Chapter 3

Methodology

3.1 Site description

3.1. 1 General physiography

The study site is located in the highland of eastern watershed area of sub-district Mae Soi, Chomthong District at about 75 km SW of Chiang Mai Province, northern Thailand (**Fig-1**). The study is confined to two streams arising in the same watershed area of other major streams of Mae Tim and flow in river Ping at Ban Sob Soi along with other streams. The two streams Huay Mak Nun A and B were chosen based on similar ecological conditions with different land use patterns in their surroundings. Preliminary field surveys revealed the two streams to be more similar in terms of width, depth, velocity but have different substrate composition. The sampling sites were chosen based on similar elevation. Sites 1 and 2 are located at 1100, 1200 respectively in stream A. Sites 3, 4, 5 are located at the elevation of 1100, 1150, and 1200 m respectively stream B. Water in stream A appears a muddy brown color where as water in stream B is a gave muddy black color during the rainy season.

3.1.2 Land use pattern

The general land use pattern surrounding the two stream is different. Stream B is surrounded by some remaining forest patch at its upper regime and flows near a village (Ban Huay Mak Nun) where it virtually joins stream A and flows together.

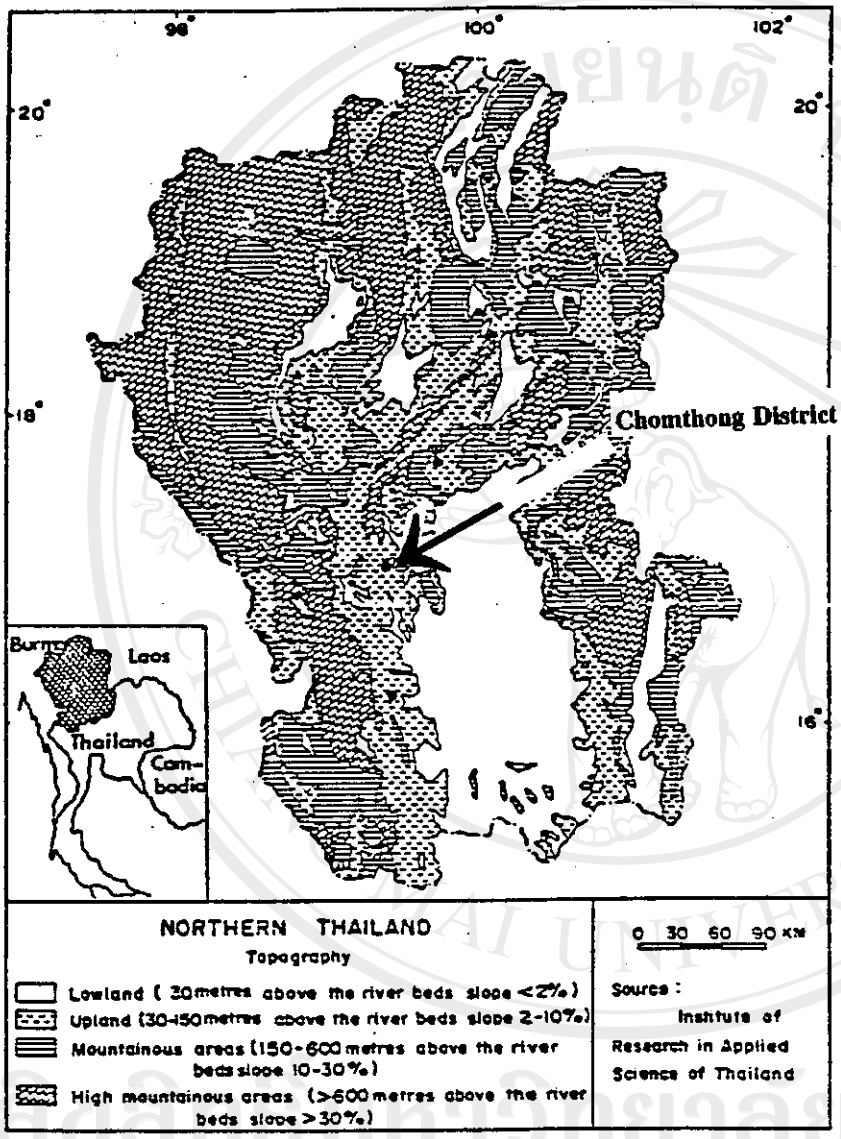

Fig 1 Location of Chomthong District in Northern Thainad

The sites 4 and 5 are comparatively more pristine than site 3 in stream B. Site 3 is located near the village where anthropogenic impacts from domestic cattle and other human activities like washing and bathing seems to have influenced the site. The forest surrounding stream B still serves for fuel, fodder and constructing