# CHARPTER 3
# MATERIALS AND METHODS

## 3.1 Study sites

Thailand is located between 5° N and 20° N and between 97° E and 106° E (Figure 1). It is the tropical region with a prominent seasonal monsoon. The south-west monsoon causes heavy rainfall from May to November. The north-east monsoon follows, and under its influence rains subside and a cool season develops, in which the weather is dry until February. The premonsoon ensues with increasing rain as the season progresses (March-April). Monsoon duration and intensity vary annually and regionally. Annual rainfall typically exceeds 1,200 mm, with greater amounts in the east and south (Jones *et al*., 2000). Five study locations in four water resources were selected for this study. All of them were different in terms of the usefulness of the water at each water resource. The four localities are located in Chiang Mai Province, northern Thailand and were a fish pond, two locations of Houy Yuak Reservoir, one of which was at the open water and the other was at the small pond, the reservoir of Mae Kuang Udomtara Dam, and the last locality was the Sakon Nakhon sewage oxidation pond, located in Sakon Nakhon Province, north-eastern Thailand. All these locations were the study sites for collecting samples for phytoplankton study, analysis of physico-chemical and some biological parameters of water and sediment and microcystin analysis, twice a season over three seasons from the rainy season in August 2003 to the summer in May 2004. The surface bloom scum in the prawn pond in Chiang Rai Province was collected only one time in March 2004 for microcystin analysis to compare microcystin variants and amounts in the scum with those from other study sites. Physico-chemical parameters of water and sediment were not investigated at the prawn pond (Figures 1, 2 and Table 1).

All samples were randomly collected from three points at approximately thirty centimeters under the water surface, then samples from the three points were

homogenously mixed and were then sepearated as three subsamples to be analysed in triplicate.

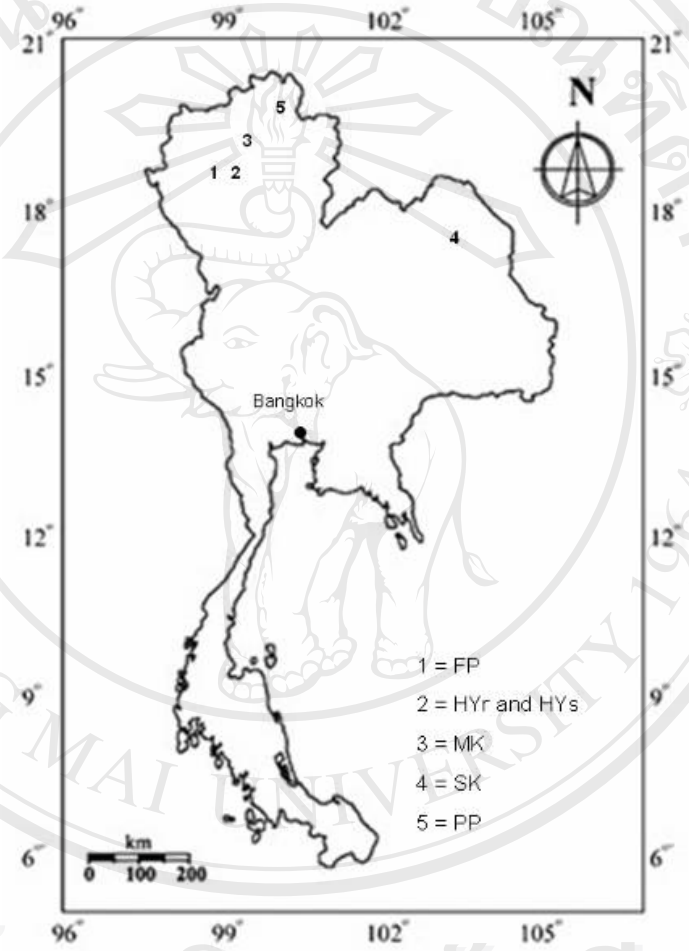


**Figure 1** Map of study water resource sites in northern and north-eastern Thailand. The fish pond (FP), the open water of Houy Yuak Reservoir (HYr), the small pond of Houy Yuak Reservoir (HYs), the reservoir of Mae Kuang Udomtara Dam (MK), Sakon Nakhon sewage oxidation pond (SK) and a prawn pond (PP).

