Chapter 3
Materials and Methods

3.1 Site description

The Mae Sa Watershed is situated in Mae Rim District, Chiang Mai Province. Part of the watershed lies within Doi Suthep–Pui National Park. Doi Suthep–Pui National Park was established in 1981and has an area of 261 km^2. Doi Suthep (elevation 1,601 m a.s.l.) and Doi Pui (1,685 m a.s.l.) are part of a geologically ancient ridge forming the western boundary of the Ping River Valley. The forests on Doi Suthep–Pui can be divided into deciduous and evergreen forest types. Some 2,000 mm of rain fall on the park each year, mostly from May to October. The dry season comes between November and March. The average annual temperature recorded near Phuphing Palace is 20°C, with maximum and minimum average temperatures of 24° C and 17° C respectively.

Five sites were studied monthly for a period of 18 months, from April 1998 to September 1999. The sites were selected along the Mae Sa stream (Fig. 1). The name and details of each site are given in Table 5.

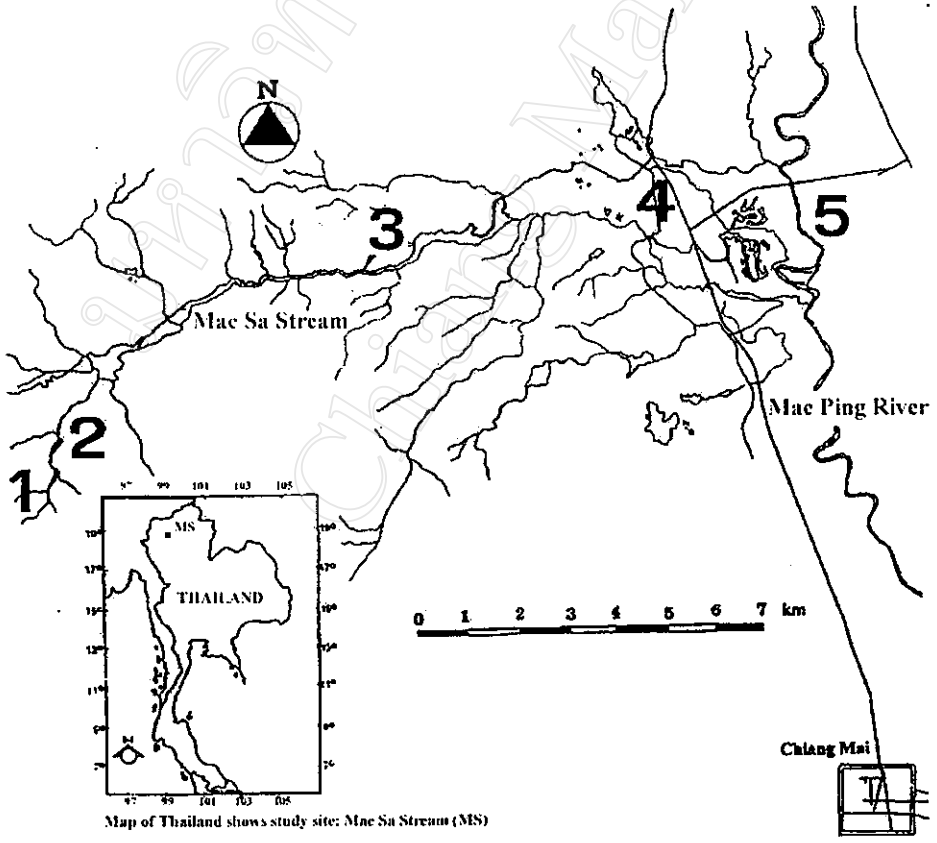

Figure 1. Map of the Mae Sa Stream showing the sampling sites (1-5).

Table 5. Site names, altitudes (m a.s.l.) and descriptions.