material for the villagers nearby. Stream A flow through one of the big agricultural fields with intensive land use pattern mainly with opium substitute cash crop cabbage (*Brassica oleracea* L. , Cruciferae). With expanding cabbage growing fields, following a traditional slash and burn method, degrading environmental condition can be noticed from the soil profiles of agricultural fields which give an appearance of hilly slope of 20 to 30 % , unsuited for any kind of cultivation surrounding stream A. The soil is sandy loam with very high concentration of phosphorous and potassium, with medium organic contents, very acidic. The pH ranged from 4.6 to 5.7 (**Appendix -1).**

From the surveys carried out by Chomthong District Hospital/Agricultural Extension Office during May 1991, it was reported that numerous of pesticides were in agricultural practice in the highlands of Chomthong District. The survey were mainly confined to Ban Huay Khanun, Ban Huay Som Poi, Ban Mae Luang, Ban Nam Lat and Ban Lum Tai. The report revealed that the most common pesticides used was Apron, Ambush, Ecomax , and Tamaron. The other pesticides used were reported to be Dithane, Apron, Antracol, Lannate, Folidon, Diethr-Phos, Sumi- Sidrin, Phosdrin, Hecodil, etc. The frequency of pesticide spray were reported to be every 7- 15 days during the crop growing season.The use of insecticides was reported to be all around the year, mostly during December to March, during the crop growing season. Residue analysis carried out in the initiation of Chomthong District Hospital/Agricultural Extension Office on different substrates like water, soil, vegetables and fruits growing around the area, no organochlorine and organophosphorous were reported to have

