CHAPTER 3
MATERIALS AND METHODS

3.1 Distribution of *Microcystis aeruginosa* and microcystins in prawn and fish ponds.

3.1.1 Studied sites

Ten aquaculture ponds (Table 3.1) of similar size were randomly selected. Four prawn ponds were located in Thoeng District, Chiang Rai Province (Figure 3.1). Four fish ponds were located in Pan District, Chiang Rai Province and two other fish ponds were located at Sansai District, Chiang Mai Province (Figure 3.2 – 3.3).

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Table 3.1 Location, size and type of feed of experimental ponds

Type of organism	Number of Ponds	Location	Pond size (m^2)	Type of feed
Giant freshwater prawn	4	Thoeng District, Chiang Rai	4,800	Commercial feed
Nile tilapia	6	Pan District, Chiang Rai and Sansai District, Chiang Mai	3,200	Commercial feed

3.1.2 Sampling of *Microcystis aeruginosa* and microcystins in prawn and fish ponds.

3.1.2.1 Sampling of water, prawn and fish for analysis.

Twenty liters of water samples from each pond were taken through plankton net (mesh size 10 μm). Phytoplankton were then collected and preserved by adding 0.7 mL of Lugol's solution to 100 mL of sample (Greenberg *et al.*, 1992). One liter of water sample from each pond was collected for some water quality and microcystins analysis.

Figure 3.1 Prawn ponds, Thoeng District, Chiang Rai Province

A) Pond No. 1 sampling site altitude 19° 40′ 010″ N, 100° 19′ 088″ E (P1)

B) Pond No. 2 sampling site altitude 19° 39′ 711″ N, 100° 19′ 014″ E (P2)

C) Pond No. 3 sampling site altitude 19° 40′ 533″ N, 100° 18′ 042″ E (P3)

D) Pond No.4 sampling site altitude 19° 39′ 118″ N, 100° 19′ 213″ E (P4)

