CHAPTER I

INTRODUCTION

Water pollution

Contamination of water resources in northern Thailand caused by domestic and/or industrial effluents and a run-off of agricultural fertilizers into water resources is a serious problem in water quality, resulting in increasing concentrations of nitrogen and phosphorus and the bloom of phytoplankton in reservoirs. This phenomena is called "eutrophication". The eutrophic water and favorable conditions such as high temperature and light intensity can promote periodic massive surface accumulations of blue-green algae or cyanobacteria (Carmichael, 1994). These cyanobacteria produce potent toxin, which can cause domestic and wild animal death (Skulberg *et al.*, 1984; Carmichael *et al.*, 1985) and hazards to human health (Codd *et al.*, 1999). Microcystins are the potent hepatotoxins primarily produced by *Microcystis aeruginosa* Kütz. (*M. aeruginosa*), the dominant toxic cyanobacteria and most widespread blue green algae in Chiang Mai's reservoirs (Peerapornpisal *et al.*, 2001). It has been reported that microcystins inhibit protein phosphatases 1 and 2A (Honkanen *et al.*, 1990; MacKintosh *et al.*, 1990) and consequently hyperphosphorylation of cellular proteins leads to cytotoxicity (Eriksson *et al.*, 1987).

Cyanobacteria

Cyanobacteria or blue-green algae are a diverse group of photosynthetic, prokaryotic organisms found in nutrient-enriched fresh, brackish and sea water throughout the world. According to the current taxonomy, 150 genera of 2,000 species, of which at least 40 are known to be toxigenic, have been identified (Chorus and Bartram, 1999). Many common cyanobacteria genera, such as *Microcystis*, *Cylindrospermopsis*, *Anabeana*, *Oscillatoria* and *Nodularia* are capable of producing potent toxins, which cause serious water quality problems (Carmichael, 1992). Occurrences of freshwater toxic cynobacteria have been reported in many places, e.g. Brazil, Canada, Europe, India, Japan, China, South Africa and the U.S.A. (Carmichael, 1989)

including Thailand (Mahakhant *et al.*, 1998; Rattanachot, 1998; Peerapornpisal *et al.*, 1999). Cyanobacteria may accumulate in surface water supplies as "blooms" and may concentrate on the surface as blue-green "scums" and "mats" (Codd, 2000).

***Microcystis aeruginosa* Kütz.**

The genus *Microcystis* has great variability in size, shape and cell arrangement of the colony, which is used to define its species and forms. This genus does not produce accessory cells such as akinetes and heterocysts. *Microcystis* is most often identified by morphology-based taxonomy, with the system of Komárek and Anagnostidis (1986) frequently used. There are five species of *Microcystis* recognized throughout the world (Watanabe *et al.*, 1996). However, the most successful and widely-found species is *Microcystis aeruginosa* Kütz.

Microcystis aeruginosa Kütz. cells have a round or oval shape. Cell diameters vary between 4.4-5.5 μm which facilitates separation into two groups: the large cell type (L-type) and small cell type (S-type). Most of these cells grow as single cells in colonies covered with a gelatinous sheath. These colonies are more or less firm, measuring up to several millimeters and amorphous in shape. There are tightly aggregated gas vacuoles within the cells appearance of yellowish or blackish under a microscope (Watanabe *et al.*, 1996).

Factors affecting cyanobacterial blooms

Cyanobacteria grow remarkably well and form water blooms when optimum conditions converge: quiet or mild wind; and tepid water (15 to 30 degree celsius) is neutral to alkaline (having a pH of 6 to 9) and harbors an abundance of nutrients nitrogen and phosphorus (Carmichael, 1994).

Light intensity Cyanobacteria contain chlorophyll *a* as a major pigment for harvesting light and conducting photosynthesis. They also contain other pigments such as phycobiliproteins, which include allophycocyanin (blue), phycocyanin (blue) and phycoerythrine (red). These pigments harvest light in the green, yellow and orange part of the spectrum (500-650 nm), which is hardly used by other phytoplankton species (Mur *et al.*, 1999).

Buoyancy Many planktonic cyanobacteria can distribute widely in water bodies because they contain gas vacuoles, the aggregates of gas-filled vesicles (Walsby, 1981). A gas

vesicle has a density of about one tenth that of water and, thus, gas vesicles can give cyanobacterial cells a lower density than water. These gas vesicles are buoyant in the water column and therefore light intensity, which are optimal for their growth, is found.

