CHAPTER 3

MATERIALS AND METHODS

The study of diversity of benthic diatoms during October 2011 to September 2012 for water quality index establishment of Wang River was carried out. Samples were collected monthly by 10 study sites located along the mainstream and 2 study sites located in reservoirs.

3.1 Site descriptions

The Wang River is altogether 460 kilometers long (Hydro and Agro Informatics Institute, 2011). The waters flow from north to south and this river has its source in the Phi Pan Nam Range in Wiang Pa Pao District, Chiang Rai Province. One of the principal settlements along the river is Lampang, which is situated on the north bank of a curve in the river. From Lampang, the river flows southwards passing by Thoen District into Tak Province. It joins the Ping River near Ban Mae Salit, Ban Tak District, and north of the town of Tak. Nowadays, there are two large irrigation projects located on this river, including the Kew Lom Dam which is located about 30 kilometers from the city of Lampang in a northern direction. The other is the Kew Kor Ma Dam, which is located in Jae Hom District on the northern part of the Wang River.

In this study, the physico-chemical parameters of the water along with diatom samples were collected from 12 sampling sites and were selected under the criteria of geological and land use differences, from upstream areas to downstream areas of the Wang River. Furthermore, the effects of the human activities taking place at each sampling site were also recorded. A map of the sampling sites (Figure 5), their description and topographical details (Table 8) are listed below.

3.1.1 Wang Kaew Waterfall, Wang Nuea District, Lampang Province (Site 1)

There is the upstream area along the Wang River, most areas of this sampling site are surrounded by forests as this site is located in Doi Luang National Park. In addition, this area is located in the limestone mountainous area and the site has been slightly

affected by recreation activities and other human activities of a nearby hill tribe village. The water level of this site is shallow throughout most of the year. Most of the substrates the samples were collected from included cobblestones.

3.1.2 Ban Rong Kor, Wang Nuea District, Lampang Province (Site 2)

This is a mainstream collection site. This sampling site is surrounded by rice fields and small communities. The width of the stream is about 10 meters. The water depth is considered shallow water, as it is not higher than 1.20 meters. Most samples were collected from substrates involving cobblestones and gravel.

3.1.3 Kew Kor Ma Dam, Chae Hom District, Lampang Province (Site 3)

This site is located in a standing water area, which is located on the waste rock dam and is surrounded by the forest area. Normally, this site was not disturbed by human activities such as agriculture, recreation or land settlement activities. The water level of this site depended on the dam operation, and most substrates collected included cobblestones.

3.1.4 Ban Hui Sanao, Chae Hom District, Lampang Province (Site 4)

This area is a running water site which is located downstream of the Kew Kor Ma Dam, about 1 kilometer away from the spillway where the water was released. Most of the area around this site is made up of cornfields and paddy fields with a small community area settled nearby. The type of substrates that samples were collected from included cobblestones and gravel. Nevertheless, the water level in this site was dependent on the time period of the operation of the dam gates.

3.1.5 Ban Pan Pong Chai, Chae Hom District, Lampang Province (Site 5)

This mainstream site was located between the two large reservoirs of two major dam constructions: Kew Kor Ma Dam and Kew Lom Dam. The area of this site consisted of rice fields and agricultural areas along with residential communities. The water level is considered shallow throughout the year. The substrates found at this site were primarily comprised of cobblestones and gravel.

3.1.6 Kew Lom Dam, Mueang District, Lampang Province (Site 6)

Kew Lom Dam is a sampling site that presented standing water. The dam was constructed of a concrete wall. The area around this site consisted of forests; however, the recreational activity of raft touring was the main activity which affected the water quality at this site. The cobblestones and concrete wall were the substrates from which the diatom samples were collected at this site.

3.1.7 Ban Sop Mai, Mueang District, Lampang Province (Site 7)

This site presented the running water and was which located below the Kew Lom Dam and far from the spillway of the dam by around 2 kilometers. A small community and agricultural area were the main utilization sites of this area. Nevertheless, the water level of this site was affected by the spillway operation. Most samples were collected from substrates at this site, which were made up of cobblestones.

3.1.8 Ban Tha Kao Noi, Mueang District, Lampang Province (Site 8)

This is the running water site that was located within the city limits of Lampang. This site was closely associated with an urban area and a fairly densely populated community is located near this area, which is the main cause of water contamination through domestic waste discharge. At this area, the water level was mostly high in the rainy season and very shallow in the dry season. The substrates samples collected from this sampling site were taken from substrates consisting of gravel.