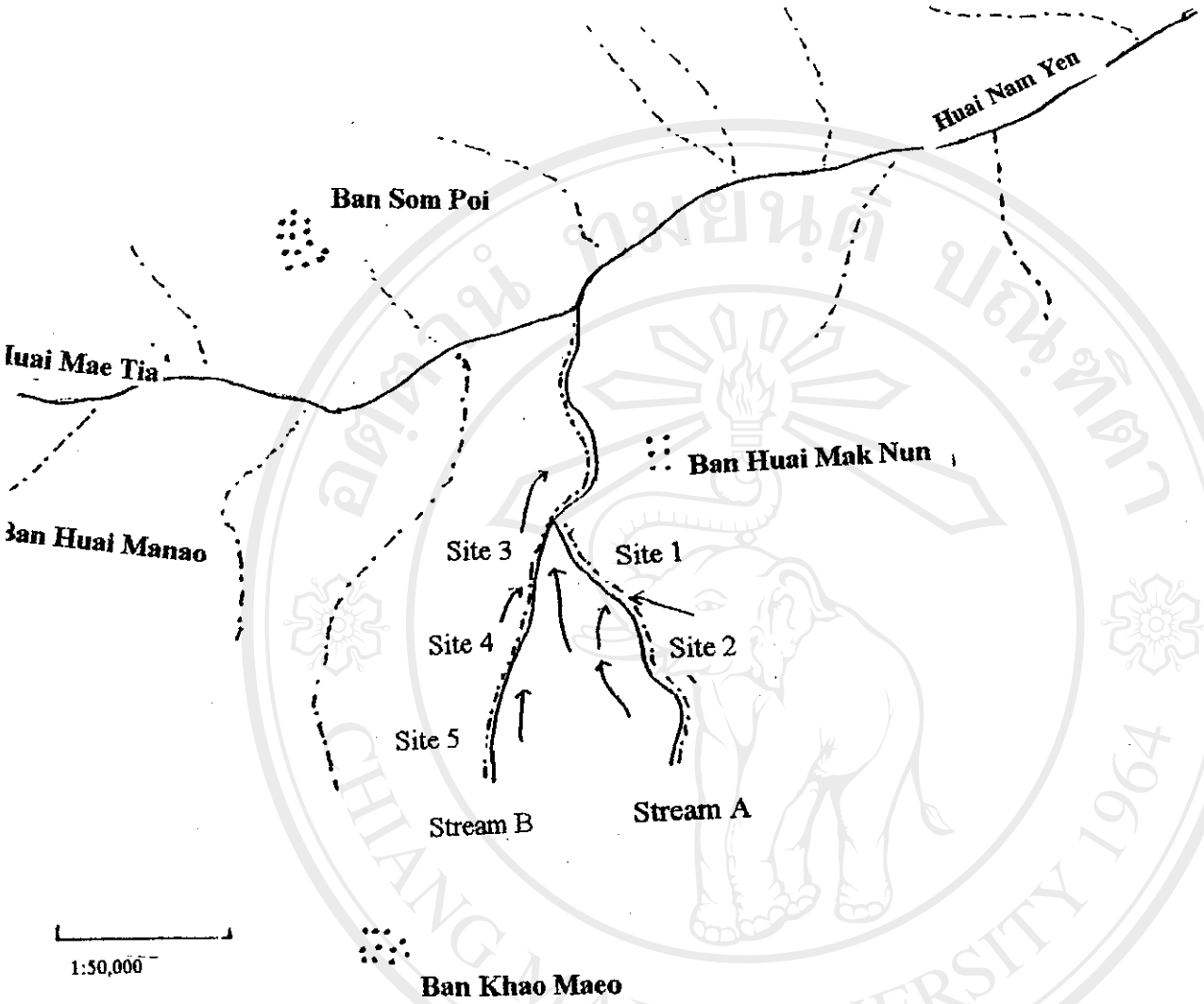

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Fig 2 Location of study sites in the Mae Soi watershed area of Chomthong District

been detected. 0.04 to 0.11 mg/kg of methiocarb was reported from cabbage samples collected from dealers, hilltribes and markets. 75.58 % among the people surveyed were reported to adopt safety measures by reading the instructions carefully before they use the pesticides. Muscular ache, skin allergy and vomiting symptoms were reported among the people surveyed.

3. 1.3. Topography and vegetation

The topography of the area includes bed rock made up of granitic with limestone along southern and north -eastern parts of the area .

The vegetation of the area includes pine trees (*Pinus kesiya)* and evergreen hardwood trees dominated by Oaks (Fagacea), e.g. *Castanopsis acuminatissima*, C. A*rmata*(Roxb.) Spach, *Lithocarpus elegans* (B1.) Hatus.ex Soep., *Quercus lanata* Sm, Q. *Semiserrata* Roxb.Other common species are suchas: *Schima wallichi* (DC.) Korth, and *Ternstroemia gyenthera* (W.& A.) Bedd.(both Theaceae), *Spondias axillaris* Roxb.(Anacardiaceae*)* , *Eugenia albiflora* Duth.ex Kurz (Myrtaceae), *Sarcosperma arboreum* Bth.(Sapotaceae), *Helicia nilagirica* Bedd.(Proteaceae*)*, *Cinnamonum camphora*(L.) Nees & Eberm.(Lauraceae), *Sapium baccatum Roxb.* (Euphorbiaceae),, *Engelhardia spicata* Lechen. Ex.B1.Var. spicata (Juglandaceae) etc (Maxwell,1994).

3.1.4. Climate

The rainfall in the area follows the south west monsoon from May to October The dry season is from November to April. The average annual rainfall is 700+ mm. The mean monthly rainfall is shown in the Table -1.

Table 1: Maximum monthly rainfall at Chomthong District, 1992- 1993.