Nutrients Because cyanobacterial blooms often develop in eutrophic lakes, it was originally assumed that they required high phosphorus and nitrogen concentrations. This assumption was maintained even though cyanobacterial blooms often occurred when concentrations of dissolved phosphate were the lowest. Experimental data have shown that the affinity of many cyanobacteria for nitrogen or phosphorus is higher than that for many other photosynthetic organisms. This means that they can out-compete other phytoplankton organisms under conditions of phosphorus or nitrogen limitation (Chorus and Bartram, 1999).

In addition to their high nutrient affinity, cyanobacteria have a substantial storage capacity for phosphorus. They can store enough phosphorus to perform two to four cell divisions, which corresponds to a 4-32 fold increase in biomass. However, if total phosphate rather than only dissolved phosphate is considered, high concentrations indirectly support cyanobacteria because they provide a high carrying capacity for phytoplankton. High phytoplankton density leads to high turbidity and low light availability, and cyanobacteria are the group of phytoplankton organisms that can grow best under these conditions (Chorus and Bartram, 1999).

Temperature Maximum growth rates are attained by most cyanobacteria at temperatures above 25°C (Robarts and Zohary, 1987). These optimum temperatures are higher than those for other phytoplankton. This can explain why most cyanobacteria bloom in temperate and boreal water bodies during the summer.

***Microcystis aeruginosa* Kütz blooms**

M. aeruginosa blooms have been reported both in fresh, brackish and sea water, e.g. Alberta lakes and farm dugouts in Canada (Kotak *et al.*, 1993), Wisconsin lakes in the U.S.A. (Repavish *et al.*, 1990), Portuguese freshwaters (Vasconcelos *et al.*, 1996), Jacarepaguá Lagoon in Brazil (Magalhães *et al.*, 2001), Hartbeespoort Dam in South Africa (Wick and Thiel, 1990), Lake Ouberia in East Algeria (Nasri *et al.*, 2004), the Murray river in Australia (Hayes and Burch, 1989), Daechung reservoir in Korea (Oh *et al.*, 2001), Taihu Lake, Haimen and Fusui in

China (Shen *et al.*, 2003; Ueno *et al.*, 1996), Kasumigaura lake in Japan (Takamura and Yasuno, 1984) and Laguna de Bay in the Philippines (Cuvin-Aralar *et al.*, 2002).

Surveying and monitoring of *Microcystis aeruginosa* Kütz. is being carried out in Thailand, and microcystins are important water resources for public water supplies in several parts of the country.

Khlongdee (1991) reported that *M. aeruginosa* was the dominant species of algae in a reservoir at Chiang Rai Water works during April-October 1990. The maximum quantity of algae was found in May when there was a high level of nutrients.

Mulsin (1997) found a dominant species of toxic algae, which was *M. aeruginosa*, in the reservoir at Mae Kuang dam, Chiang Mai province during August 1995-July 1996. The toxic algae's growth correlated with the amount of total phosphorus in the winter. Peerapornpisal *et al.* (1999) also found a proliferation of *M. aeruginosa* in this reservoir throughout their investigation during July 1996-January 1998. The factor affecting the proliferation was the amount of soluble reactive and total phosphorus.

Mahakhant *et al.* (1998) investigated the microcystin content and composition in water blooms collected from Kang Krachan dam in Phetchaburi province, Mae Kuang dam in Chiang Mai province and a husbandry pond in Bangkok. The dominant species of cyanobacteria found was *M. aeruginosa.* The water blooms contained 0.7-0.8 mg of microcystin.g^{-1} dried sample. Microcystin-LR and –RR were found as the major microcystins in these reservoirs.

Proongkiat (1999) found the phytoplankton, *M. aeruginosa*, in the reservoir at Mae Ngat Somboonchol dam, Chiang Mai province during August 1997-July 1998.

