CHAPTER 5
DISCUSSION

5.1 Distribution of *Microcystis aeruginosa* and microcystins in prawn and fish ponds in Chiang Rai and Chiang Mai provinces.

5.1.1 *M. aeruginosa,* microcystin producing blue green algae and some physico-chemical properties of water.

The water qualities ranged from clean- moderate (oligo-mesotrophic status) to moderate-polluted (meso-eutrophic status). The nutrients in both ponds decreased slightly in July 2006 because of rainy season.

High amount of *Microcystis* spp. especially, *Microcystis aeruginosa* Kützing was found in prawn ponds. Other blue green algal taxa which are known to be MC producing genera were also found in this investigation. They were *Anabaena* sp., *Cylindrospermopsis curvispora* Watanabe, *M. wesenbergii* Komárek, *M. ichthyoblabe* Kützing, *M. flos-aquae* (Wittrock) Kirchner ex Forti and *Oscillatoria* sp. Among toxic blue green algae, *Microcystis aeruginosa* is the most frequently bloom. *M. aeruginosa* which was the most abundant species, was found in various sampling sites because of their assistance properties supported their growth (Mur *et al*., 1999). Similar to Falconer (2005) reported that *M. aeruginosa* has been recorded worldwide. Its distribution ranges from cold, temperate climates to tropical environments.

M. aeruginosa is often dominant under nutrient-rich conditions, especially where there is a significant supply of ammonia, although it also forms blooms in less polluted water (Sivonen and Jones, 1999). It was found that the presence of *Microcystis* could not be related strictly to the level of eutrophication. This genus is found in mesotrophic, eutrophic and hypertrophic waters or even oligo-mesotrophic water (Mur *et al*., 1999). In general, blue green algae do not have as high demand for phosphorus as do other phytoplankton. They can store polyphosphate sufficient for two to four cell divisions in phosphate deficient water (Mur *et al*., 1999) and can migrate vertically to a depth where phosphate availability is higher. *Microcystis* is one of the blue green algae that can utilize these advantages, with a large capacity for

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phosphorus storage and high variable buoyancy (Falconer, 2005). This study found that the highest amounts of *M. aeruginosa* were found in July 2006 but no relationships between the cyanobacterium and environmental parameters. The occurrence of blue green algal population in various water resources did not related to physico-chemical parameters or water quality significantly. Similar to many research which had been carried out on the relationship between nutrients, phytoplankton growth and relative abundance of blue green algae, did not find the relationship between blue green algal cell and nutrients (Oliver and Ganf, 2000).

The temperature of prawn and fish ponds ranged from 29.88 ± 0.54 and 27.63 ± 1.05 ^{o}C respectively. In general, the blue green algae will bloom only above 25 ^{o}C, These optimum temperatures are higher for green algae and diatoms (Padisak, 1997). Whereas this study found green algae as dominant species in many sampling sites.

Besides, the composition of *Microcystis* spp. was different in some sites. The dominance of organisms depends not only on the weather but also on the specific geochemical conditions of the lake. In any year or season, individual water bodies have their own population of blue green algae and algae. If there are no major changes in these conditions, toxic blooms are likely to reoccur annually in those lakes that have a history of toxic blooms (Wick and Thiel, 1990; Ekman-Ekbom *et al*., 1992). Similar to the abundance of *M. aeruginosa,* it was also found in prawn pond throughout the investigation.

5.1.2 Microcystins in fish and prawn samples

Total MCs were analyzed by ELISA Microcystin Plate Kit. ELISA which are screening method, have been developed for MCs and saxitoxins and the results show good correlation with traditional methods of analysis such as high performance liquid chromatography (HPLC). Although, later studies have shown variable cross-reactivities (Chu *et al*., 1989; Mecalf and Codd, 2004) and can underestimate the concentration of some MC variants (Msagati *et al*, 2006) but the procedure has been used successfully to determine the content of toxins in environmental samples and allow detection of microcystis-LR to concentration below 1 $\mu g.l^{-1}$ which is the provisional guideline value for this toxin in drinking water as derived by the WHO

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(WHO, 2006). Analysis of MC using commercial ELISA was done by Magalhães *et al*. (2001) and Kankaanpää *et al.* (2005).