Site names	Altitudes (m)	Description	Co-Ordinates
1. Kong Hae Village, Pong Yang Subdistrict, Mae Rim District	1,075	agricultural and residential area	18°51′18″N 98°48′38″E
2. Entrance to Kong Hae Village, Pong Yang Subdistrict, Mae Rim District	1,000	agricultural and residential area	18°51′44″N 98°48′42″E
3. Mae Sa Elephant Camp, Pong Yang Subdistrict, Mae Rim District	550	tourist attraction	18°53′53″N 98°52′48″E
4. Cholaprathan Bridge, Mae Sa Subdistrict, Mae Rim District	330	residential area	18°54′16″N 98°56′33″E
5. Mae Sa Luang Village, Mae Sa Subdistrict, Mae Rim District	340	agricultural and residential area	18°53′31″N 98°58′22″E

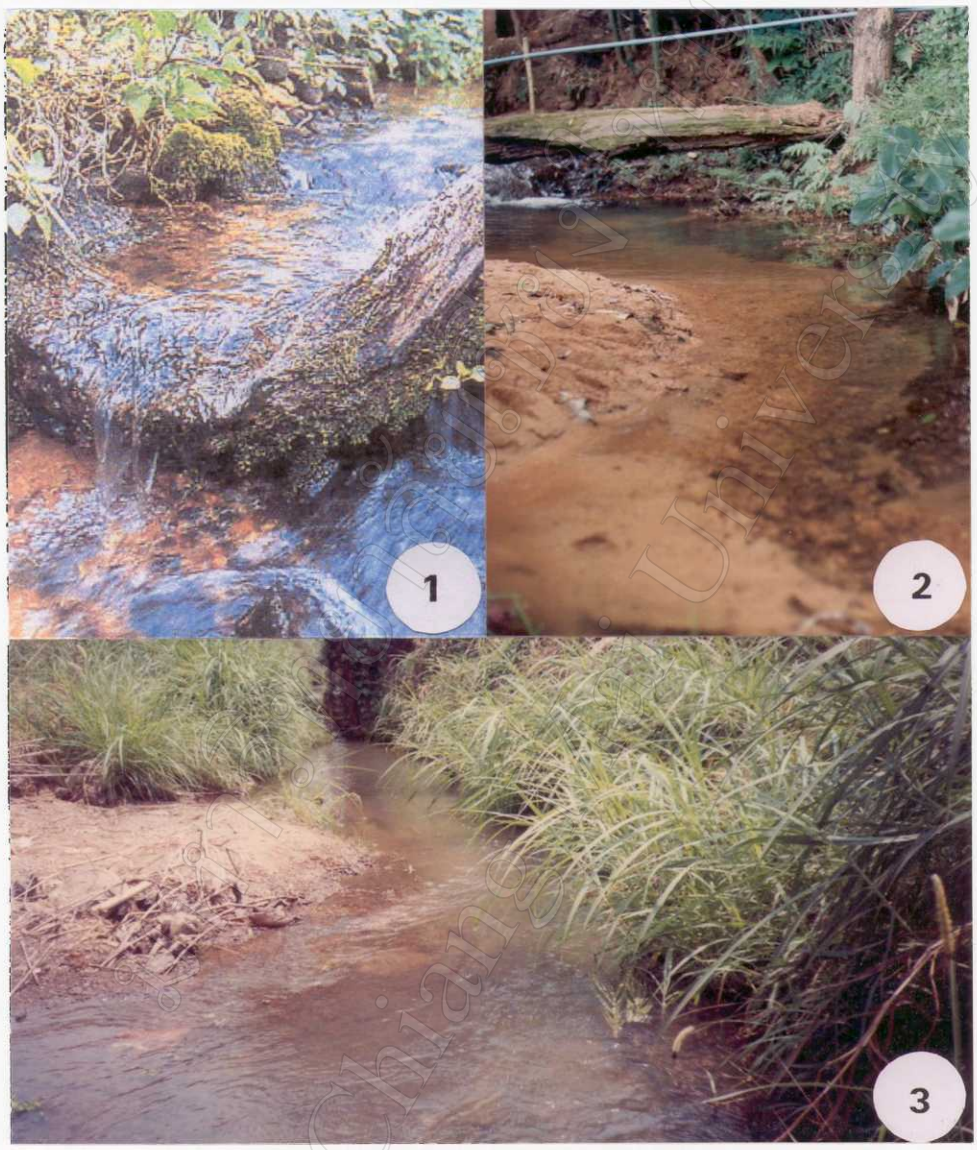

Figure 2. Sampling sites at Mae Sa Stream, Doi Suthep-Pui National Park, Chiang Mai, Thailand.
1-site 1 (Kong Hae Village)
2-site 2 (Entrance to Kong Hae Village)
3-site 3 (Pang Chang Elephant Camp).

Figure 3. Sampling sites at Mae Sa Stream, Doi Suthep-Pui National Park, Chiang Mai, Thailand.
4-site 4 (Cholapratarn Bridge)
5-site 5 (Mae Sa Loung Village)

3.2 Water sampling procedure

3.2.1 Collect the water sample using a polyethylene bottle for the following purposes:

3.2.1.1 Alkalinity analysis by using Phenolpthalein methyl orange indicator method (APHA, AWWA and WEF, 1998)

3.2.1.2 Turbidity analysis by using turbidimeter

3.2.1.3 Silica analysis by using Molybdosilicate Method (APHA, AWWA and WEF, 1998)

3.2.1.4 Iron analysis by using Phenanthroline Method (APHA, AWWA and WEF, 1998)

3.2.1.5 Nutrient analysis as follows:

a) nitrate nitrogen by using Cadmium Reduction method

b) nitrite nitrogen by using Colorimetric method

c) ammonium nitrogen by using Phenate method

d) soluble reactive phosphorus (SRP) and total phosphorus (TP) by using Ascorbic acid method

(APHA, AWWA and WEF, 1998)

3.2.2 Collect the water sample using a glass bottle for the following purposes:

3.2.2.1 Dissolved oxygen (DO)

3.2.2.2 Biochemical oxygen demand (BOD_5)

Analyze DO and BOD_5 by using Azide modification method (APHA, AWWA and WEF, 1998).