Month	1992 (mm)	1993 (mm)
January	0	1.5
February	13.8	0
March	0	.5
April	12.1	41.4
May	5.7	78.2
June	23.5	7.1
July	3.9	48.2
August	50.2	28.3
September	48.6	51.3
October	47.5	65.2
November	31.3	0
December	60.5	0

Source: Data processing sub-division , climatology division, meteorological department, Chiang Mai, 1995.

3.2. Physico-chemical analysis of water quality parameters

Water quality analysis was done following the standard procedure for analysis of surface water quality (APHA ,AWWA , 1989). Nine parameters viz.- temperature, velocity, dissolved oxygen (D.O), alkalinity, pH, nitrate-nitrogen, ammonia- nitrogen, soluble reactive phosphorous, and conductivity were measured in the field and laboratory.

3.2.1 Field parameters

Some physical parameters like air and water temperature, velocity and chemical parameters like D.O, conductivity , pH were measured in the field during each sampling time.

a) **Physical parameters**

I) **Temperature**

The water temperature was recorded at a depth of about 10 cm. with mercury thermometer. The temperature was recorded in degree centigrade.

II) **Velocity**

The water velocity was measured using a velocity meter (current meter, model 2100-1514, Swoffer Instrument, Inc. USA), following the stated instruction in the instrument manual.

b) **Chemical parameter**

III) **Conductivity, pH**

The conductivity and pH of the stream water was measured with a conductivity meter (LF-196) and pH meter (pH-196 T). The calibration was done before the sampling (model-WTW. GmbH. Weniheim. Germany).

1v) **Dissolved oxygen (D.O) and percent saturation (% Sat)**

The dissolved oxygen and oxygen saturation was measured using a portable D.O. meter (OXI- 86). The instrument was calibrated each time before taking the sample (Model- WTW. GmH. Wenheim, Germany).

3.2.2 Laboratory parameters

b) **Chemical Parameters**

I) Alkalinity

Alkalinity was measured by titration, using pheneopthalin as the indicator Total alkalinity was calculated using the formula:

$$\text{Total alkalinity (mg /CaCO}_3\text{)} = \frac{\text{x mL 0.02 N H}_2\text{So}_4\text{ x 50,000}}{\text{ml sample}}$$

where, x = tritrate value (pH = 4.5)

N = Normality of the acid

ml sample: volume of water sample taken

ii) Nitrate (NO_3-N)

The Nitrates were measured with spectrophotometer following cadmium reduction method using powder pillow (NitraVer-5).The measurement was done at 500 nm wavelength.

iii) Ammonia (NH_3-N)

The ammonia was analyzed using a spectrophotometer following the Nessler method at a wavelength of 425 nm. The values were recorded as direct values from spectrophotometer.

iv) Soluble phosphorous (PO_4-P)

Phosphate powder pillow-PhosVer 3 was used following the ascorbic acid method for the analysis of phosphorous in the water sample, using spectrophotometer, at the wavelength of 890 nm. The values were recorded as a direct reading from the instrument.

3.3 Analysis of organochlorine pesticides

3.3.1 Principle

The analysis of organochlorine pesticides were done with a view of detecting them in the sediments as they belong to persistence group. Moreover, the detection of this group of pesticide was expected to give idea about the present trend of organochlorine pesticide use in that area.

The analysis of organochlorine pesticides in sediment (approximately 20 gms) were extracted by sonication and cleaned up using solid phase extraction (C_{18}) cartridges (500 mg). The pesticides were eluted with iso-octane 0.1 µl of the elute was injected into GC with capillary column and ECD for qualitative and quantitative analysis (Dao, et al., 1989).

3.3.1 Sampling

The samples were collected from 0-10 cm of sediment. Approximately equal volumes of sediment were randomly dug out confining within an area of

100 m^2 in each sampling site. Each of the samples were roughly mixed. About 500 gm of the sediment were wrapped in aluminum foil and transferred to the laboratory for preservation and analysis. The sampling was done mainly for two seasons in June and November only. The collected samples were preserved and stored at -20 ^0C in the freezer until the laboratory analysis was done during November and December 1994 at the Research Health Institute, Chiang Mai University.

3.3.2 Sample preparation

This part includes homogenization, extraction, clean up and identification. The procedure for the sample preparation is shown in fig. 3 and 4.