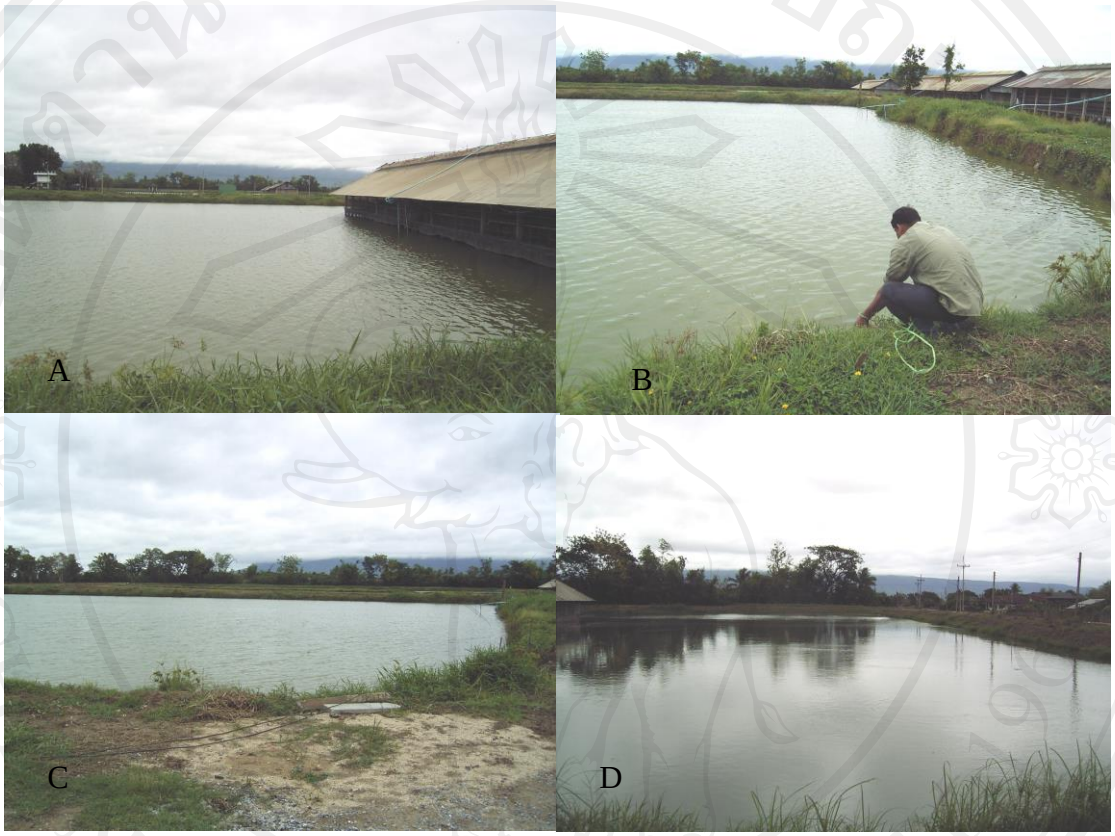

Figure 3.2 Fish ponds, Pan District, Chiang Rai Province

A) Pond No. 1 sampling site altitude 19° 50′ 014″ N, 99° 48′ 079″ E (T1)

B) Pond No. 2 sampling site altitude 19° 51′ 029″ N, 99° 47′ 808″ E (T2)

C) Pond No. 3 sampling site altitude 19° 51′ 622″ N, 99° 48′ 070″ E (T3)

D) Pond No.4 sampling site altitude 19° 50′ 039″ N, 99° 47′ 055″ E (T4)

Figure 3.3 Fish ponds, Sansai District, Chiang Mai Province
Upper) Pond No. 1 sampling site altitude 19° 56′ 014″ N, 99° 7′ 079″ E (T5)
Lower) Pond No. 2 sampling site altitude 19° 56′ 029″ N, 99° 7′ 808″ E (T6)

Three tilapias and giant freshwater prawns of marketable size were collected from each pond and stored in ice box during transportation (Figure 3.4). Both water and meat samples were stored -20 °C until microcystin analysis.

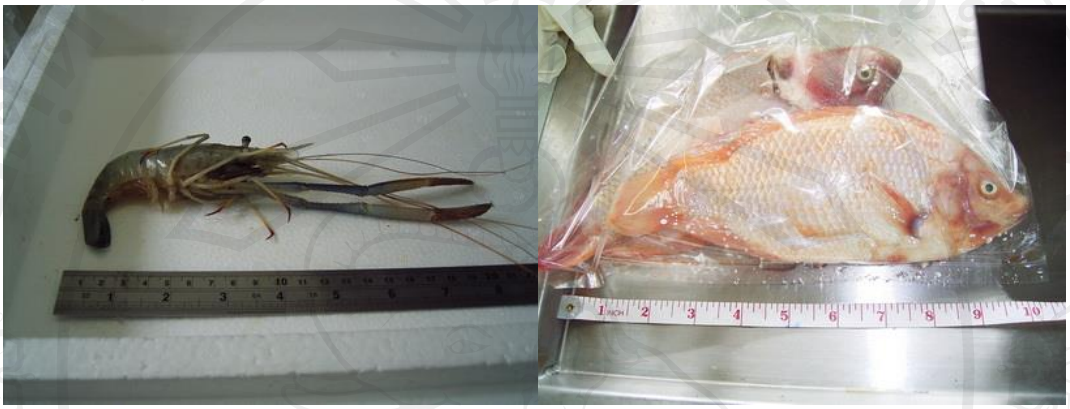

Figure 3.4 Prawn and fish samples

3.1.2.2 Determination of some physico-chemical properties of water.

Some water quality parameters were measured in the field and laboratory. The water samples were stored in polyethylene bottles and kept in ice box.

The parameters which were measured in the field were water temperature, pH, conductivity and dissolved oxygen.

i) Temperature was measured with thermometer.

ii) pH was measured with a pH meter (WTW).

iii) Conductivity meter, WTW. was used for measuring conductivity.

iv) Dissolved oxygen was measured by iodometric method type azide-modification (Greenberg *et al*., 1992).

Other parameters i.e. nitrate-nitrogen, ammonium nitrogen and soluble reactive phosphorus, were measured in the laboratory.

v) Nitrate-nitrogen was determined by cadmium reduction method (Greenberg *et al*., 1992)

vi) Ammonium nitrogen was determined by nesslerization method (Greenberg *et al*., 1992).

vii) Soluble reactive phosphorus was determined by ascobic acid method (Greenberg *et al*., 1992).