Prommana (2000) studied the diversity of toxic algae in planktonic form in some water resources of wetland, in Chiang Mai and the Lumphun basin during June-December 1999. Four species of toxic algae were found, in the Division Cayanophyta and the most abundant was *M. aeruginosa.*

Peerapornpisal *et al.* (2002) studied the toxic algae, *Microcystis aeruginosa* Kütz., in Mae Kuang Udomtara Reservoir, Chiang Mai province and Kwan Payao, Payao province during December 1999-May 2001. *M. aeruginosa* was found throughout the investigation. A high amount of microcystin was found in microcystin-RR followed by microcystin-LR and –YR, but it was lower than the WHO guideline concentration for drinking water (1 $\mu g.L^{-1}$).

Pekkoh (2002) found blooming of the toxic algae, *M. aeruginosa*, in the reservoir at Mae Kuang Udomtara Dam, Chiang Mai province during April 1999-September 2000. Microcystin-RR was found in the highest amount (0.15 $ng.L^{-1}$), but it was also lower than the WHO guideline level.

Prommana (2002) reported that *M. aeruginosa* was the dominant species with the highest number of cyanobacteria in Kwan Payao, Payao province in April 1999. This blooming correlated with the amount of total iron and water temperature.

A small amount of *M. aeruginosa* was found by Panuvanitchakorn (2003) in Lamtakong Dam, Nakhon Ratchasima province, and it increased during March 2001. The greatest presence of toxin was in microcystin-RR.

Kiatpradub (2003) found contamination of *M. aeruginosa* in Bang Phra reservoir, Chonburi province during April 2000-March 2001. Moreover, the number of *M. aeruginosa* cells correlated positively with the concentration of microcystins. The only type of microcystin detected in this reservoir was microcystin-RR.

Ngearnpat (2003) found a very low number of *M. aeruginosa* cells (0-16 $cells.mL^{-1}$) in Nong Han Reservoir, Sakol Nakhon province during April 2000-March 2001. Two types of microcystins were found, microcystin-RR and –LR at concentrations of between 1.6-40.4 $ng.L^{-1}$.

Kiatpradub (2003) found a massive growth of the blue-green algae, *M. aeruginosa*, in Huay Yuak reservoir, Chiang Mai province during November 2001-March 2002. The blooming of this algae showed a positive correlation with the temperature and ammonium nitrogen in this reservoir.

Ruangyuttikarn *et al.* (2004) detected microcystin-LR as the major toxin in cyanobacterial blooms in Huay Yuak reservoir, Chiang Mai province in September 2001. The cyanobacterial cells contained 1.86 mg of microcystin-$LR.g^{-1}$ dried cells.

Microcystins

Microcystins are classified as a family of toxins produced by the species of freshwater cyanobacteria, primarily *M. aeruginosa*, and also other *Microcystis* species and other genera, namely *Anabaena*, *Oscillatoria* and *Aphanizomenon*. Microcystins were formerly known as "the fast death factor" or "cyanoginosin" (Carmichael *et al.*, 1988). They are monocyclic

heptapeptides composed of D-alanine at position 1, two variable L-amino acids at position 2 and 4, γ-linked D-glutamic acid at position 6, and 3 unusual amino acids: β-linked D-*erythro*-β-methylaspartic acid (MeAsp) at position 3; (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4, 6-dienoic acid (Adda) at position 5; and *N*-methyl dehydroalanine (MDha) at position 7 (Sivonen and Jones, 1999). About 65 structural variants of microcystins have been characterised (Sivonen and Jones, 1999), which differ primarily in the two L-amino acids at position 2 and 4 and methylation/demethylation on MeAsp and MDha. One of the microcystins that most commonly occurs is the highly toxic microcystin-LR, where the variable L-amino acids are leucine (L) and arginine (R). Its structure is shown below.