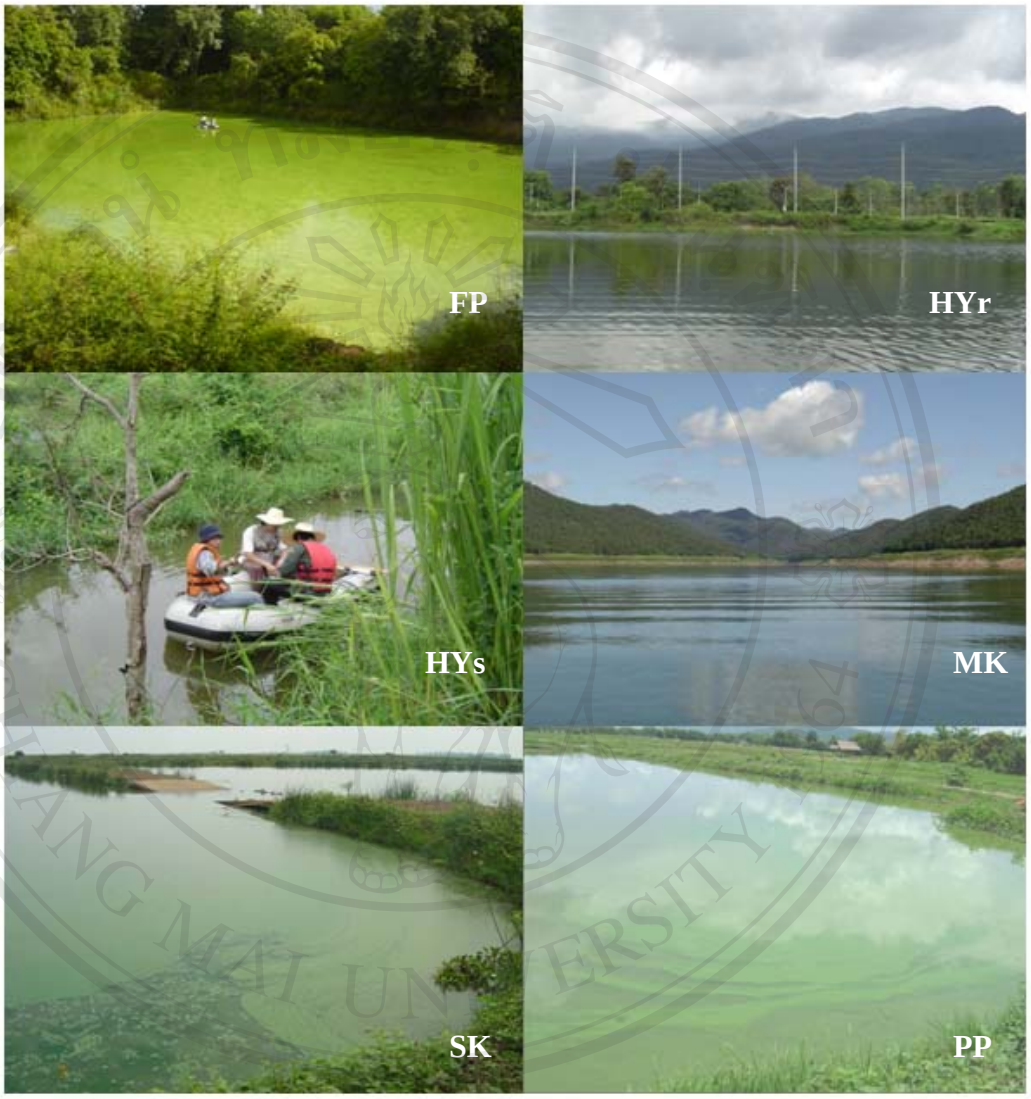


**Figure 2** The areas of six study locations in five water resources in northern and north-eastern Thailand. The fish pond (FP), the open water of Houy Yuak Reservoir (HYr), the small pond of Houy Yuak Reservoir (HYs), the reservoir of Mae Kuang Udomtara Dam (MK), Sakon Nakhon sewage oxidation pond (SK) and a prawn pond (PP).