3.1.9 Ban Nong Jok, Ko Kha District, Lampang Province (Site 9)

This site stands along the main river, which is surrounded by the residential community and sugarcane factory. In addition, there was a fermented fish store located near the sampling site. Usually, the water velocity around this sampling site was found to be very low due to the effects of the check dam. Most samples taken from this site were collected from substrates comprised of tree sticks and the leaves of aquatic plants.

3.1.10 Ban Mae Thod, Thoen District, Lampang Province (Site 10)

This running water site is located near the small residential community, which was

surrounded by an agricultural area. The paddy fields and cornfields were responsible for the main activities affecting this site. Nevertheless, before the river runs in to this area, it has flowed through the mountainous and forested areas of the Mae Wa National Park. Most samples collected from this site were taken from substrates consisting of cobblestones.

3.1.11 Ban Mae Chiang Rai, Sam Ngao District, Tak Province (Site 11)

This running water site is located on the border between Tak and Lamprang Provinces. A small village is located along this river along with the spreading agricultural area. Most samples collected from this site were taken from substrates comprised of gravel.

3.1.12 Ban Pak Wang, Ban Tak District, Tak Province (Site 12)

This running water site is located on the mouth of the Wang River where this river joins with the Ping River. This collection point stands below the Bhumipol Dam by about 30 kilometers. The water level of this site is affected by the regular release of water from the Bhumipol Dam. The tilapia fish cage culture, longan tree planting enterprise, cornfields and waste discharge from a nearby small village made up the human activities that impacted on the river water in this area.

3.2 Investigation of benthic diatoms

3.2.1 Collection of benthic diatoms

The benthic diatom samples were collected from stones or other substrates at each site by brushing the substrate with a toothbrush. For each composite sample, 3-5 samples were brushed using a 9 cm^2 plastic template. The benthic diatoms on the surface of the selected stones were brushed and rinsed with distilled water, until the cutout area was completely cleared. Each sample was pooled in a small bottle, transferred to plastic container and labeled with the relevant details: site name, location code, date, and replicate number. The samples were kept in a cool box at 5-7 ^oC. (Renberg, 1990; Rott *et al.*, 1997 and Kelly *et al.*, 1998).

Table 9 Sampling sites and their topography

Site	Location	Latitudes and Longitude	Altitudes (m)	Land Utilization
1	Wang Kaew Waterfall, Wang Nuea District, Lampang Province.	19°18″992′N 99°39″765′E	573	Waterfall, Recreation area
2	Ban Rong Kor, Wang Nuea District, Lampang Province.	18°59″438′N 99°37″256′E	341	Paddy field
3	Kew Kor Ma Dam, Chae Hom District, Lampang Province.	18°38″538′N 99°37″739′E	357	Reservoir
4	Ban Hui Sanao, Chae Hom District, Lampang Province	18°47″100′N 99°37″847′E	323	Floodgate, Paddy Field
5	Ban Pan Pong Chai, Chae Hom District, Lampang Province	18°40″178′N 99°33″247′E	255	Paddy field
6	Kew Lom Dam, Mueang District, Lampang Province	18°31″431′N 99°37″490′E	288	Reservoir
7	Ban Sop Mai, Mueang District, Lampang Province	18°30″471′N 99°38″759′E	241	Floodgate, Paddy field
8	Ban Tha Kao Noi, Mueang District, Lampang Province	18°17″466′N 99°28″204′E	218	Urban area.
9	Ban Nong Jok, Ko Kha District, Lampang Province	18°11″394′N 99°23″817′E	191	Urban area
10	Ban Mae Thod, Thoen District, Lampang Province	17°45″111′N 99°13″807′E	191	Agricultural area, Small community
11	Ban Mae Chiang Rai, Sam Ngao District, Tak Province	17°22″044′N 99°13″812′E	121	Agricultural area Small community
12	Ban Pak Wang, Ban Tak District, Tak Province	17°07″383′N 99°03″740′E	111	Mouth of river, Agricultural area