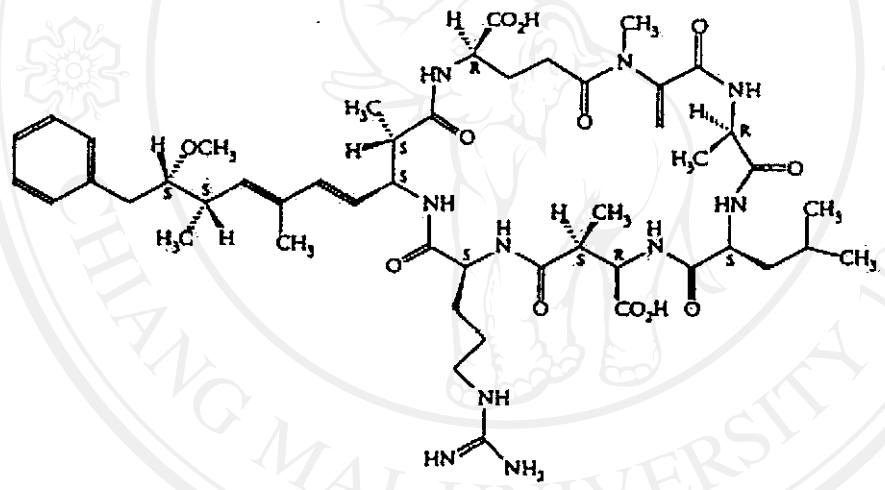

Figure 1 Structure of microcystin-LR

The highest toxicity of microcystins is microcystin-LR with LD50 i.p. or i.v. in mice and rats ranging from 36 to 122 $\mu g.kg^{-1}$, while the inhalation of toxicity in mice is similar: the median lethal concentration time (LCT50) = 180 $mg.min.m^{-3}$, LD50 = 43 $\mu g.kg^{-1}$ (Stoner *et al.*, 1989; Creasia, 1990; Miura *et al.*, 1991).

Microcystins are well known hepatotoxins that cause hepatocytes, the functional cells of the liver, to shrink. This shrinkage allows the blood carried by the vessels to seep into the liver tissue leading to local tissue damage, hepatic heamorrhaging (Runnegar *et al.*, 1991). The symptoms of poisoning from microcystin exposure include stomach cramps, vomiting, diarrhoea, fever, headache, pains in the muscles and joints and weakness (Dillenberg and

Dahnel, 1960). Similar symptoms of skin, eye and throat irritation, and allergic response to microcystins in water have also been reported (Ressom *et al.*, 1994). Death can occur within a few hours to a few days after initial exposure (Falconer *et al.*, 1981) and may be preceded by coma, muscle tremors and forced expiration of air (Beasley *et al.*, 1989). Death is most likely from heamorrhagic and hypovolumic shock (Carmichael, 1992).

Human health risk from exposure to cyanobacterial cells and toxins arises through three routes: direct contact with exposed parts of the body, including sensitive areas such as the ears, eyes, mouth and throat; accidental uptake of water containing cells by swallowing; and uptake of water containing cells by inhalation. The most likely route of exposure is via oral ingestion. However, there has been no toxicokinetic studies with orally administered microcystins. After administering radiolabelled toxins in mice and rats, microcystins appear to be transported by bile acid transporter into both the small intestines (Dahlem *et al.*, 1989) and liver (Runnegar *et al.*, 1991). About 70% of the toxin is rapidly localized in the liver (Brooks and Codd, 1987). The kidney and intestines also accumulate significant amounts of microcystin-LR (Robinson *et al.*, 1991).

The liver plays a large and important role in the detoxification of microcystins (Brooks and Codd, 1987) by being involved in the covalent conjugation to glutathione (Pflugmacher *et al.*, 1998; Takenaka, 2001; Gehringer *et al.*, 2004). Detoxification products have been seen in the urine, faeces and liver cytosolic fractions (Robinson *et al.*, 1991).

Microcystin is excreted rapidly, with 75% of the total excretion occurring within 12 hours. The remaining 24% of the administered dose is excreted after 6 days, about 9% via the urinary route and 15% slowly (1% per day) via the fecal route (Robinson *et al.*, 1991).

Toxicity of microcystins

Poisoning of livestock from drinking water containing cyanobacteria was first observed at Lake Alexandrina in South Australia in 1878. In this case, the toxin found was nodularin (Falconer, 1992). Since then, there have been many reports of poisoned sheep, cattle, horses, pigs, dogs, fish, rodents, amphibians, birds and other animals by cyanobacterial toxins, including microcystins (Codd *et al.*, 1989). Growth of cyanobacterial blooms is encouraged by an increase of nutrients such as nitrates and phosphates in the water; these nutrients may be derived from

human wastes such as sewage and detergents, industrial pollution, run-off of fertilizers from agricultural land, and the input of animal or bird wastes from intensive farming (Bell and Codd, 1994).