**Table 1** Basic information of six study localities in five water resources in northern and north-eastern Thailand.

| Water resource | Code | Location | Elevation (m) | Approximated water surface area ($m^2$) | Approximated average water volume ($m^3$) |
|---|---|---|---|---|---|
| Fish pond | FP | N18° 43’ 053” E 098° 55’ 039” | 332 | 2,772 | 4,796 |
| Houy Yuak Reservoir (open water) | HYr | N18° 49’ 051” E 098° 58’ 010” | 329 | 62,500 | 181,250 |
| Houy Yuak Reservoir (small pond) | HYs | N18° 49’ 051” E 098° 58’ 010” | 329 | 70 | 61 |
| Reservoir of Mae Kuang Udomtara Dam | MK | N 18° 56’ 054” E 099° 07’ 077” | 359 | 11,800,000 | 83,720,500 |
| Sakon Nakhon sewage oxidation pond | SK | N17° 09’ 612” E 104° 09’ 771” | 144 | 40,000 | 51,200 |
| Prawn pond | PP | N 19° 40’ 044” E 100° 19’ 088” | 362 | 10,000 | 20,000 |

## 3.2 Phytoplankton study

### 3.2.1 Sampling of phytoplanktons

Sampling of phytoplankton samples for density determination and species identification was collectively done by mixing from three points of each waterbody. A ten-litre-water-sample collected around 30 cm under the water surface from each waterbody was filtered to yield about 100 ml using a plankton net (mesh size = 10 micrometers) and was preserved with 1% by volume of Lugol’s solution.

### 3.2.2 Identification and biovolume study

3.2.2.1 Identification of phytoplankton species was carried out (e.g. Prescott, 1970; Huber-Pestalozzi, 1955, 1968, 1983, Komarek and Anagnostidis, 1999 and Peerapornpisal, 2005) including those relating to the identification of tropical phytoplanktons.

3.2.2.2 Cell or filament count of all phytoplanktons was done by precipitating the samples and observing them under an inverted microscope using the method of Utermöhl,1958.

3.2.2.3 Cells of *Microcystis aeruginosa* are packed in an irregular colony and covered with a gelatinous sheath. They therefore could be separated with an ultrasonic bath (Bransonic® model 2210, frequency 47 kHz ±6%) and were then counted using a counting chamber slide or haemacytometer and calculated as the number of cells $ml^{-1}$ for biovolume estimation.

3.2.2.4 Each phytoplankton biovolume of each species was measured and calculated by Microsoft Excel.

## 3.3 Environmental factor study

### 3.3.1 Physico – chemical parameters of water at the sampling sites

3.3.1.1 Analysis of some physico-chemical parameters were done at the sampling stations. The depth of waterbodies and Secchi depth, depth of the water to which the sunlight could get through, was measured with a Secchi disc.

3.3.1.2 Water temperature was measured with a thermometer and pH was measured with a pH-meter (WTW® model pH 330).

3.3.1.3 The electro-conductivity was measured with a conductivity meter (WTW® model LF 330).

3.3.1.4 Dissolved oxygen (DO) was measured by Azide modification method by APHA, AWWA and WEF, 1992.

### 3.3.2 Laboratory investigation of chlorophyll-a, total bacterial plate count and some chemical parameters of water samples

3.3.2.1 Chlorophyll a was measured by the method of ISO 10260.

3.3.2.2 Total bacterial plate count was analysed by APHA, AWWA and WEF, 1992.

3.3.2.3 Alkalinity was measured by methyl orange indicator method by APHA, AWWA and WEF, 1992.

3.3.2.4 $BOD_5$ was measured using Azide modification method according to APHA, AWWA and WEF, 1992.

3.3.2.5 Soluble reactive phosphorus or available phosphorus (orthophosphate ion: $H_2PO_4^-$ and $HPO_4^{2-}$) was measured by Bray II method (Bray and Kurtz, 1945) and Molybdenum blue method (Murphy and Riley, 1962).

3.3.2.6 Nitrate-nitrogen was measured by Salicylic acid method (Anderson and Ingram, 1993). Ammonium-nitrogen was measured by Salicylate-hypochlorite method (Baethgen and Alley, 1989).

