CHAPTER 3

Materials and Methods

3.1 Sampling sites

The sampling sites were located between 18.18˚ and 19.47˚ N of the Equator and with a longitude ranging from 98.57˚ to 99.54˚ E of Greenwich (Figure 3.1). Thailand is on the Indochina Peninsula in a tropical zone. Thailand experiences three seasons annually. The rainy season (from mid-May to mid-October) is influenced by the southwest monsoons. The cool-dry season (from mid-October to mid-February) is influenced by the northeast monsoons, and the hot-dry season (from mid-February to mid-May) (Thai Meteorological Department, 1994).

The samples were collected from different water bodies including lakes, reservoirs, dams, fish ponds, garden ponds, ponds, field ponds, sewage ponds, ditches, rice fields, and ephemeral water bodies located throughout the geographic range of Northern Thailand in March 2009 (Figure 3.2). In total, samples from 31 sampling sites were collected in Chiang Mai, Chiang Rai, Lamphun, Lampang, and Phayao Provinces.

Among them, 13 sampling sites, which presented a high diversity of euglenoids, were selected for the collection of the samples, while the physico-chemical parameters were analyzed every month from April 2009 to March 2010. Moreover, the ecological data, such as altitude and ordination, topography (size, shape and depth), catchment areas, presence of aquatic plants and utilization of sampling sites of each sampling site were recorded. The list of sampling sites together with some general data is shown (Table 3.1).

Table 3.1 Location and description of sampling sites

Sampling sites	Codes	Ordinations	Characteristics
1. Chiang Mai			
1.1 Pond 1*	AG	18°47'36.00" N 98°57'40.02" E	Garden pond at Chiang Mai University, shallow, transparent, with Lotus (plants)
1.2 Pond 2*	UM	18°46'55.98" N 98°57'5.46" E	Fish pond at Wat Umong, mostly with Catfish
1.3 Pond 3*	MJ1	18°53'55.80" N 99°0'59.94" E	Fish pond at Maejo University, with Mekong Giant Catfish
1.4 Mae Kuang Udom Thara Dam*	MK	18° 56'36.1' N 99°8'5.90" E	Dam built across the Mae Kuang River
1.5 Pond 4	PK	18°47'36.48" N 98°57'54.72" E	Garden pond in the park naer Chiang Mai University Hall
1.6 Pond 5	MJ2	18°53'54.90" N 99°0'59.88" E	Fish pond at Maejo University, with Mekong Giant Catfish
1.7 Pond 6	MJ3	18°53'55.38" N 99°1'2.94" E	Fish pond at Maejo University, with Mekong Giant Catfish
1.8 Pond 7	MJ4	18°53'56.28" N 99°0'57.90" E	Fish pond at Maejo University, with Mekong Giant Catfish
1.9 Ephemeral water body	CV	18°48'27.42" N 99°14'3.96" E	Ephemeric water body on canvas at a biogas farm
1.10 Pond 8	ST	18°46'18.66" N 99°16'16.56" E	Sewage pond at Sutach Farm, waste from a chicken farm
2. Lampang			
2.1 Pond 1*	KL	18°19'8.22" N 99°28'0.54" E	Fish pond at Khelang Nakorn School, with Nile Tilapia
2.2 Pond 2*	TJ	18°18'37.80" N 99°29'41.94" E	Fish pond atWat Phra Jao Tanjai, with Nile Tilapia, with Water Hyacinth
2.3 Ricefield	RF	18°18'54.12" N 99°26'22.50" E	Ricefield on the road side
2.4 Pond 3	AY	18°19'46.56" N 99°22'33.60" E	Garden pond in the area of Anantayot Park
2.5 Pond 4	WK	18°18'8.76" N 99°30'32.52" E	Garden pond near Wat Phra Kaew Don Tao Suchadaram
3. Lamphun			
3.1 Ditch 1*	LL	18°32'44.04" N 99°1'57.60" E	Ditch in front of Lanlao restaurant

* = The collection of samples every month over a single year, from April 2009 to March 2010

Table 3.1 (continued)