Human illnesses attributed to cyanobacterial toxins come in three categories: gastroenteritis and related diseases, allergic reactions and irritation, and liver diseases (Bell and Codd, 1994). Until recently, there has been no definite documentation on human deaths due to cyanobacterial toxins, although circumstantial evidence links mortalities with consumption of fish containing these toxins. However, Jochimsen *et al.* (1998) reported the case of a haemodialysis unit in Caruaru, Brazil, which took water for dialysis solutions from a reservoir contaminated with cyanobacteria. Forty-three patients out of 130 who developed toxic hepatitis died. HPLC revealed the presence of microcystin-LR and related toxins in the water sources and clinics, and analysis of the patient's liver and serum by immunoassays confirmed the presence of microcystins at the levels associated with acute or lethal toxicity (Pouria *et al.*, 1998; Azevedo *et al.*, 2002).

The development of primary liver cancer in China has been linked to long-term chronic exposure to microcystins. People who drink water from a pond or ditch containing low levels of microcystins have a higher mortality rate from hepatocellular carcinoma than those who drink water from a well that does not contain any microcystins (Ueno *et al.*, 1996).

Cytotoxicity of microcystins

Microcystins are a potent and specific inhibitor of serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A), with an irreversible covalent bond (Honkanen *et al.*, 1990; MacKintosh *et al.*, 1990; Toivola *et al.*, 1994; Guzman *et al.*, 2003). PP1 and PP2A are the most abundant phosphatases in mammalian tissues. Microcystin-LR inhibits the purified PP1 and PP2A with IC50 = 1.7 nM and 0.04 nM, respectively (Honkanen *et al.*, 1990). Hepatotoxicity of microcystins is a result of the hyperphosphorylation of all three cytoskeletal proteins (microtubules, intermediated filaments and microfilaments) and the redistribution of these proteins. Consequently, hepatic lobular and sinusoidal capillary architecture are lost extensively with hepatocytes necrosis and hepatic hemorrhage.

There are only a few studies on *in vitro* cytotoxic assays that involve the exposure of permanent cell lines and primary cultured rat hepatocytes to purified microcystins (Eriksson *et al.*,1987; Henning *et al.*, 1992; Chong *et al.*, 2000).

Eriksson *et al.* (1987) investigated the effects of a cyclic peptide toxin, which was isolated from *M. aeruginosa*, on cell morphology and ion transport in human erythrocytes; isolated rat hepatocytes, and mouse fibroblasts (3T3). The results showed no cytotoxic effects in erythrocytes or fibroblasts. However, in hepatocytes, the toxin induced morphological alterations at a concentration of about 50 nM and increased in both phosphate and potassium efflux at concentrations of about 0.1 and 1 nM, respectively.

Henning *et al.* (1992) investigated the cytotoxic effects of crude extracts of *M. aeruginosa* strain PCC 7806 and purified microcystin-LR on Chang liver cells by using the Lactate Dehydrogenase (LDH) released assay. The results did not show any detectable cytotoxic effects of purified toxin on Chang liver cells.

Chong *et al.* (2000) studied the cytotoxicity of microcystin-LR on eight permanent cell lines. Results from the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay indicated that purified microcystin-LR induced a toxic effect on human oral epidermoid carcinoma (KB) and rat Reuber H35 hepatoma (H-4-II-E) cell lines after 96 h incubation at toxin concentrations of greater than 18.75 $\mu g.mL^{-1}$.

Carcinogenicity of microcystins

There has been some evidence of carcinogenicity of microcystins in animal studies. Ito *et al.* (1997) observed neoplastic nodules in mice liver treated with microcystin-LR by the i.p. route over 28 weeks. After 100 i.p. injections of a sublethal dose (20 $\mu g.kg^{-1}$) of microcystin-LR, neoplastic nodules were observed without the use of an initiator.

The tumor promotor activity of microcystins was assessed in Fischer 344 rats dosed with microcystin-LR at levels below acute toxicity, after initially being primed with the liver-specific tumor initiator diethylnitrosamine (DEN). Then, the rats were repeatedly treated with 1 or 10 $\mu g.kg^{-1}$ of microcystin-LR, twice a week for 5 weeks. The result demonstrated an increased number of placental glutathione S-transferase (GST-P)-positive foci per square centimeter of the

liver tissue when compared to the lower dose group and the control animals (Nishiwaki-Matsushima *et al.*, 1992).