3.3.2.7 The trophic level of the water resource was classified using some parameters which included the chlorophyll-a level and Secchi depth, according to Lorraine and Vollenweider (Lorraine and Vollenweider, 1981) as well as chlorophyll-a content and dominant phytoplankton species, according to Wetzel (Wetzel, 1983). In addition, the AARL-CMU Scores were applied for classification of the trophic level of water resource. The dominant phytoplanktons were used for classification of the trophic level according to AARL-PP score (Peerapornpisal, 2006). Some parameters which were the electro-conductivity, DO, $BOD_5$, nitrate-nitrogen, ammonium-nitrogen, soluble reactive phosphorus and chlorophyll-a, also were used for classification of the trophic

level according to AARL-PC score (Peerapornpisal, *et al*. 2004) as described in the APPENDIX A.

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### 3.3.3 Laboratory analysis of sediment samples

3.3.3.1 About 2 kilograms of each sediment sample were collected from one point of each water resource at the same time with sampling phytoplankton samples, using a grab sampler.

3.3.3.2 The bacteria in sediments were analysed by total plate count method (APHA, AWWA and WEF, 1992)

3.3.3.3 Sediment pH and texture were measured according to Soil Survey Division Staff (1993).

3.3.3.4 Organic matter content were measured according to the wet oxidation method (Soil Survey Division Staff, 1993).

3.3.3.5 Soluble reactive phosphorus or available reactive phosphorus (orthophosphate ion: $H_2PO_4^-$ and $HPO_4^{2-}$) was measured by Bray II method (Bray and Kurtz, 1945) and Molybdenum blue method (Murphy and Riley, 1962).

3.3.3.6 Nitrate-nitrogen was measured by Salicylic acid method (Anderson and Ingram, 1993). Ammonium-nitrogen was measured by Salicylate-hypochlorite method (Baethgen and Alley, 1989).

## 3.4 Microcystin analysis

### 3.4.1 Microcystins in cyanobacterial scum

Lyophilized cyanobacterial scum samples from five study locations in four water resources in northern and north-eastern Thailand including the prawn pond were extracted with methanol and vortex mixed at room temperature for 3 minutes and sonicated for another 4-minute period. The samples were then centrifuged at 2,000X g for

2 minutes and filtered through a 0.45 µm filter (Chrom Tech ®). The supernatants were injected into the HPLC system. The purity was confirmed by Water ® 996 photodiode array detector. The column was a C 18 Water ® Symmetry ® C18 column (3.9 mm internal diameter x 150 mm, 5µm particle size) maintained at 40 °C, with a mobile phase of acetonitrile/Milli-Q water, using a linear gradient from 25% acetonitrile to 75% acetonitrile over 45 minutes. Chromatograms were monitored at 238 nm for indentification and quantification of the microcystins. (Lawton *et al*., 1994; Metcalf, *et al,* 2000a; 2000b). The HPLC detection limit was 10 ng in 25 µl of injection in column.

Microcystin-LR used in this study was purified from lyophilized *Microcystis* PCC 7813 as described by Lawton *et al*. (1995).

### 3.4.2 Microcystins in water

Ten litres of each water sample collectecd around 30 cm under the water surface from five study locations in four water resources in northern and north-eastern Thailand apart from the prawn pond, were filtered through a GF/C disc under vacuum, then the water was cleaned up by solid phase extraction (SPE). C18 solid phase extraction cartridges (Sep-Pak® Cartridges, Waters Corparation, Milford, Massachusetts, USA) were prepared by conditioning them with 10 ml methanol followed by 20 ml deionised water and taking care to ensure that the cartridges did not become dry at any time. The methanol and water eluates were discarded. The filtered water samples were then flown through the cartridge by peristatic pump. After that, the cartridges were washed with 20 ml of 30% methanol. The cartridges were then eluted with 3 ml of 80% methanol. These were collected in a glass tube and dried on a hot block (45° C) under a gentle stream of nitrogen gas. Samples were resuspended in 80% methanol and filtered by a 0.45 µm filter (Chrom Tech ®). The supernatants were injected into the HPLC system for indentification and quantification of the microcystins.

### 3.4.3 Microcystins in sediment

The sediment samples were collected in Loch Rescobie in Scotland, UK firstly for the experiments of microcystin extraction from the sediment to compare the effects of solvents and application of microwave plus untrasonication on microcystin extraction from the sediment. Secondly, for the experiments of microcystin sorption onto sediment to study the characterization of microcystin sorption onto the sediment.