3.1.2.3 Identification and counting of *M. aeruginosa* and phytoplankton.

Morphological classification of blue green algael samples were done under compound microscope (Olympus model CH30RF200). The character and color of each colony, appearance of sheath, differentiation, arrangement, shape and dimension of cells and filaments were used for identification.

The identification of phytoplankton species was carried out using related texts such as Komárek and Komáková-Legnerová (2002a), Komárek and Komáková-Legnerová (2002b), Komárek and Anagnostidis (2005) and Hindak (2008). Phytoplankton quantity was evaluated by using whole count technique. Cells of *M. aeruginosa* were counted with a haemacytometer. Biovolume of phytoplankton was evaluated according to Rott (1981).

3.1.2.4 Analysis of microcystins

I. Extraction of microcystins

Microcystins were extracted after Kankaanpää *et al.* (2005) with modification. Fish and prawn tissues were dissected and freeze-dried at -20 °C in 24–72 hours before extraction and ELISA analysis.

One mL of 100% methanol was added into 2-5 g fish and prawn tissues for extraction overnight. The extracts were centrifuged at 12,000 rpm for 30 min and the supernatants were concentrated to 150 µl with a heat block (50 °C, overnight), and centrifuged at 12,000 rpm for 30 min before ELISA analysis.

Microcystin content of *Microcystis* and water sample were extracted by freeze-thaw method for 3 times and then centrifuged at 14,000 x g for 5 minutes. The supernatant was also analysed by ELISA (Albay *et al*., 2003).

II. Microcystin analysis by ELISA assay

ELISA Microcystin Plate Kit (Catalog No. EP022), ENVIROLOGIX INC© was used and performed in accordance with the manufacturer's instructions. A standard curve was constructed using three calibrators (0.16, 0.5 and 2.5 µg/L, respectively) supplied with the kit. The absorbance at 450 nm was measured with a

microplate reader (Spectra MR, DYNEX Technologies). The microcystin concentration in each extract was expressed as MC-LR equivalent.

3.2 The accumulation of microcystins in fish and prawn samples in demonstrated ponds

The study was carried out at Faculty of Fisheries Technology and Aquatic Resources, Maejo University. Nile tilapia (*Tilapia nilotica*) and giant freshwater prawn (*Macrobrachium rosenbergii*) samples was cultivated in two typed of demonstrated ponds.

A) Earthen pond 7.0 x 14.2 square meters with three pens (1x2.5 square meters were constructed at the center of each pond with water depth of 0.80 meter) (Figure 3.5).

Figure 3.5 Earthen ponds with pens

B) Cement ponds were used after the promoting the mass of *Microcystis* in earthen pond was unsuccessful. Nile tilapia about 5 cm in size and giant freshwater prawn about 5-7 cm in size were obtained from the Faculty of Fisheries Technology and Aquatic Resources, Maejo University. The fish were cultured in 3 cement ponds, 1.5 m x 1.5 m and the depth of 0.50 m containing green water 0.30 m deep, 30 fish in

each pond. Feeding treatments were; 1) Green water system (Tr.1). 2) Green water system with 18-30x10^6 cells.L^{-1} *M. aeruginosa* from natural pond and combined with commercial pellet feed (Tr.2). 3) Green water system with 18-30x10^6 cells.L^{-1} *M. aeruginosa* (Tr.3)

The prawns were cultured in two cement ponds of similar size, 30 prawns were in the pen (0.45 m x 0.45 m with water depth of 0.30 m) made of blue net (mezh size 2 mm) attached to the pond (type A), 30 prawns were outside the pen (type B). Feeding treatments were: Green water system (Tr.1) and green water system with *M. aeruginosa* combined with commercial pellet feed (Tr.2) (Figure 3.6).

Figure 3.6 Demonstrated ponds with pens

Completely randomize design (CRD) with duplicate treatments were carried out. Both fish and prawn were cultured for 2 months. Water samples were collected every two weeks to determine the amount of *M. aeruginosa,* phytoplankton and microcystins.