Both prawn and fish samples were found to be contaminated with MCs. The amount of MCs in the prawn samples was higher than fish samples. Although it seemed to be correlated with cell counts of *M. aeruginosa*, there was no correlation between *Microcystis* cell number and MCs in prawn and fish. In the ponds that *M. aeruginosa* was formerly present, MCs were detected in spite of no *Microcystis* cells were found at the sampling date. It means that if toxic *Microcystis* occurred in the water bodies, MCs might be found. MC molecules are found to be very stable (Falconer, 2005). Although MCs are susceptible to breakdown by aquatic bacteria found naturally in river and reservoirs, degradation of MC can be as short as two days or more than three weeks (Sivonen and Jones, 1999). Similar to Edwards *et al*. (2008) found that rate of degradation of MC-LR, LF and nodularin in water samples ranges from a half-life of four to eighteen days depending on the water body, climatic condition, and the concentration of dissolved MC and in some cases, the previous bloom history of a water reservoir. Similar to this study, the information from the prawn farm's owner shown that the bloom of *M. aeruginosa* had happened before.

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

A) Earthen pond

Promoting of *Microcystis aeruginosa* bloom by adding dry chicken manure was not successful. One year of cultivation (May 2007 – April 2008) shown that the dominant species of phytoplankton in earthen ponds were *Euglena* spp. and *Oscillatoria* spp.

Dry chicken manure at the rate of 200 kg/rai/week enriched nutrients to the pond. The condition was suitable for not only *M. aeruginosa* but also *Euglena* spp. and *Oscillatoria* spp. They could be growing under high concentration of nutrients better than *Microcystis* (John *et al*., 2002). Another factor that influenced the blooming of *Microcystis* is light. The water in earthen pond is not clear due to its turbidity. This characteristic affected the ability of light harvesting. Light availability has a strong influence on the dominant species of blue green algae and the depth at

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which they occur. Like algae, blue green algae contain chlorophyll *a* as a major pigment for harvesting light and conducting photosynthesis. They also contain other pigments such as phycobiliproteins which include allophycocyanin, phycocyanin and phycoerythrine. These pigments harvest light in the green, yellow and orange part of the spectrum (500-600 nm.), which are hardly used by other phytoplankton species (Mur *et al*., 1999). Light requirements of blue green algae vary among species (Oliver and Ganf, 2000). The growth of *Oscillatoria* is inhibited when exposed for extended periods to light intensities above 180 $\mu E.M^{-2}S^{-1}$. It has been shown to adapt to low light (Mur *et al*., 1999; Scheffer, 1998), whereas *Microcystis* photosynthesizes at optimal rates and resists photoinhibition in high surface irradiances. At high light intensities, the biomass of *Microcystis* increased rapidly but in water with high turbidity *Oscillatoria* have better chances of out-competing *Microcystis* (Sivonen and Jones, 1999).

After 3 months of cultivation (May – July 2007), *M. aeruginosa* was not discovered. Therefore the experiment for promoting the mass of *M. aeruginosa* was designed. Six treatments were created. Chicken manure at the rate 0-150 kg/rai/week were added to find out the optimum of nutrients for *Microcystis*. But the scum of *M. aeruginosa* never appear all the period of cultivation even though adding *M. aeruginosa* culture was done. *Microcystis* do not have as high demand for phosphorus as do other phytoplankton. In addition, they can store polyphosphate sufficient for two to four cell divisions in phosphate deficient water (Mur *et al*., 1999) and can migrate vertically to a depth where phosphate availability is higher by using buoyancy property (Falconer, 2005) but high light intensity also require for this ability.