Sekijima *et al.* (1999) elucidated the synergism between aflatoxin B1 (AFB1) and microcystin-LR. DEN-initiated rats were exposed to both AFB1 and microcystin-LR. The results showed a synergistic increase in the development of GST-P-positive foci in rats initiated with AFB1 and subsequently exposed to microcystin-LR at 1 or 10 $\mu g.kg^{-1}$. This result supported the tumor promoting activity of microcystins.

Guideline value for microcystin-LR in drinking and recreational water

Due to growing concern about the human health effects of cyanobacterial toxins, especially via drinking water, the World Health Organization (WHO) has suggested a provisional guideline for microcystin-LR in drinking water of 1 $\mu g.L^{-1}$. However, Ueno *et al.* (1996) proposed a figure of 0.01 $\mu g.L^{-1}$, based on a possible correlation of primary liver cancer in certain areas of China with the presence of microcystins in the water of ponds/ditches, rivers and some shallow (but not deep) wells.

Tolerable daily intake (TDI) is the amount of a potentially harmful substance that can be consumed over a lifetime with negligible risk of adverse health effects. The WHO (1998) has recommended a TDI value of 0.04 $\mu g.kg^{-1}$body weight an average adult body weight of 70 Kg per day for microcystin-LR, which is ingested from drinking water.

In the year 2001, the National Health and Medical Research Council, Agriculture and Resource Management Council of Australia and New Zealand suggested, in revised Australian drinking water guidelines, that the cyanobacterial cell numbers could be used to provide a preliminary orientation to the potential hazard to public health. As indication of a highly toxic population of *M. aeruginosa*, a cell density of approximately 6,500 $cells.mL^{-1}$ is equivalent to the guideline of 1.3 $\mu g.mL^{-1}$ microcystin-LR. However, there are insufficient data to give guidance for the equivalent indicative cell numbers of other cyanobacterial species.

In recreational water, health impairment from cyanobacteria exposure must be differentiated between the chiefly irritative symptoms and the potentially more hazardous of exposure to high concentrations of cyanobacterial toxins, particularly microcystins. Individual sensitivities vary greatly. Therefore, a series of guideline values in recreational water

environments associated with incremental severity and probability of health effects has been defined as follows; at the cell density of 20,000 cells.mL^{-1} (which is equivalent to 2-4 μg of microcystin.L^{-1} that can cause irritative or allergenic effects (Pilotto *et al.*, 1997). Thus, the authorities should provide risk information for visitors to swimming areas and be informed in order to initiate further surveillance of the sites. The level of 100,000 cells.mL^{-1} (which is equivalent to 20 μg of microcystin.L^{-1}, with an average toxin content of 0.2 pg.cell^{-1}) represents a guideline value for a moderate health alert. Intervention is recommended to trigger effective public information campaigns to educate people on how to avoid of cyanobacterial contact. An intensified monitoring programme should also be implemented.

Extraction of microcystins

Because the cyanobacterial structure has a sturdy cell wall, it functions as an effective barrier against microcystin leakage into the environment. It is only when a bloom collapses, or is treated with an algicide such as copper sulfate, and cells die, that microcystins are released in surrounding water in significant concentrations (Lam *et al.*, 1995). An effective extraction of intracellular toxin (microcystin) is necessary for microcystin analysis.

Extraction of microcystins with the solvent system, n-butanol-methanol-water (5: 20: 75, v: v: v), developed by Siegelman *et al.* (1984), and its slight variation, have been used widely and found to give good results (Brook and Codd, 1986; Krishnamurthy *et al.*, 1986; Meriluoto and Eriksson, 1988).

The use of 5% acetic acid for the extraction of lyophilised cells also provides satisfactory results, because excellent toxin recovery is obtained and pigment extraction limited (Harada *et al.*, 1988a).

Lawton *et al.* (1994) found that the extraction efficiency of methanol prior to both 5% acetic acid and n-butanol-methanol-water provided a good recovery result. Five percent of acetic acid was especially weak in extracting more hydrophobic microcystins, and the yields were only 10-20% compared to those from methanol extraction. n-Butanol-methanol-water extraction was about 15% less efficient than methanol extraction.

Fastner *et al.* (1998) extracted cyanobacteria with either pure methanol, water, 5% acetic acid, 75% aqueous methanol or a sequential extraction using methanol followed by water.