Sampling sites	Codes	Ordinations	Characteristics
3.2 Pond 1*	BL	18°36'41.70" N 99°2'3.18" E	Field pond in front of Bualuang restaurant
3.3 Pond 2*	LP1	18°38'7.62" N 99°3'3.42" E	Field pond near agriculture areas, on the road side
3.4 Moat	MT	18°34'31.80" N 99°0'13.68" E	Moat in Lamphun
3.5 Ditch 2	LP2	18°38'6.66" N 99°3'2.58" E	Ditch near agriculture areas
4. Chiang Rai			
4.1 Pond 1*	CC1	19°16'2.46" N 99°30'42.60" E	Fish pond in the area of Cabbage & Condom Inn and Restaurant, with a Carp
4.2 Pond 2*	BY1	19°47'7.20" N 99°44'40.38" E	Garden pond at the Pa Ko Dam Tobacco station, shallow
4.3 Hui Yah Khamma Reservoir	WM	19°10'57.30" N 99°29'41.46" E	Reservoir for the preservation of freshwater fish
4.4 Ditch	CC2	19°16'2.46" N 99°30'42.60" E	Ditch in the area of Cabbage & Condom Inn and Restaurant
4.5 Pond 3	BY2	19°47'5.52" N 99°44'39.24" E	Garden pond at the Pa Ko Dam Tobacco station, shallow, with ducks
5. Phayao			
5.1 Ditch*	AS	19°10'17.16" N 99°54'4.38" E	Ditch in front of Kindergarten Phayao School,
5.2 Pond 1*	PS1	19°10'16.80" N 99°54'35.82" E	Fish pond at Phayaopitthayakom School, with fishpens
5.3 Pond 2	PS2	19°10'14.70" N 99°54'35.82" E	Garden pond in area of Phayaopitthayakom School
5.4 Pond 3	FP	19°9'31.62" N 99°54'36.00" E	Fish pond in area of Provincial Waterworks Authority
5.5 Kwan Phayao Lake	KP	19°10'6.48" N 99°53'42.96" E	Lake in the valley of the Ing River
5.6 Pond 4	PC	19°5'26.16" N 99°49'29.28" E	Garden pond in area of Phayao College of Agriculture and Technology

* = The collection of samples every month over a single year from April 2009 to March 2010