Considering on phytoplankton history of the ponds was also important. The earthen ponds at Maejo University were used for the research of aquaculture in many times. The dominant phytoplankton species which had been found frequently in these ponds were green algae, euglenoids and filamentous blue green algae (Tawong, 2008; Whangchai *et al*, 2008). The blooming of euglenoids had been appeared regularly. So when the water properties were appropriate for bloom formation, *Microcystis* were not out-competed these organisms.

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B) Cement pond

5.2.1 *Microcystis aeruginosa* and phytoplankton in cement ponds

This study was conducted in short period. Nile tilapias were cultured during July – August 2007 and May – June 2008. While prawns were cultured during September – November 2008. Species compositions of phytoplankton including the numbers of *M. aeruginosa* were investigated. Dominant species of phytoplankton excluding *M. aeruginosa* were similar in each treatment because of using same green water. The water without *M. aeruginosa* was detected before adding to the ponds.

The mass of *M. aeruginosa* were added to the pond water (approximately 18 – $30x10^6$ cells.L^{-1}). After adding, high numbers of *Microcystis* could be detect easily by eyes.

Both fish and prawn cultivation, Tr. 1 has lowest amounts of phytoplankton. Tr. 2 and Tr. 3 have higher amounts because not only biomass of *M. aeruginosa* was added but also phytoplankton associated with *M. aeruginosa*. However considering the weight of aquatic organisms it was not affected their growth.

5.2.2 The growth of fish and prawn and microcystin contents in their meat

Total weight of Nile tilapia was slightly increased whereas high MC contents in fish samples which were cultured about 1 month were detected. Samples cultured in Tr. 2 and Tr. 3 which contained high amounts of *M. aeruginosa* also contained high amounts of MCs.

Total weight of giant freshwater prawn was slightly increased in Tr. 1. To study the effect of their consumption behavior on the microcystin accumulation, two types of cultivation were studied in the same pond. Due to microcystins can interact with humic and fulvic substances, suspended particulate matter or sediments. Prawns are bottom dweller. They consume feed which falls to the bottom of the pond. So if prawn are forced to avoid of feeding at the bottom by pen. It could be lower accumulating toxin. Unfortunately, promoting of *M. aeruginosa* bloom by adding dry chicken manure to earthen pond was not successful. The experiment has to conducted in cement pond which has no sediment. It was shown that MC contents from both types of cultivation were similar.

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MC contents in prawn meat were higher than fish meat. It might be caused the prawns were cultured in longer period than fish. Moreover a number of studies have demonstrated that MCs can be excreted quickly by fish. Soares (1999) showed that 48% of the total MCs ingested by *Tilapia rendalli* were eliminated by feces during a 30-day experiment.

The organ which accumulated the toxin is also important. Liras *et al*. (1998) reported that Crayfish accumulated the toxins in the hepatopancreas and were thus suspected to be able to carry the toxin further up in the food chain. The results of Kankaanpää *et al.* (2005) are similar to their findings. If extensive, toxic blue green algal blooms should emerge, prawns would accumulate a fraction of the toxins in their hepatopancreas. Whereas the objective of this research focused on the accumulation of MCs in fish and prawn meat which could be affected on human consumption directly, therefore MC contents in the hepatopancreas were not analysed.

Due to the extraction method used (100% MeOH) and detection with ELISA, this research reports MC values that are an integration of numerous forms of MC, including variants and detoxification conjugates. ELISA (EnviroLogix) has good cross reactivity with many variants of MC (Carmichael and An, 1999) and MC that has been biotransformed (detoxified) at the Mdha residue (N-methyldehydroalanine) with glutathione, cysteine–glycine, or cysteine (Metcalf *et al*., 2000). However, because MC-LR, the most potent MC variant with an LD_{50} of 50 $\mu g.kg^{-1}$ in mice, was used as the standard for this assay, results are reported as MC-LR equivalents (Kotak *et al*., 1995).

MC contents were detected from the samples of control experiment which should be MC-free.