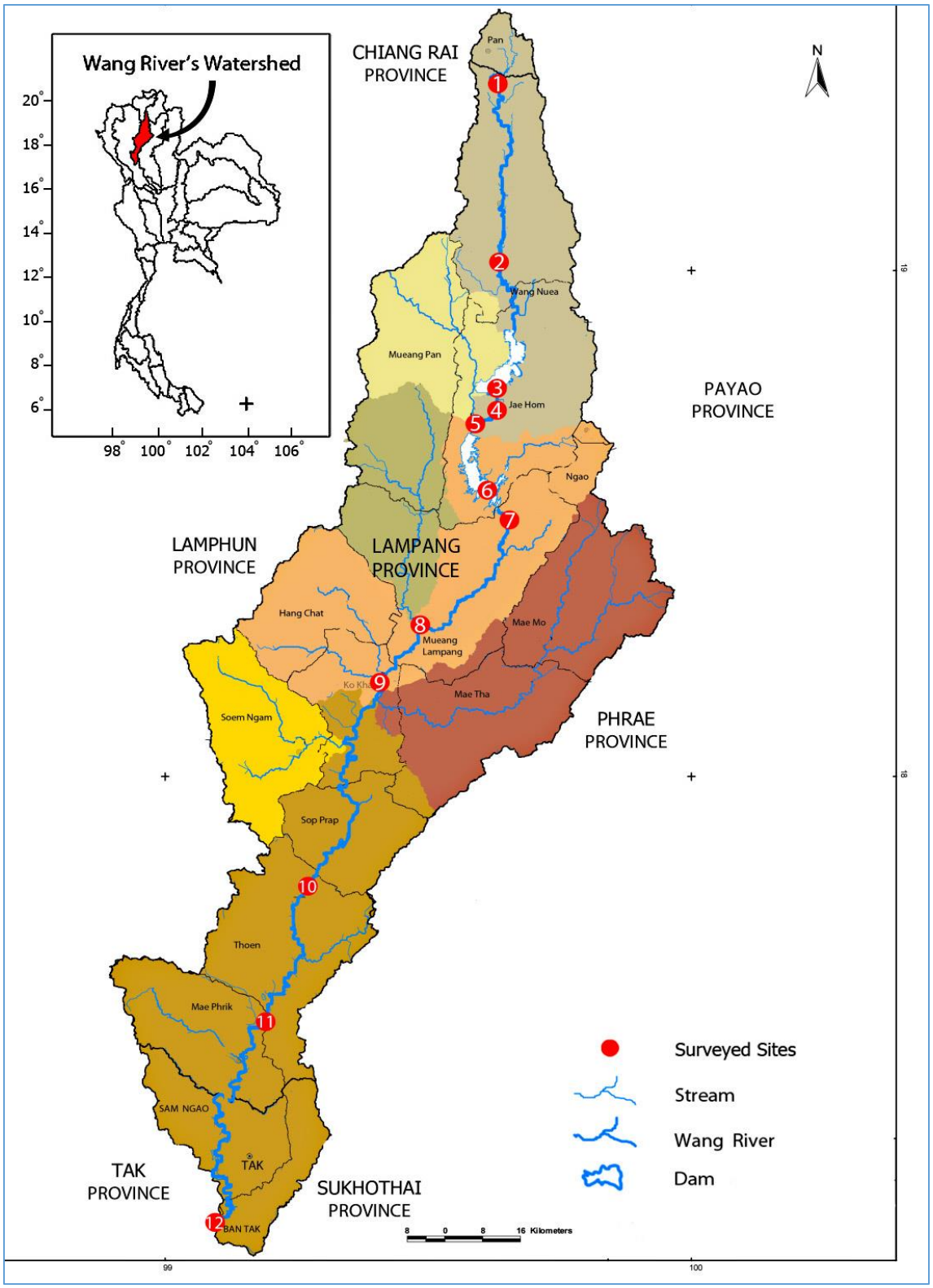

Figure 5 Map of Thailand and twelve sampling sites along the Wang River for the assessment of the diversity of benthic diatoms and the establishment of a water quality index during the period of October 2011 to September 2012