#### 3.4.3.1 Microcystin-LR extractions from Scottish sediment

The 100 g dry weight of freeze-dried sediment collected from Loch Rescobie, Scotland, was suspended in 500 ml Milli-Q water and was then double sterilised by autoclave. After that it was homogeneously mixed by magnetic stirrer for 1 hr. An amount of 0.5 ml of the suspended sediment was divided into each Eppendorf and was then spiked with 10 µl of 5 µg $ml^{-1}$ microcystin-LR. The samples were vortex mixed for 1 minute and were shaken for 1 hr. After that, the samples were centrifuged at 16,000 rpm for 10 minutes and the supernatants were discarded. Whilst, the pellets were resuspended with 1 ml of each solvent (Steriled Milli-Q water, methanol, 0.1%TFA in methanol, 5% acetic acid in 0.1% TFA-methanol and steriled Milli-Q water with 5 minute microwave exposure plus 1 minute sonication). They were then vortex mixed for 1 minute and was then shaked for 1 hr. The samples were centrifuged at 16,000 rpm for 10 minutes. The supernatants were analysed by ELISA (Metcalf *et al*., 2000a; 2000b). The negative control and the positive control also were done with no spiking microcystin-LR in sediments and spiking microcystin-LR in sterilised Milli-Q water, respectively.



#### 3.4.3.2 Microcystin-LR sorption onto Scottish sediment

(1). Microcystins in sterilised and non-sterilised environmental sediments

The 100 g wet weight of sediment sample collected from Loch Rescobie, Scotland, was resuspened in 0.5 l Milli-Q water and was homogeneously mixed. The sediment was then divided by 50 ml into each 100 ml flask for 6 flasks. Three sample flasks were double sterilised with the autoclave to stop and prevent any biodegradation of microcystins by bacteria in the sediment. Both sterilised and non-sterilised sediment samples were mixed by being magneticly stirred for 6 hrs. During the mixing, 1 ml of sample was taken at 0 minute, 1 minute, 10 minutes, 1 hr and 6 hrs for centrifugation to separate the supernatant and sediment. The supernatants were analysed for total microcystins by ELISA (Metcalf *et al*., 2000a; 2000b).

(2). The effect of microcysin-LR concentration on microcystin sorption onto the sediment

The 100 g wet weight of sediment sample collected from Loch Rescobie, Scotland, was resuspened in 0.5 l Milli-Q water and was homogeneously mixed. The suspension of sediment was double sterilised with the autoclave to stop and prevent any biodegradation of microcystins by bacteria in sediment. Two ml of 3 microcystin-LR concentrations which were 1, 2 and 3 μg $ml^{-1}$, and were then spiked into the samples which were subjected to overnight shaking. The samples were centrifuged with 16,000 rpm for 10 minutes. The supernatants were freeze-dried then were resuspended with 2 ml 70% methanol and were then centrifuged. The supernatants were filtered by a 0.45 μm filter and then were injected into the HPLC system for indentification and quantification of the microcystins. The control also was done with no spiking microcystin-LR.



(3). The effect of sediment concentration on microcystin-LR sorption onto sediment

Amount of 1.25, 2.50 and 5.00 g dry weight of freeze-dried sediment samples collected from Loch Rescobie, Scotland, were suspended in 25 ml steriled Milli-Q water and were double sterilised with the autoclave and after that were homogeneously

mixed by magnetic stirrer for 1 hr. The suspended sediment was then divided by 0.5 ml into each Eppendorf. An amount of 10 µl microcystins-LR 5 µg $ml^{-1}$ was spiked into the sediment Eppendorf. The sediment Edppendorf was then vortex mixed for 1 minute and shaken overnight. The samples were centrifuged at 16,000 rpm for 10 minutes and the supernatants were analysed by ELISA (Metcalf *et al*., 2000a; 2000b). The negative control and the positive control also were done with no spiking microcystin-LR in sediments and spiking microcystin-LR in sterilised Milli-Q water, respectively.