Seventy-five percent of methanol was appropriate for the extraction of lyophilized cyanobacterial field samples because of the better reproducibility and faster procedure.

Analysis of microcystins

Several methods such as bioassay, immunoassay and chemical assay techniques are now available for the detection and analysis of microcystins, but none are considered by official agencies as a standard method.

The mouse bioassay has been a typical first test for toxicity in screening water bloom material and laboratory cultures or cell extracts. Its advantages are easy use, low cost and detection within a few hours assuming that laboratory test mice are readily available. The disadvantages of the mouse bioassay are its inability to detect low level amounts of toxins, especially in fully prepared drinking water, and failure to distinguish between the homologues of different toxins (Carmichael, 1992). In addition, it is desirable to minimize the use of laboratory test animals. This has led to the investigation of other bioassays, including zooplankton, some invertebrates like larvae of mosquito and brine shrimp (Turell and Middlebrook, 1988; Kiviranta *et al.*, 1991; Demott *et al.*, 1991). These biotests are not always easy to inteprete, as the invertebrates also react to compounds other than microcystins.

The protein phosphatase bioassay is another screening method for the quantification of microcystin-LR in water samples (Lambert *et al.*, 1994). This method is sensitive to subnanogram levels of microcystins in finished and raw water samples. It is a quick method, and many samples can be quantified in a few hours. However, it is not specific to microcystins and will indicate the presence of other substances inhibiting protein phosphatases.

A rapid immunoassay for the detection of microcystins is the Enzyme linked immunosorbent assay (ELISA) using polyclonal antibodies. This method successfully detects low level toxin amounts with a detection limit of 0.2 $ng.mL^{-1}$ (Chu *et al.*, 1990). It is likely that methods using monoclonal and polyclonal antibodies raised against a single toxin (e.g. microcystin-LR) will have problems of cross-reactivity with other microcystins.

Chemical detection assays for cyanobacterial toxins have also been developed along with the methods being used to isolate and purify toxins. These include high performance liquid chromatography (HPLC), thin layer chromatography (TLC), mass spectrometry (MS)

(Meriluoto, 1997), nuclear magnetic resonance spectrometry (NMR) and capillary electrophoresis (CE).

High performance liquid chromatography (HPLC)

Liquid chromatography (LC) is the most widely used method for microcystins separation, quantification and identification, mainly by reversed-phase C_{18} column with UV detection (Krishnamurthy *et al.*, 1986; Harada *et al.*, 1988; Meriluoto and Eriksson, 1988; Watanabe *et al.*, 1988; Lawton *et al.*, 1994; Edwards *et al.*, 1996; Aguete *et al.*, 2003). This method relies on retention time for identification and microcystin standards are required. However, while the chemical methods that identify common parts of microcystins are useful for screening purposes, they can not estimate the toxicity.

Reversed-phase C_{18} HPLC of microcystins involves the use of a gradient mobile phase consisting of acetonitrile in water. The chromatographic efficiency of this mobile phase has been considerably improved by the addition of perfluorinated alkyl carbocxylic acids such as trifluoroacetic acid (TFA). Although gradient elutions offer superior resolving power, many experiments still use isocratic mobile phases because of their simpler and more accurate instrumentation.

Guo *et al.* (1986) successfully predicted the retention times of small linear peptides in reversed-phase HPLC by using gradient elution of water-acetonitrile-0.1% TFA.

Siegelman *et al.* (1984) worked with toxin-LR (most likely microcystin-LR) from laboratory and field samples of *M. aeruginosa* by using HPLC. The HPLC procedure that consisted of ethanol-n-butanol-ammonium acetate extraction, was transfered to C_{18} HPLC with UV detection at 238 nm (mobile phase: 26% acetonitrile and 500 mM ammonium acetate). This procedure was useful for hepatotoxin (probably microcystin) quantitation.

Although these methods have been widely used, microcystin-LR and –YR were co-eluted under the above HPLC conditions (Harada *et al.*, 1988b). Kungsuwan *et al.* (1988) used methanol-0.1% TFA as a mobile phase to separate microcystin-LR and –YR.

However, separation of microcystins can be difficult at neutral pH by isocratic reversed-phase HPLC, which has led to the use of acidic mobile phases (Harada *et al.*, 1988a; 1990) or a basic mobile phase (Ruangyuttikarn *et al.*, 2004).