3.2.3 Take measurements of some physical and chemical parameters of the water's quality

3.2.3.1 Water depth measurement taken in the middle of the stream width.

3.2.3.2 Substrate investigation by assessing the percentage of substrate type (Boulder, Gravel, Sand, Silt, Bedrock or Clay) from one side of the stream to another within a section of 5 parts along the stream width. Five sections in 5 meter lengths of each sampling site should be measured (Robert , 1996).

3.2.3.3 Water temperature and air temperature measurement by use of thermometers

3.2.3.4 Gage stream current by using a velocity meter (Swoffer model 2100)

3.2.3.5 Record altitude measurement with a GPS Receiver (Batch Meridian XL)

3.2.3.6 Conductivity measurement taken by using a conductivity meter (electrode kit of WTW company)

3.2.3.7 pH measurement by using a pH meter (electrode kit of WTW company)

3.2.3.8 Total dissolved solids or TDS measurement by using a conductivity meter (electrode kit of WTW company)

3.3 Benthic diatoms gathering and slide preparation procedure

3.3.1 Collecting of the benthic diatoms sample (Rott *et al.*, 1997; Krammer and Lange-Bertalot, 1986 and Barber and Haworth, 1981)

3.3.1.1 Epilithic diatom samples scraped from 5–10 stones per site with a toothbrush. The surface area of the selected stones should be estimated. Epipelic diatom samples are taken by skimming the mud surface with a spoon.

3.3.1.2 Make a compound sample in a polyethylene bottle. Store the sample in a cool and dark storage until microscopy and preparation will be done. Fix with Lugol ' s solution 1 ml per 100 ml sample or with 2% formalin solution.

3.3.2 Preparating of the benthic diatoms sample (modified from Rott *et al.*, 1997)

3.3.2.1 Separation of the diatom samples from sedimentation using centrifuge to isolate diatom cell from gravel and sand. Centrifuge the samples at 2,500–3,000 rpm. for 15 minutes. Remove the brown portion above the sediment and put it into a test tube.