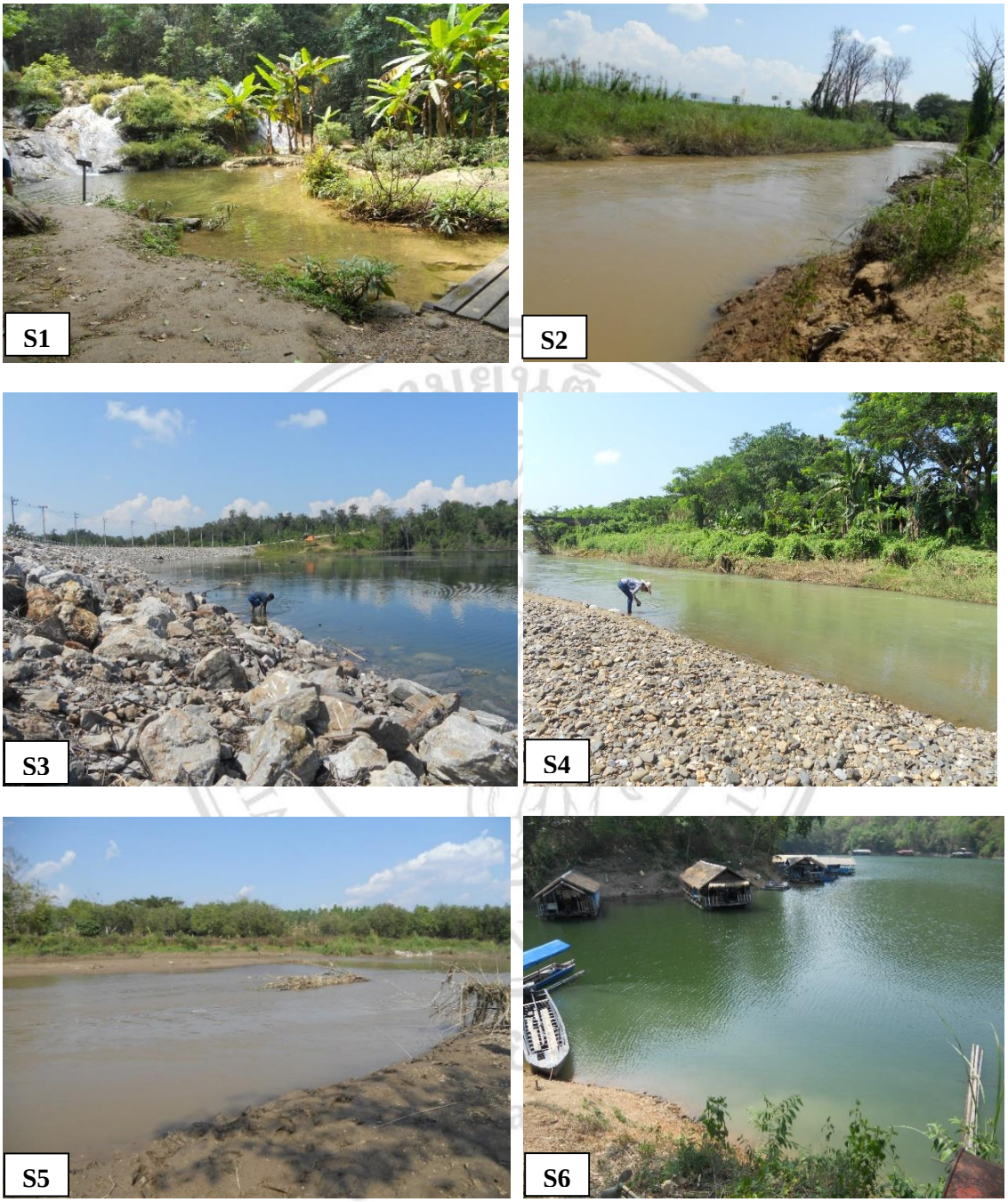

Figure 6 Sampling sites along the Wang River from October 2011 to September 2012

(S1) - Wang Kaew Waterfall, Wang Nuea Dist, Lampang Province
(S2) - Ban Rong Kor, Wang Nuea District, Lampang Province
(S3) - Kew Kor Ma Dam, Chae Hom District, Lampang Province
(S4) - Ban Hui Sanao, Chae Hom District, Lampang Province
(S5) - Ban Pan Pong Chai, Chae Hom District, Lampang Province
(S6) - Kew Lom Dam, Mueang District, Lampang Province

Figure 7 Sampling sites along the Wang River from October 2011 to September 2012
(S7) - Ban Sop Mai, Mueang District, Lampang Province
(S8) - Ban Tha Kao Noi, Mueang District, Lampang Province
(S9) - Ban Nong Jok, Ko Kha District, Lampang Province
(S10)-Ban Mae Thod, Thoen District, Lampang Province
(S11)-Ban Mae Chiang Rai, Sam Ngao District, Tak province
(S12)-Ban Pak Wang, Ban Tak District, Tak province