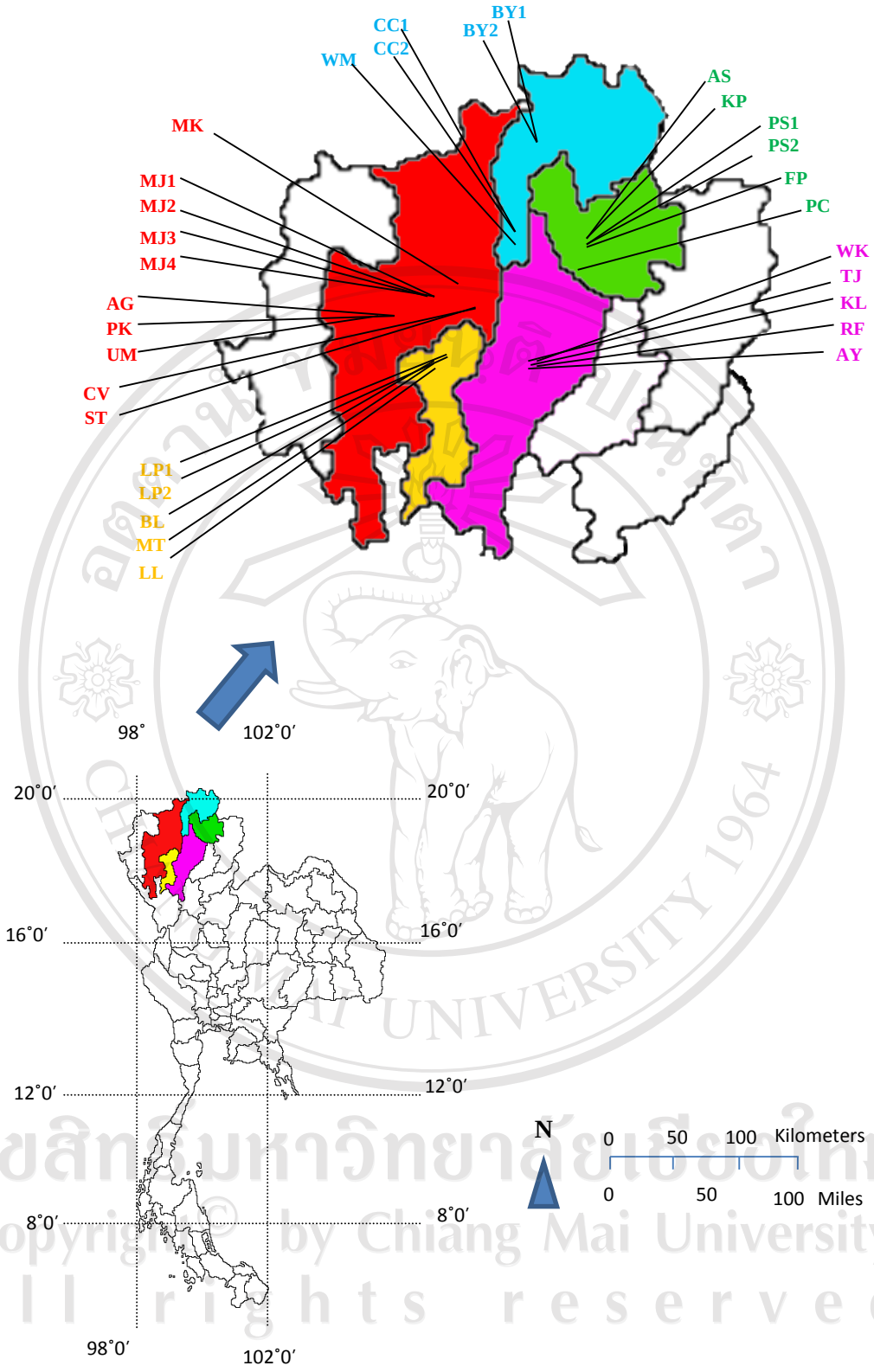

Figure 3.1 Map of Thailand and the Northern area showing 31 sampling sites in Thailand

Figure 3.2 Examples of different sampling sites: Mae Kuang Udom Thara Dam (a), ditch in front of Lanlao Restaurant (b), a pond at Maejo University (c), a ditch in front of Kindergarten Phayao School (d), a garden pond at the Pa Ko Dam Tobacco Station (e), a garden pond at Chiang Mai University (f), a rice field on the roadside (g), and a fish pond at Wat Umong (h)

3.2 Collection of euglenoids

3.2.1 The material was taken from about 10 liters of open water with a plankton net (10 µm pore size). Additionally, a high diversity of euglenoids was studied from aquatic plants, and from the sediment with a slime aspirator by a random method (Figure 3.3).

3.2.2 All materials were placed in plastic flasks (ca. 100 ml) and divided into two parts: one preserved with Lugol's solution, and the other transported as fresh material.

3.2.3 Samples were studied in the laboratory with an Olympus CX31 light microscope.

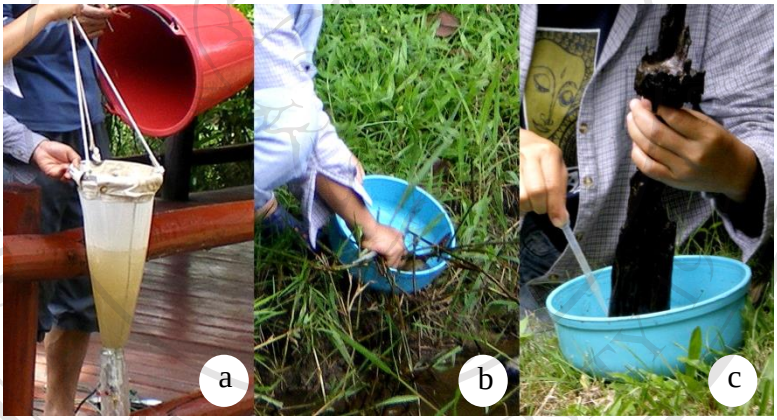

Figure 3.3 Collection of euglenoids: filtration through a plankton net (a) use of a slime aspirator and by scraping from aquatic plants (b, c)

3.3 Identification and the counting of euglenoids

The general taxonomic system followed that of Leedale (1967) using six orders (Eutreptiales, Euglenales, Rhabdomonadales, Sphenomonadales, Heteronematales, and Euglenamorphales) of the Division Euglenophyta (Leedale, 1967).

Taxonomic studies were based on live and preserved materials observed with LM an Olympus BX51 and a Nikon Eclipse E600 with Nomarski phase contrast. For SEM, samples

were prepared according to the procedures described by Bozzola and Russell (1991) and then studied with a Hitachi S-4700 SEM in the Scanning Microscopy Laboratory of Biological and Geological Sciences, Jagiellonian University, Kraków, Poland.

The samples were identified according to Conrad (1935), Huber-Pestalozzi (1955), Pringsheim (1956), Prowse (1958), Akiyama (1977), Kudo (1977), Starmach (1983), Conforti and Tell (1986a, 1986b), Yamagishi (1992, 2010), Wołowski (1998, 2011), Zhixin (1999), Kosel-Fetzmanm (2002), Rosowski (2003), Wołowski and Hindak (2005), and Ciugulea and Triemer (2010). Publications related to the identification of tropical and sub-tropical species were also used in identifying the samples.

The euglenoids presented in the samples were counted in cell/liter unit (Pals *et al*., 2006). The abundance of species, expressed in terms of the member of its specimens, was estimated using a five-degree scale (Wołowski, 1998), as follows:

+ - The species occurred only as a single specimen at least on one slide;
1 - The species occurred as up to 6 specimens, on almost every slide (spare);
2 - The species occurred on every slide but not on all visible fields (frequent);
3 - The species occurred on every slide and visible field (very frequent);
4 - The species formed a bloom on the surface of the water, visible to the naked eye (in masses)

3.4 Water quality analysis

The water samples were collected in polyethylene bottles for nutrients and physico-chemical analysis. The bottles were kept in a cool box (5-7 ˚C) for analysis in the laborabory. The times of sample collection, which were divided into two ranges, were the morning (8.00-12.00 am) and the afternoon (13.00-18.00 pm).