3.3.2.2 Samples are cleaned by boiling the sample with concentrated acid or oxidizing agents (hydrochloric acid, nitric acid, hydrogen peroxide, potassium permanganate or potassium dichromate) for 15–30 minutes. Boil for half an hour on a hot plate. Wash the samples after all cleaning steps, rinse frequently with distilled water and centrifuge at 2,300–3,000 rpm. for 5 minutes.

3.3.3 Slide preparation

3.3.3.1 Prepare samples for light microscope investigation by placing 1–4 drops of a cleaned sample, depending upon the density of the sample, on a round coverslip. To reduce concentration, samples may be dispersed in distilled water . Dry samples on a hotplate with gentle heat (about 50–100 $^{\circ}$C) to fix diatoms to the glass. Add 1–2 drops of Naphrax or Dyrax on the labeled slide. Then place the coverslide face down on a prepared slide. Replace the slide on the hotplate for 1–2 seconds to remove toluene. After that, cool the slide. Gently tap the coverslip with a forcep tip to remove any bubbles (modified from Rott *et al.*, 1997). The mounts should finally be dried at room temperature overnight before being observed under a light microscope in oil immersion, at 100X magnification. Light micrographs can be made with an Olympus BX-40 microscope.

3.3.3.2 Prepare samples for SEM micrographs by dropping the cleaned diatom sample on a coverslip and drying it on a hotplate. Keep it in a desiccator overnight and fix it on a stub (Nopanit, 1984). Scanning electron micrographs can be made with a JEOL JSM-840A microscope, operated at 8–20 KV. Black and white film should be used (Kodak Verichromepan ISO 125).

3.3.4 Identification

3.3.4.1 The taxonomic classification system of the Süßwasserflora Mitteleuropas by Krammer and Lange-Bertalot (1986, 1988, 1991a, 1991b), Krammer (1986, 1992, 1997a, 1997b), Lange-Bertalot and Krammer (1989), Lange-Bertalot (1993, 1995) and Reichardt (1984), Huber-Pestalozzi (1942), Hustedt (1937) were followed. In some cases, however, the relevant keys in books or theses of some tropical studies, such as Foged (1971,1974, 1975, 1976), Podzorski and Hakansson (1987), Vyverman (1991) and Benavides (1994) were used. Some small *Gomphonema* species were classification following Reichardt (1997). The hand-drawing procedure followed Barber and Carter (1996). Fresh material of some species were investigated following Cox (1996). The features of the diatom frustule were described in English following Barber and Haworth (1981) and Kelly (2000). Structural data presented such as diameter, length, width, striae, striae frequency in 10 μm and other features (raphe, puncta, areolae, fibulae, nodule, septa, costae,

stigmata, rib, spine, wing and canals) were observed under light and scanning electron microscopes (Krammer and Lange-Bertalot, 1986, 1988, 1991a, 1991b; Krammer, 1986, 1992, 1997a, 1997b; Lange-Bertalot and Krammer, 1989; Lange-Bertalot, 1993, 1995; Reichardt, 1984; Huber-Pestalozzi, 1942; Hustedt, 1937; Foged, 1971, 1974, 1975, 1976; Podzorski and Hakansson, 1987; Vyverman, 1991; Benavides, 1994; Reichardt, 1997; Barber and Carter, 1996; Cox, 1996; Barber and Haworth, 1981; Kelly, 2000). A good quality microscope equipped with a mechanical stage and a 100X oil-immersion lens is required for all details.

3.3.5 Counting procedure

3.3.5.1 Permanent slides of epilithic diatoms should be counted and these figures should then be applied to the Diatoms Index (DI) for final calculation of water quality assessment. Counting begins with the relative counts that concentrate on relative portions of the species, until a total count of 100-300 specimens is reached, however the variability of the taxa in the counts depends on their dispersion and this can be checked by an ascending series of counts from 100 to 1,000. (modified from Rott *et al.*, 1997)

3.3.6 Fresh material investigation and illustration procedure

3.3.6.1 Although the taxonomy of diatoms are based on the morphology of the silica cell wall, other features such a chloroplast shape and number position within the living cell can used in the identification process (Cox, 1996). Screen the samples starting with living samples and continue with cleaned material and the mounted slides.