3.2.2 Process of benthic diatom cleaning

The diatom samples were cleaned by concentrated acid digestion method. Separation of the diatoms samples from sediment was done using a centrifuge to isolate diatom cells from the gravel and sand. The samples were centrifuged at 2,500 rpm for 15 minutes. The diatom cells (brown layer between supernatant and solid particles) were removed in a test tube. The samples were boiled in strong acid (HNO_3) and oxidizing agents (hydrogen peroxide) for 30-45 minutes to clean them. The samples were rinsed 4-5 times with de-ionized water after all cleaning steps in order to adjust the pH value to 7. The cleaned samples were mounted using the mounting agent. The mounting process involved dropping 2-3 drops of a cleaned and dried sample on a hotplate to fix the sample to a cover slip. A mounting agent solution such as Naphrax was added on the slide. The cover slip was covered on a prepared slide on a hotplate to remove toluene. The permanent slide was used for the counting and identifying processes (Rott *et al.*, 1997; Kelly *et al*., 1998).

3.2.3 Benthic diatoms identification and counting

For LM observation and morphometry, a SKE microscope (Nikon, Tokyo, Japan) with a ×70 oil immersion objective or a BX51 microscope (Olympus, Tokyo, Japan) with a ×100 oil immersion objective were used. Observed images were captured with a CH30RF200 digital camera (Olympus, Tokyo, Japan). Some species were studied using a scanning electron microscope. The samples were identified according to Ross *et al*. (1979)**,** Krammer and Lange-Bertalot (1986, 1988, 1991a, 1991b), Lange-Bertalot (2001), Kelly and Haworth (2002), Peerapornpisal (2004) and Kobayashi *et al* (2006). For the process of diatom counting, which was done under a compound microscope following the quantitative estimation, the counting was done by using Haemacytometer method and involved the application of the diversity index for this study and diatoms displaying a high abundance (>1%) were selected to establish the Wang Diatom Index (Kelly and Whitton, 1995).

3.3 Water samples analysis

Water samples were collected in polyethylene bottles. The bottles were kept in a

cool box at 5-7 ^{o}C, while the physico-chemical analysis was conducted by the following methods:

3.3.1 Water and air temperature were recorded using a thermometer.

3.3.2 Turbidity was measured using a turbidity meter.

3.3.3 Conductivity was measured with a conductivity meter (electrode kit of WTW Company).

3.3.4 pH were recorded using a pH meter (electrode kit of WTW company).

3.3.5 Alkalinity analysis was conducted by phenolphthalein methyl orange indicator method (Eaton *et al*., 2005).

3.3.6 Dissolved oxygen (DO) and biochemical oxygen demand (BOD) were conducted by the azide modification method (Eaton *et al*., 2005).

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

- Nitrate nitrogen analysis was conducted by the cadmium reduction method
- Ammonium nitrogen analysis was conducted by the nesslerization method.
- Soluble Reactive Phosphorus (SRP) analysis was conducted by the ascorbic acid method.

3.4 Data analysis

3.4.1 Trophic status and water quality

The trophic status and water quality of the water was evaluated for the main parameters (conductivity, DO, BOD, ammonium nitrogen, nitrate nitrogen and SRP) according to Lorraine and Vollenweider (1981), Wetzel (2001), Peerapornpisal *et al.* (2004) and the Pollution Control Department (2010).

3.4.2 Statistical analysis

The determination of any significant differences between the groups of benthic diatom assemblages and the datasets of water quality were done using ANOVA.

Pearson's correlation was also applied to see if certain variables were significantly correlated (Green, 2010).

Cluster analysis was used to analyze the similarity percentage between the samples on the log of the transformed water quality data using the Multivariate Statistical Package (MVSP) software with UPGMA cluster method. The environmental variables of the clusters were then tested for significance relationship with the ANOVA single factor method. Principal Component Analysis (PCA) was applied and the log ratio was used to transform the diatom data using MVSP with axes extracted using Kaiser's rule. Canonical Correspondence Analysis (CCA) was applied to assess water quality and to analyze the diatom data with regard to any relationship (Ter Braak and Verdonschot, 1995)

3.4.3 Diversity index (DI)

The Shannon-Wiener Diversity Index was used to calculate all the diatom data sets. The Shannon-Wiener Diversity Index (*H'*) was designed on the basis of species richness and evenness (E) in terms of the abundance of the species (Pinder, 1999; Stiling, 2002), and the values were calculated by the following formula:

$$H' = -\sum_{i=1}^{s} pi.\ln pi$$

Where *pi* refers to the proportion of individuals in the sample that belong to the *I* th of *s* taxa.