ELISA is frequently used because of its high sensitivity. A disadvantage of ELISA is cross reactivity with detoxication products that are formed in the metabolization of toxins, like the conjugates of MC and glutathione (GSH). These conjugates have been shown to have no or a much reduced toxicity (Metcalf *et al*., 2000). In vivo, the blue green algal hepatotoxins can be metabolized by phase II detoxification enzymes in a reaction with cysteine and GSH catalyzed by GSH-S-transferase (Kondo *et al*., 1996; Pflugmacher *et al*., 1998; Wiegand *et al*., 1999). These conjugates can still be readily detected by ELISA, because their affinity to

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antibodies is not changed by conjugation with GSH, cysteine-glycine, and cysteine (Metcalf *et al*., 2000), so that ELISA measurements may give an overestimation of toxin concentrations present. Conjugates can be detected using LC-MS, but this has been carried out only in some of the studies reported (see e.g. Sipia *et al*., 2002; Karlsson *et al*., 2003). Because ELISA suffers from cross reactivity, some studies on biota in the Baltic Sea have introduced the term TEH (total extractable hepatotoxins) which includes the non-toxic or less toxic biotransformation products. TEH almost unchangeable exceeds the concentrations of untransformed hepatotoxins in biota (Ibelings and Chorus, 2007).

The amount of toxin in tilapia and prawn has been found to reach levels as high as 0.85 and 3.02 $\mu g.kg^{-1}$, respectively, so that in a typical meal an adult could be exposed to 1 and 75 of times the seasonal TDI.

5.3 Controlling of *Microcystis aeruginosa* and microcystins by using Effective Microorganisms (EM)

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 MCs. Declining of *M. aeruginosa* cells showed in similar amounts with the amounts of MCs were slightly changed. No correlation was found between concentration of EM and amounts of *M. aeruginosa* or MCs. EM may not have MC degrading organism including antialgal ability from bacteria and cyanophage. EM consists of the following five families of micro-organisms:

Lactic acid bacteria: these bacteria are differentiated by their powerful sterilising properties. They suppress harmful micro-organisms and encourage quick breakdown of organic substances.

Yeasts: these manufacture anti-microbial and useful substances for plant growth. Their metabolites are food for other bacteria such as the lactic acid and actinomycete groups.

Actinomycetes: these suppress harmful fungi and bacteria and can live together with photosynthetic bacteria.

Photosynthetic bacteria: these bacteria play the leading role in the activity of EM. They synthesize useful substances from secretions of roots, organic matter and/or

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harmful gases by using sunlight and the heat of soil as sources of energy. The metabolites developed by these microorganisms are directly absorbed into plants. In addition, these bacteria increase the number of other bacteria and act as nitrogen binders.

Fungi that bring about fermentation these break down the organic substances quickly. This suppresses smell and prevents damage that could be caused by harmful insects.

There was no document which presents that those organisms have ability to degrade MC and/or inhibit *Microcystis.* They might not have degradation pathway and genes coding the MC-degrading enzymes.

It is now known that MCs can be degraded by aquatic bacteria but only a few bacterial strains with the ability to degrade MCs have been isolated and characterized (Edwards and Lawton, 2009). They are *Sphingomonas* sp., *Pseudomonas* sp. (Jone *et al*., 1994; Isuji *et al*., 2006), *Paucibacter toxinivorans* (Rapala *et al*., 2005), *Burkholderia* (Lemes *et al.*, 2008), *Methylobacillus* (Hu *et al*., 2009), *Sphingosinicella microcystinivorans* (Maruyama *et al*., 2006*), Arthrobacter* spp., *Brevibacterium* sp. and *Rhodococcus* sp. (Manage *et al*., 2009).

Antialgal substance producing microorganisms were reported. They are *Alcaligenes denitrificans* and *Streptomyces neyagawaensis* (Salomon *et al*., 2003; Choi *et al*., 2005).

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