophyll \( a \) constitutes, on the average, 1.5 % of the dry weight organic matter of the algae) (APHA 1995).

3.8 Sediment sampling and analyses

The core samples of sediments were collected using gravity type sediment corer of 50cm length. The sampling stations were selected based on the criteria that they were near the urban and domestic effluent discharge points, agricultural dewatering areas, places of tourism activities, industrial discharge points and estuarine region. A total of eight sediment cores, including five from southern region and three from northern part, were collected for the present study. The details of the sampling stations are further discussed in chapter 6.

3.8.1 Preservation of sediment sample

The collected sediment cores were cut into slices of approximately 2-4cm length from the field itself and properly labeled. The samples were brought to the laboratory using ice bags and stored in a deep freezer unit until the drying procedure (UNEP 1985).

3.8.2 Methodology of Sediment Analyses

The various physicochemical parameters like pH, electrical conductivity, total alkalinity, chloride and sulphate were estimated following soil analysis procedures (Jackson 1973) and APHA (APHA 2006).
3.8.2.1 Mechanical analysis

Using the hydrometer method the weight percentages of sand, silt and clay in the sediments were calculated and classified the sediments into various textural classes using the ternary model. For this a known weight (40g) of the sediment was taken, washed with acid to remove the carbonates, then washed with water to remove the soluble impurities and treated with Hydrogen peroxide to oxidize the organic matter completely. The sediment was then dispersed in a dispersing medium (3.6% Sodium hexametaphosphate +0.8% Sodium carbonate), transferred in to the sedimentation cylinder, made up to a volume of 1000ml with distilled water and shaken well. After 4 minutes and 120 minutes the hydrometer was dipped into the cylinder and noted the scale reading. From these readings and that of the blank percentages of sand, silt and clay were calculated. Classification into various textural classes were done using the ternary model.

3.8.2.2 pH

The pH of the sediment was determined using 1:10 soil water (w/v) suspension. 10g of air-dried sediment was mixed with 100ml of distilled water in a beaker. The solution was stirred at least 5 times over a 30 minutes period and allowed the sediment and water to reach equilibrium. The sediment suspension was shaken and inserted the electrodes into it. The pH meter was standardized with known buffer solutions.

3.8.2.3 Electrical conductivity

Electrical Conductivity of the sediment was determined using 1:10 sediment water (w/v) suspension. The sample was put overnight and inserted the conductivity meter into it.

3.8.2.4 Alkalinity, Sulphate and Chloride

The sediment water suspension was used for the estimation of the total alkalinity, sulphate and chloride content in the sediments. The sample was filtered
through 0.45 micron filter paper and the filtrate was analyzed using the procedures explained for water samples.

3.8.2.5 Organic carbon

A portion of the sediment was oven dried, ground and sieved. 0.25g of the material was taken for the estimation of organic carbon using the Walkley Black’s method. In this method the nascent oxygen formed by the reaction between potassium dichromate and concentrated sulfuric acid oxidizes the organic carbon in the sediment. The organic carbon content in the sediment was then estimated by titrating the rest of potassium dichromate with ferrous ammonium sulphate. The organic matter was computed by multiplying with a factor of 1.723.

3.8.2.6 Exchangeable cations

Ammonium acetate was used to extract the exchangeable sodium, potassium, calcium, and magnesium. The extract was filtered through 0.45 micron filter paper and the filtrate was used for the estimation. Estimation of exchangeable sodium and potassium was done by flame photometry using Flame Photometer. Exchangeable calcium and magnesium was estimated by complexometric titration using EDTA.

3.8.2.7 Phosphorous

Inorganic Phosphate-P was extracted using 1N HCl and determined colorimetrically using Spectrophotometer. The sediment samples were digested using HClO₄-HNO₃ acid mixture was used for total phosphorous measurement. For the determination of hydrolysable form, the sample was extracted using 0.01N sulfuric acid. Organic form was determined as the difference between total and sum of inorganic and hydrolysable form.

3.8.2.8 Kjeldahal Nitrogen

The total nitrogen content of the sediment was determined using digestion with sulfuric acid followed by distillation and titration with hydrochloric acid. The distillate
was collected in 4% boric acid. Titration value of a blank solution of boric acid and mixed indicator has determined. From the titral value the percentage of nitrogen was calculated.

3.8.2.9 Heavy metals

The concentration of different heavy metals in the sediment sample was estimated using digestion technique followed by analysis using AAS. One gram of dry sediment was taken in a 250 ml beaker added 6 ml con. HNO₃ and 2 ml of perchloric acid (HClO₄), the beaker was closed and kept at room temperature for about 2 hours. The above mixture was heated in an oven at 100°C for one hour and evaporated the sample over a hotplate. The residue was diluted with 20 ml of 6% 1:1 mixture of HNO₃ and HClO₄ and heated the mixture on a hot plate for about 10 mints. The sample was filtered the sample through Whatmann No.41 filter paper, into a 50 ml. standard flask and adjust the volume with the same acid mixture. A blank was prepared by the same procedure. The amount of heavy metals were determined from the filtrate with the help of atomic absorption spectrophotometer (AAS, Thermo Scientific M-Series).