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

Where S is the number of species

3.4.4 Establishment of Wang Diatom Index for water quality assessment

In preparation of the establishment of the Wang Diatom Index, the previous diatom indices were applied, such as the Trophic Diatom Index (TDI) (Kelly and Whitton, 1995), the Saprobic Index (Rott *et al.*, 1997), the Mae Sa Index (Pekthong, 2002), the Ping and Nan Diatom Index (Kunpradid, 2005), the River Diatom Index (RDI) (Tang *et al.*, 2006), the Mekong Diatom Index (Suphan, 2009), the Thailand Diatom Index (Leelahakriengrai, 2011) and the Yom Diatom Index (Yana, 2014).

The methods used to prepare the Wang Diatom Index were applied from the weighted averages approach (WAs) created by Kelly and Whitton (1995) and Kelly (2000). Benthic diatoms with a high relative abundance (>1%) at each site were selected to establish the Benthic Diatoms Index. An estimation of the average indicator values of each species was arrived at by multiplying the indicator value of each species across the sampling site during every month and was based on the weighted averages approach (WAs). WAs were calculated based on the indicator value method (IVM) (Dufrene and Legendre, 1997)

WAs were calculated from the formula below:

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

Where WA_{ik} = the average of taxon *i* for water quality factor *k*

X_{ij} = The value of the environment k variables in site j in a month

Y_{ik} = the percent relative abundance of taxon *i* at site *j* in a month

Z_{ij} = indicator value of of taxon *i* at site *j* in a month (IV_{ij})

$$IV_{ij} = RA_{ij} \times RF_{ij}$$

Where IV_{ij} = Indicator Value of species i at sampling site j in a cluster 1

$$RF_{ij} = S_{ij}/S_j \quad (2)$$

Where RF_{ij} = Relative frequency of species i in a cluster

S_{ij} = the number of sites in a cluster where species i is present

S_j = the total number of sites in that group

$$RA_{ij} = A_{ij}/A_i \quad (1)$$

Where RA_{ij} = Relative abundant of species i at sampling j in a cluster

A_{ij} = the mean abundance of species i at sampling j in a cluster

A_i = the sum of the mean abundance of specie i in a cluster

WAs were calculated based on the water property variables and the abundance of organisms present at each site. The major environmental factors,

including BOD, nitrate nitrogen, ammonium nitrogen and SRP, were divided into seven classes (Table 11) according to Lorraine and Vollenweider (1981), Wetzel (2001) Peerapornpisal *et al.* (2004), Jones and Medrano (2006) and Pollution Control Department (2010). Indicator values were averaged from 4 major environmental factors and compared with the trophic status in Table 10.

Table 10 The seven categories of BOD, ammonium-nitrogen, nitrate-nitrogen and SRP for Index calculation.

Scores	**1**	**2**	**3**	**4**	**5**	**6**	**7**
BOD ($mg.l^{-1}$)	0.5	0.5-1.0	1.0-2.0	2.0-4.0	4.0-10.0	10.0-20.0	>20
Nitrate -N ($mg.l^{-1}$)	<0.01	0.01-0.19	0.20-0.39	0.40-0.79	0.80-1.90	2.0-10.0	>10.0
Ammonium-N ($mg.l^{-1}$)	<0.01	0.01-0.19	0.20-0.39	0.40-0.59	0.60-0.99	1.0-5.0	>5.0
SRP ($mg.l^{-1}$)	<0.01	0.02-0.04	0.05-0.06	0.07-0.19	0.20-0.99	1.0-3.0	>3.0
Trophic Status	hyper-oligo trophic	oligo trophic	oligo-meso trophic	meso trophic	meso-eutrophic	eutrophic	hyper-eutrophic

Source: modified from Kelly (2000), Lorraine and Vollenweider (1981), Wetzel (1983) and Peerapornpisal *et al.* (2004)

3.5 Research facility

3.5.1 Place of study and identification

The process of sampling identification was performed at the Applied Algal Research Laboratory, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand and Department of Biology, Tokyo Gakugei University, Tokyo, Japan**.**

3.5.2 Study Period

The time period for the sampling investigation took place from October 2011 to September 2012. Data analysis and publication were done over the period of September 2012 – December 2014.