3.3.2.1. Homogenization

The sediment samples were mixed thoroughly prior to the analysis. Four aliquots were prepared for each sample. The first aliquot was oven dried at 105 ^0C about 24 hours until the weight of the sample was constant. Second, third and fourth aliquots were taken each for blank sample, sample with internal standard, and with mixed standard .

3.3.2.2 Extraction

Extraction of the samples was done by using 4 mL of ethyl acetate (EA.) and 30 mL methanol (MeOH). The sonication was done for 60 minutes. The extract was

filtered and the filtrate was rinsed twice with 5 ml of MeOH. The filtrate was put in a centrifuge machine for about 20 minutes. Then the combined supernatant (~ 44 ml) was diluted with 44 ml of deionised water to get a 1: 1 mixture eluate (organic solvent : water).

3.3.2.3. **Clean up**

The sample clean up was done by using a C_{18} column. The column was conditioned by using 2 ml of each of n-Hexane, EA, MeOH, and deionised water (d H_2O) using vacuum manifold. Sample loading was done at the flow rate of 6-8 ml / min. and interference components were washed out with 2 x 1 ml 25 % ACN / d H_2O. The analyte was eluted with 2 x 0.5 ml iso-octane and 1 µl of eluate was applied to capillary GC-ECD for analysis.

3.3.2.4 **Identification**

The sample chromatograms were compared with the standard chromatograms. Relative retention time was used for qualitative identification of different pesticides. 50 µl of 0.2 µg/ml Aldrin was used as a reference and internal standard .

3.3.3. Gas chromatographic determination of pesticides

The Sample extracts were injected into GC column under following operating conditions:

Column: HP-1 (25 m x 0.32 m.m.x 1.05 µm film thickness)

Carrier : Helium(He), 57.2 cm / sec , flow rate 3.6 ml /min, calculated at 85 ^{0}C, 15 psi.

Oven : 85 ^{0}C

Injector: 200 ^{0}C

Detector : ECD 300 ^{0}C

3.3.4 Apparatus

1. Diverse glasswares - beakers, conical flasks, pipettes, volumetric flask, test tubes etc.

2. Analytical balance - OHAU SCALE CORP, USA.

3. Oven -Model- Heraus, Germany

4. Sonicator -Model- 8845-4, Ultrasonic cleaner, Chicago.

5. Centrifuge machine -Model- K, AMPS3.5, 3/4 HP. USA.

6. Gas Chromatograph (HP 5890 Series II) with HP 7673 Auto- sampler / injector and datastation (HP Chemstation System).

3.3.5 Chemicals

All the solvents used were pesticide residue grade.

EA, MeOH, n- Hexane, 25% ACN / H_2O, Iso-Octane

Surrogate standard: 0.2 μg / ml Aldrin

Pesticide Standard : β-HCH, HCB, γ -HCH, Heptachlor, Hept. Epoxide, o, p'- DDE, p, p'-DDE, Dieldrin, p, p'-DDD, o, p' -DDT, p, p'- DDT.

SEDIMENT

(4 ALIQUOTS)

Oven dry weight

(105 ^{0} C-24 hr)

	Blank Sample	*Internal standard*	*Spiked sample* (For Recovery)
1. Ethyl Acetate	50 µl	----------	----------
2. Surrogate (0.2 µg /ml) Aldrin	---------	50 µl	50 µl
3. Pesticide standard	---------	---------	50 µl
4. Ethyl Acetate	4 ml	4ml	4ml
5. Methanol	30	30	30
6. Sonication	60'	60 '	60 '
7. Centrifuge (3000 rpm)	20'	20'	20'

Fig. 3 Flow chart diagram for extraction of organochlorine pesticides in sediments

Sample Clean Up by SPE

SPE Column (C_{18}) - Conditioning

↓ 2 x1 ml n - hexane

↓ 2 x1 ml Ethyl Acetate

↓ 2x1 ml d H_2O

Sample addition

↓ Sediment Extract [88 ml organic solvent + deionised water (1:1)]