3.3.1 Physico-chemical analysis

1) Water and air temperature by a thermometer

2) Conductivity measurement by a conductivity meter (electrode kit of WTW Company)

3) pH measurement by a pH meter (electrode kit of WTW Company)

4) Dissolved oxygen (DO) by azide modification method (Eaton *et al*., 2005)

5) Alkalinity analysis by the phenolpthalein methyl orange indicator method (Eaton *et al*., 2005)

6) Chlorophyll *a* analysis (Eaton *et al*., 2005)

7) Turbidity analysis by portable datalogging spectrometer (HACH DR/2010)

8) Transparency measurement by Secchi disc

9) Light concentration measurement by Lux meter (Tecpel 530)

3.3.2 Nutrient analysis (Eaton *et al*., 2005)

1) Nitrate nitrogen analysis by cadmium reduction method

2) Ammonium nitrogen analysis by Nesslerization method

3) Soluble reactive phosphorus analysis (SRP) by ascorbic acid method

3.5 Data evaluation

3.4.1 Trophic status and water quality:

The trophic status and water quality were classified following the methods of AARL-PC score and AARL-PP score (Peerapornpisal *et al*., 2004, 2007) by altering the amounts of biochemical oxygen demand, conductivity,

ammonium nitrogen, nitrate nitrogen, soluble reactive phosphorus, and chlorophyll *a* to calculate the water quality status.

3.4.2 Diversity index (DI):

The species diversity index (H') and evenness (E) were calculated following the Shannon Diversity Index (Odum, 1971).

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

When H' = Shannon' s diversity index

Pi = Proporion of species I in the community

S = Number of spercies

$$E = H' / \ln s$$

When E = Evenness

H' = Shannon's diversity index

S = Number of species

3.4.3 The similarity between the sampling sites and certain water physico-chemical parameters is described below.

The sampling sites and certain water physico-chemical parameters were analyzed by multivariate statistical package (MVSP) for Windows with cluster analysis (CA). Then, each water physico-chemical parameter of the segregated groups from cluster analysis were consequently computed by the commercially avilable statistical package, SPSS for Windows using one way analysis of variance (ANOVA) and the Tukey test.

3.4.4 The correlation between certain physico-chemical parameters and their correlation with euglenoid communities was noted.

Certain physico-chemical parameters and euglenoid communities were computed by the commercially available statistical package, SPSS for

Windows. The bivariate correlations procedure was computed. Pearson's correlation coefficient were used together with principal component analysis (PCA) and canonical correspondence analysis (CCA), using the multivative statistical package (MVSP) for windows.

3.4.5 The influence of the seasons on the physico-chemical parameters and euglenoid communities is presented as follows.

The influence of the seasons on the physico-chemical parameters and euglenoid communities was computed by commercially avilable statistical package, SPSS for Windows using randomized complete block design (RCBD) and the Tukey test.

3.4.6 Euglenoids as bioindicators is described below.

The methods used to prepare the euglenoid index were applied Palmer (1969), Slàdeček and Sládečková (1996), and Peerapornpisal (1996). A total 32 taxa of euglenoids that were selected from 3 groups including those with the highest relative abundance (>1%), 30% occurrence of euglenoid taxa in the algae total of each site, and the euglenoid taxa blooms of each site were selected to establish a euglenoid index. The estimation of the indicator values was based on a weighted averages approach (WAs). WAs were calculated based on water quality variables and the abundance of organisms from each site. The major environmental factors including BOD, nitrate nitrogen, ammonium nitrogen and SRP were classified to seven classes (Table 7) according to Lorraine and Vollenweider (1981), Wetzel (2001), Peerapornpisal *et al*. (2004), Jones and Medrano (2006), Pollution Control Department (2010), and Leelahakriengkra (2011). Indicator values were averaged from 4 major environmental factors and compared with the trophic status in Table 7. WAs were calculated using the formula below:

$$WA_{jk} = \frac{\sum_{i=1}^{n} (X_{ij} \cdot Y_{ik})}{\sum_{i=1}^{n} X_{ij}}$$

WA_{jk} = The weight average of taxon *j* at water quality factor *k*

X_{ij} = The percent relative abundance of taxon *j* at site *i*

Y_{ik} = The level of factor *k* at site *i*

n = The number of sites which found taxon *j*

The sample index was calculated from the formula below:

$$\text{Sample index} = \frac{\Sigma \text{ Relative abundance x Average indicator values}}{\Sigma \text{ Relative abundance}}$$