3.4.3.3 Microcystin-LR sorption onto Thai environmental sediment samples

The 2 g dry weight freeze-dried sediments collected from five study locations in four water resources in northern and north-eastern Thailand apart from the prawn pond, were suspended in 10 ml Milli-Q water and were then double sterilised by autoclave after that they were homogeneously mixed by magnetic stirrer for 1 hr. The 0.5 ml of the suspended sediment was divided into each Eppendorf and was then spiked with 10 µl of 5 µg $ml^{-1}$ microcystin-LR. The samples were vortex mixed for 1 minute and were shaken for 1 hr. After that, the samples were centrifuged at 16,000 rpm for 10 minutes. The supernatants were analysed by ELISA (Metcalf *et al*., 2000a; 2000b). The negative control and the positive control also were done with no spiking microcystin-LR in sediments and spiking microcystin-LR in sterilised Milli-Q water, respectively.

3.4.3.4 Total microcystins in Thai environmental sediment samples

The 2 g dry weight freeze-dried sediments collected from five study locations in four water resources in northern and north-eastern Thailand apart from the prawn pond, were suspended in 10 ml Milli-Q water and were then double sterilised by autoclave after that they were homogeneously mixed by magnetic stirrer for 1 hr. The 0.5 ml of the suspended sediment was divided into each Eppendorf and was then vortex

mixed for 1 minute and was shaken for 1 hr after that the samples were centrifuged at 16,000 rpm for 10 minutes. The supernatants were analysed by ELISA (Metcalf *et al*., 2000a; 2000b) for total microcystin (A). Whilst, the pellets were resuspended with 1 ml steriled Milli-Q water, they were then vortex mixed for 1 minute and then shaken for 1 hr. The samples were centrifuged at 16,000 rpm for 10 minutes. The supernatants were analysed by ELISA (Metcalf *et al*., 2000a; 2000b) for total microcystins (B). Total microcystins in sediment was the sum of total microcystins (A) and (B).

### 3.5 Analysis of correlations among environmental factors, phytoplankton species composition and microcystins

The Multivariate Statistical Package (MVSP) was used for statistical analysis. Principle Component Analysis (PCA) was used for analysis of similarity of environmental factors among water resources and seasons so the physico-chemical parameters (water volume, depth, temperature, conductivity, pH, DO, $BOD_5$, Secchi depth, alkalinity, nitrate-nitrogen, ammonium-nitrogen and soluble reactive phosphorus) and some biological parameter (chlorophyll a) were used as the variables. Detrended Correspondence Analysis (DCA) was used for analysis of similarity of the species composition among water resources and seasons so the species number and distribution were used as the variables. The correlations among environmental factors and species composition in all localities of each waterbody were done by Canonical Correspondence Analysis (CCA) which uses the same basic algorithm as CA (Correspondence Analysis), but the ordination is constrained to fit to a second data matrix containing environmental factors. The second matrix must have the same number of observations (rows) as the species data, but need not have the same number of variables or columns (Shaw, 2003).

All study scopes which were done in each study site of this study were shown in the Table 2.

**Table 2** Conclusion of study scopes which were done in each study site. (√) represents the study scope which was done.

| Study scope / Site | Phytoplankton study | | | Environmental factor analysis | | Microcystin analysis | | | Multiple-correlation analysis | Duration of Sampling |
|---|---|---|---|---|---|---|---|---|---|---|
| | Species identification | Cell/ filament Counting | Biovolume analysis | Water quality | Sediment quality | Microcystin in scum | Microcystin in water | Microcystin in sediment | | |
| Fish pond | √ | √ | √ | √ | √ | √ | √ | √ | √ | August 2003 – May 2004 |
| Houy Yuak Reservoir (Open water) | √ | √ | √ | √ | √ | √ | √ | √ | √ | August 2003 – May 2004 |
| Houy Yuak Reservoir (Small pond) | √ | √ | √ | √ | √ | √ | √ | √ | √ | August 2003 – May 2004 |
| The reservoir of Mae Kuang Udomtara Dam | √ | √ | √ | √ | √ | √ | √ | √ | √ | August 2003 – May 2004 |
| Sakon Nakhon sewage oxidation pond | √ | √ | √ | √ | √ | √ | √ | √ | √ | August 2003 – May 2004 |
| Prawn pond | - | - | - | - | - | √ | - | - | - | March 2004 |