3.9 Methodology for Isotopic analysis

3.9.1 Collection of water samples

The precipitation samples were collected from three sampling stations which are marked in the area map. Samples were collected daily as and when there was rain. A composite sample was prepared after the end of each month by pooling the samples collected at daily/weekly /whenever rain during the month. Care was taken to prevent air entry into the collecting vessel to avoid exchange tritium from air into the sample. Water samples were collected during 2007 to 2008, daily basis, during the summer, southwest monsoon and northeast monsoon periods. Surface water samples from 20 representative stations of Vembanad Lake were collected for ²H and ¹⁸O measurement in three seasons, pre-monsoon, monsoon and post-monsoon. Samples from the depth were also collected in two seasons. The sampling stations were marked in the area map. Thirteen groundwater samples including samples from shallow open well, deep bore well and
a small pond were also collected from the basins of the Vembanad Lake, which is marked in Figure 5.3. Large volumes, around 20 litters of surface water samples were collected in the pre-monsoon season for the determination of $\delta^{34}$S. A total of 56 samples including surface and bottom water, from different stations of Cochin estuary were collected during different time intervals in pre-rinsed Tarson bottles. A core sediment sample was collected from the middle of the lake (C/V) to find out the sedimentation rate of the lake by finding the cesium-137 activity.

### 3.9.2 Preservation of the sample

The samples were collected in clean polythene bottles of 60 ml capacity. The bottles were filled with water samples to the maximum, leaving only a little space to account for the expansion during the transport. While filling the bottles, only a little space was left in order to prevent air, which may introduce error due to the exchange of the $^{18}$O present in the air with water. The analysis was made using the instrument Isotope Ratio Mass Spectrophotometer.

### 3.9.3 Analysis of the water samples for the stable isotope, $^{18}$O

200μl water sample was taken in a special glass vial fitted with a screw cap provided with septum, which allows the injection needle to pass through. The sample was then equilibrated with a slow stream of carbon dioxide–Helium mixture, which was passed at a rate of 50ml/min so that the $^{18}$O of water is exchanged with the $^{16}$O of carbon dioxide through isotope exchange. This is achieved by keeping the water sample agitated for a period of 18 hours.

$$H^{16}O^{18}O+C^{16}O_2=H^{16}O^{16}O+C^{16}O^{18}O$$

The equilibrated oxygen gas was then introduced in to the mass spectrometer for analyses. Helium was passed at the rate of 5ml/min, to flush out the gasses. Since the international standard was also subjected to the same equilibration, the $\delta$ value was not expected to vary. The results were analyzed using the software, Isodat NT, provided along with the system by the manufacturer.
3.9.4 Analysis of water samples for deuterium

200μl of water sample was taken in a special glass vial fitted with a screw cap provided with septum, which allows the injection needle to pass through. A platinum rode was added as a catalyst. The sample was then equilibrated with a low stream of hydrogen and helium mixture, passed at a rate of 50ml/min so that the hydrogen is exchanged, with deuterium of water samples through isotope exchange.

\[ ^2\text{H}^1\text{HO}^+^1\text{H} \rightarrow ^1\text{HO}^+^2\text{H}^1\text{H} \]

The equilibrated hydrogen gas was then introduced to the mass spectrometer for analysis.

3.9.5 Analysis of water for $^{34}\text{S}$

For the analysis of $\delta^{34}\text{S}$ of sulfates, it was precipitated as BaSO4 by adding a saturated BaCl2 solution to the filtered water sample. SO2 gas was then prepared from this and the composition of $\delta^{34}\text{S}$ was analyzed using mass spectrometer.

Stable isotopic compositions are expressed in usual d notation relative to the V-SMOW for oxygen and hydrogen isotopes, and CDT for sulfur isotopes. $\text{D (‰)} = (\text{Rsample}/\text{Rstandard}-1)*1000$, where R represents $^{18}\text{O}/^{16}\text{O}$, D/H, $^{34}\text{S}/^{32}\text{S}$. The analytical reproducibility for each standard were ±0.1‰ for $\text{d}^{18}\text{O}$, ±1‰ for dD and ±0.2 for $\text{d}^{34}\text{S}$

3.9.6 Sedimentation rate from $^{137}\text{Cs}$ activity

The sediment core was sliced at every 2 cm intervals and analyzed for $^{137}\text{Cs}$ activities. The $^{137}\text{Cs}$ activity in each oven-dried section of sample was determined by gamma-ray counting using Hyper Pure Germanium detector coupled with a 4096 multi-channel analyzer system. The detection limit for $^{137}\text{Cs}$ measurement was 0.25mBq/g and the standard counting error was less than 10% in the core sections. The analysis was carried out with the help of the facility available at Bhabha Atomic Research Centre Mumbai.