↓ flow rate = 6- 8 ml /min and washing with 2x1 ml 25 % ACN /d H_2O

Column Drying

↓ 5 minute by vacuum

SPE Elution

↓ 2x 0.5 ml 1so-octane

Capillary GC analysis (1 µl)

Fig 4 Flow Chart Diagram for analysis of organochlorine pesticide in sediments

3.4 Benthic community analysis

3.4.1 Sampling procedure

A surber sampler with 0.4 mm mesh and sample size of 22 x 22 cm^2 was used as a device in collecting the benthic community samples. The samples were collected by securing the sampler firmly on the stream bottom parallel to the water flow with the net portion downstream . Random samplings were done separately for sand, stone, and gravel substrates by agitating the substrate to a depth of 5 to 10 cm , turning over the pebbles and rubbing the big stone surfaces with a light brush . Samplings were done for three seasons-summer, rainy and early cool season i.e. from March to November, 1994.

3.4.2 Sample preservation

The macrozoobenthos collected were transferred immediately to plastic bags with sample identity number and were fixed with 10 % formalin in the field. The sorted species after identification were preserved in 70 % ethanol in small vials in the laboratory (Welch, 1992).

3.4.3. Sorting and identification

The benthic samples were washed with tap water in order to wash out the formalin. The sample information were transferred from label to data sheet. The washed samples were emptied in a shallow, white, enamel tray with water for sorting.

The samples were sorted out in smaller sub- samples in water, using a stereo microscope and a compound microscope. The benthos were analyzed up to family level. Following references were used for identification of the benthos :

1. Mccarthy (1981)
2. Needahm and Needham (1961)
3. Chu , H.F. (1969)
4. Mellanby (1963)
5. Merit, R.W. (1984)
6. Arthus Neboiss (1992)

3.5 Data analysis

Diversity indices, multivariate statistical analyses like cluster, ordination, principle component, multiple analyses of variance (MANOVA) and discriminate analysis have been recommended as appropriate tool for evaluation of benthic invertebrates with relation to anthropogenic impacts (APHA, AWWA, WPCF, 1989).

Cluster and factorial analysis have been applied to both chemical and biological parameters. Diversity, evenness, and community structure were analyzed for the benthic fauna. MANOVA was implied to find significant relationships among the variables. Correlation coefficient was worked out to see the relationship between physico-chemical, biological and substrate data. Multiple regression analysis was implied as an explanatory tool to investigate the maximum linear relationship with criterion variable. ECOSTAT computer programme was used to calculate diversity and evenness index. Computer programme SPSS win-ver 6 was used for cluster, MANOVA, correlation and regression analysis.

3.5.1 Cluster analysis

The raw data obtained from my surveys were put in the form of a matrix of site and families with number of individuals at each sampling time. Classification involved grouping of similar sampling units containing all the information needed to cluster together. This method fuses the similar sampling units into larger groups which is called a dendrogram. The standardized Z score was used, followed by hierarchical, agglomerative method employing cases with average linking. Person's correlation coefficient was used as a measure for grouping the informationally homogenous groups (Gauch, 1989).

3.5.2 Diversity Index

Diversity index is based on the information theory. The main objective of the information theory is to try to measure amount of information content of a sample (units/ individuals). It incorporates both species richness and evenness into a single value. Shannon -Wiener Index was used to calculate the species diversity at family level. Diversity indices are supposedly high at unpolluted (control) sites and low at polluted (experimental) site.

$$H' = -\sum_{I=1}^{s} Pi \log Pi$$

where, H' = information content of a sample
= index of species diversity

Pi = ni/N

S = total number of species

ni = the number of species belonging to the ith species

N = total number of individual = no. of ith species

3.5.3 Evenness

Evenness measure gives an idea about the distribution of species in a sample. An evenness index should be maximum and decrease towards zero as the relative abundance of the species diverge away from the evenness (Luidwig, 1988).
Hill's ratio has been implied for evenness measure. In this index, value of E approaches to zero, as a single species become more and more abundant (Altalo, 1981, cited in Ludwig, 1988).

$$H' = \frac{N_2 - 1}{N_1 - 1}$$

where, H' = Hill's eveness index

N_2 = number of very abundant species

N_1 = number of rare species

3.5.4 Community structure analysis

The use of community structure gives an idea about the present community existing in the ecosystem at that period of time. The number of families and their distribution pattern in the ecosystem can be taken as a measure to get an indication about health of the ecosystem.