3.2.1 Identification and Enumeration of *M. aeruginosa* and Phytoplankton

Morphological classification of *Microcystis* spp. and phytoplankton were done under compound microscope (Olympus model CH30RF200) using related texts such

as Komárek and Komáková-Legnerová [12] and Hindak [13]. Cells of *M. aeruginosa* were counted on a haemacytometer.

3.2.2 Analysis of Microcystins

M. aeruginosa cells and microcystin contents were analyzed according to the methods in 3.1.2.4. Fish and prawn samples from the beginning and end of experiment were collected for microcystin analysis.

Summarized flow chart was shown in Figure 3.7.

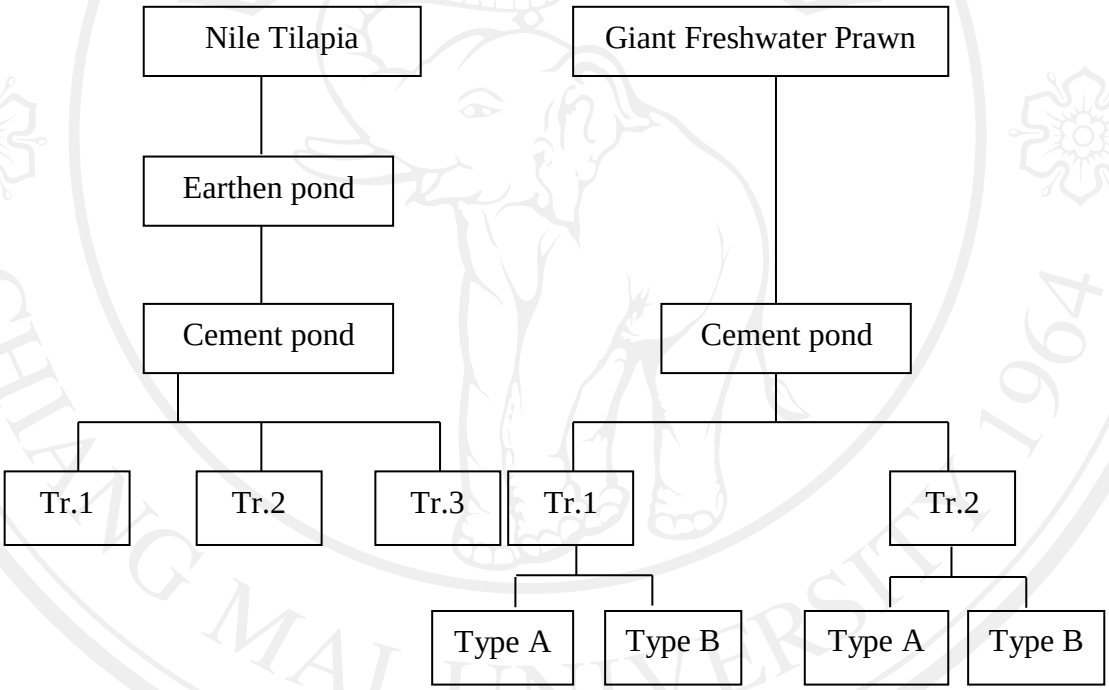

Figure 3.7 Flow chart of the study on accumulation of microcystins in fish and prawn samples in demonstrated ponds

3.3 Controlling of *M. aeruginosa* and microcystins by using Effective Microorganisms (EM)

Water sample with high concentration of *M. aeruginosa* was added in 2 liters bottle (Figure 3.8). Then commercial EM was added to the bottle. Three concentrations of EM (0.3, 0.5 and 1.0 $ml.l^{-1}$) of two commercial EM were compared with control treatment for the ability to eliminate *M. aeruginosa* and microcystins.

The samples were collected at the beginning and end of experiment. *M. aeruginosa* and microcystins were analyzed according to the methods in 3.1.2.3 and 3.1.2.4 respectively.

Figure 3.8 High concentration of *Microcystis aeruginosa*.

3.4 Data evaluation

The water quality was classified into trophic level according to the criteria of Wetzel (2001); Lorraine and Vollenweider (1981) and Surface Water Quality Standards of National Environmental Board, Thailand (1994) and Peerapornpisal *et al*. (2007) by considering some physico-chemical parameters and dominant species of phytoplankton (Appendix A).

The computer statistical package, SPSS for Windows version 14.0 will be used to perform statistical analysis of the results.