Observe the different chloroplast types on form, number and arrangement within the cells, described in the key book. Identification of Freshwater Diatoms from Live Material (Cox, 1996). Measure the length, width, thickness and process of the diatom cell under a light microscope. Make hand drawings and notes of the shape, size and structure of the specimens. Camera lucida and light photos can be used. The species that are unable to be identified by light microscope can be re-examined by SEM.

3.4 Data evaluation

3.4.1 Diversity Index (DI)

3.4.1.1 The species diversity index (H') and evenness (E) calculation follow Shannon (Odum, 1971).

$$H' = -\sum_{i=1}^{s} p_i \log p_i$$

When H' = Shannon's diversity index

p_i = Proportion of specie i in the community

s = Number of species

$$E = \frac{H'}{\ln s}$$

When E = Evenness

H' = Shannon's diversity index

s = number of species

3.4.2 Trophic Diatoms Index

3.4.2.1 Index systems are based on indicator species lists, which contain information on the autecological preferences of each species toward one ecological variable (e.g. pH, organic pollution, nutrient concentration). Information on the autecological preferences of each species is based, either on the analysis of a large number of samples (several hundreds of samples) of diatom species composition or environmental variables (e.g. Schiefele & Kohmann, 1993).The trophic index of van Dam (van Dam *et al.*, 1994) and the Saprobic index of Rott *et al.* (Rott *et al.*, 1997) are applied in counting the epilithic diatoms sample data of the Mae Sa Stream. Index value were shown in Table 20 and Table 21. Results of the calculation were presented in Table 22-23 and Figure 113-114.

3.4.3 Statistical analysis

3.4.3.1 The collected data of the counted cells and the water quality can be processed in many ways. The raw data of the cell counts should be put into the Multivariate Statistical Package (MVSP) for window version 3.1. This is to find similarities between the sample sites and which species of diatoms are dominant. This is done by using Principal Components Analysis (PCA), Canonical Correspondence Analysis (CCA), Diversity Indices were performed. For the PCA, the data was transformed to Log10 and the axes were extracted to Kaiser's rule.

3.4.3.2 The computer statistical package, SPSS for window version 6.0 is used to perform the following statistical analysis, analysis of Variance (ANOVA), Least Significant Difference (LSD), Correlation, Regression and Factor analysis are use for computering the water quality of the five sites using different chemical and biological parameters. The correlation between dominant species and some physico-chemical parameters were performed.

3.5 Mae Sa Index Development

The method used was adapted from Kelly (Kelly, 2000). With the former is a measure of the effect of nutrients (predominately phosphorus) on stream communities, whilst the latter is a more general measure of water quality, taking account of factors such as biochemical oxygen demand, ammonia and salinity alongside that of nutrients. Trophic Diatom Index is computed with the same way, using a "weight average" equation (Zelinka and Marvan, 1961 cited by Kelly, 2000). The formula for this equation is:

$$WMS = \frac{\sum avs}{\sum av}$$

Where a = relative abundance (proportion) of species in the sample

v = the indicator value (1-3)

s = pollution sensitivity (1-5) of the species

The environmental preferences of each dominant taxa which selected from PCA are summarized in the form of simple scatter with plotted for alkalinity, conductivity, nitrate nitrogen and soluble reactive phosphorus, inorder to avoid making too many generalizations. The relative abundance are indicated on the vertical axis, and the environmental determination on the horizontal axis. Patterns of the plot may show peak, indicator values depended on the spread of values around this peak (Whitton and Kelly, 1995). Sumarized information for each dominant species represented in Table 19, this will be use for calculate the Mae Sa index.