3.5.5 Lognormal distribution of benthic organisms

The logarithmic series implies that the greater number of species has minimal abundance. So that the number of species represented by single specimen is always maximal (May, 1975 cited in Krebs, 1989). Preston (1948) cited in Krebs 1989,

suggested about expressing the x axis in geometric scale rather than arithmetic scale. One of the methods suggested for assessing whether or not individual sites are disturbed is to plot the number of individual among species in geometric class of abundance (Gray, 1992). An undisturbed site will have a steep curve, cover more geometric class and have abundance of rare species represented by fewer species.

3.5.6 Principle component analyses (PCA)

PCA is a method of factor analysis. The aim of Pc analysis is the construction out of a given set of variables (Xjs (j = 1,2,3,....., n) , of new variables (pi), called principal components which are linear combinations of the Xs.

$$Pi = a_{11}X_i + a_{12}X_2 + \ldots\ldots\ldots\ldots + a_{15}X_n$$

$$p2 = a_{21}X1 + a_{22}X_2 + \ldots\ldots\ldots\ldots + a_{25}X_n$$

$$pk = a_{k1}X_1 + a_{k2}X_2 + \ldots\ldots\ldots\ldots\ldots a_{kk}X_k$$

The method is being applied mostly by using the standardized variables,

i.e. $Zj = (Xj - Xj)2/ j$

Factor loading are those values which explain how closely the variables are related to each of the factors discovered and satisfy two conditions I) Principle components are uncorrelated ii) The first PC (p_1) has the maximum variance than principle component (p_2) . Communality shows how much of the each variable is accounted by the underlying factor taken together. High values of communality means not so much of the variables is left over after whatever the factors represent is taken into consideration. Eigen values indicates the relative importance of each factor in

accounting for the set of variables being analyze (Kothari, 1992). Factor analysis was applied to Yodo river system to examine interrelationship among 24 co-variables and Chao Phrya River in Thailand (Lohani, 1985).

3.5.7 Ordination of the families

Ordination represents entities that are subjects of study (study sites or samples) in a multidimensional space that is defined by taking each attributes (usually species or environmental variables) as an axis. The distance between any two samples plotted in this space corresponds to the value of the distance measure used to represent their dissimilarity. New axes or factors, then are independent and these new axes are ordered so that the first factor accounts for most variation among all other factors extracted (Rosenberg, 1993). Fator 1 and Factor II from factorial analyses was used for ordination of benthic community .

Ordination summarizes community data of many species and many samples by collapsing the data into a single graph which summarizes the pattern in them. Ordination is useful for recognizing the pattern present in the community data (Krebs, 1989).

3.5.8 MANOVA

This method of analysis is helpful whenever more than two populations need to be investigated and to find whether the populations mean vectors are the same or not. If the population mean are not same or differ significantly, it helps in find out mean of the components separately. It follows that each of the components of observations are univariate model (one way ANOVA).

3.5.9 Correlation Coefficient

Correlation coefficient was taken for measurement of similarity to determine relationship between physico-chemical parameters and biological parameters. Pearson's -product moment correlation coefficient r was taken as similarity measures.

3.5.10 Multiple regression

Multiple regression form a linear composite of explanatory variables in such a way that it has maximum correlation with a criterion variables. The main objective is to predict the variability of dependent variable based on its covariance with all independent variables.

In this case the explanatory variables are arranged in such a way that it has maximum correlation with the criterion variable. One can predict the level of dependent phenomenon through multiple regression analysis model, given the level of independent variable. Given the dependent variable, the linear multiple regression problem is to estimate constants B1, B2....Bn and A such that the expression Y = B1X + B2 X2 + B3X3....BnXn + A provides a good estimate of an individual Y score (Kothari,1992).