Microcystins are usually monitored at 238 nm, the λ_{max} of the majority of microcystins. The typical detection limit is a few nanograms in most HPLC systems (Meriluoto and Eriksson, 1988; Harada *et al.*, 1988a, b; Lawton *et al.*, 1994).

Harada *et al.* (1988b) designed three isocratic mobile phases for microcystins; methanol-0.05% TFA (6:4), methanol-0.05 M phosphate buffer pH3 (6:4), and methanol-0.05 M sodium sulphate (1:1). Used for HPLC instead of the conventional phase, an effective combination of these mobile phases enables a rapid and precise analysis of microcystin components.

Lawton *et al.* (1994) used a gradient of water-0.05% TFA and acetonitrile-0.05% TFA, starting at 30% to 70% acetonitrile in 40 min to detect microcystins and nodularin in raw and treated water. This HPLC condition can fully separate a mixture of standards containing nine microcystins and nodularin. The observed elution order was microcystin-RR, -YR and –LR.

Mass spectrometry (MS)

Mass spectrometry, as a detection method following HPLC separation, provides a much better solution to the problem of identifying microcystins, as microcystins produce characteristic ions in their mass spectra (Kondo *et al.*, 1992). LC/MS, with various interface and ionisation configurations, has been reported for the determination of microcystins (Poon *et al.*, 1993; Lawton *et al.*, 1995; Kondo *et al.*, 1992; Tsuji *et al.*, 1994; Bateman *et al.*, 1995; Hummert *et al.*, 2000; Ruangyuttikarn *et al.*, 2004).

Fast atom bombardment mass spectrometry (FAB-MS) is a powerful technique for obtaining molecular weight information of microcystins. However, it is difficult to obtain the sequence information about constituent amino acids because microcystins are cyclic peptides.

Harada *et al.* (1996) described a new analytical method for the microcystin using frit-FAB LC/MS, which gives a rapid identification of microcystins and related compounds. The background ions of this method can be subtracted to give a mass spectra that consists of sample ions only.

Matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry has also been successfully applied as a detection method following HPLC separation (Erhardt *et al.*, 1997). Derivatisation of microcystins prior to LC/MS analysis has also been reported as a technique to assist in identifying microcystins.

From the mass spectrum, which acts as a fingerprint, a microcystin can be identified as long as a mass spectrum of an authentic standard is available. A useful extension of this technique is MS/MS detection, where the fragmentation pattern can be used to greatly assist in determining the identities of unknown microcystins (Bateman *et al.*, 1995; Lawton *et al.*, 1995). However, as with any analytical procedure, standards are necessary for accurate quantification. This will be a limitation in any method that determines the concentration of individual toxins, as LC/MS systems are not yet particularly common in routine analytical laboratories.

Objectives of the study

Huay Yuak reservoir, located at Tambon Chang Pheuk, Amphoe Muang, Chaing Mai province, was constructed by the Royal Irrigation Department, Ministry of Agriculture and Cooperatives in order to conserve water resources for recreational activities, e.g. swimming and fishing. Recently, this reservoir has become contaminated with what appears to be eutrophic phenomena, such as cyanobacterial blooms (Kiatpradub, 2003; Ruangyuttikarn *et al.*, 2004). The appearance of cyanobacterial blooms and the presence of their toxins, especially microcystins, become a threat to human health and aquatic natural resources. Therefore, the monitoring of toxic algae blooms and observation of microcystin contamination in this reservoir is very important.

The annual report of cancer registry, from Maharaj Nakorn Chiang Mai Hospital (1999), showed mortality from liver cancer as the second most frequent cause of death among Chiang Mai male cancer patients. In recent years, the rate of liver cancer and mortality from it has increased (Deerasamee *et al.*, 1999). It is possible that there might be exposure to liver carcinogens or tumor promoters such as microcystins in contaminated water.

Microcystins induce cytotoxicity by altering the structure and function of hepatocytes. The cellular damage can be measured by the MTT assay. The yellowish tetrazolium salt of MTT is reduced by mitochondrial dehydrogenase enzymes of the living and metabolically active cells to a purple formazan product. Thus, the MTT assay is the most frequently used method for testing the cytotoxicity of microcystins (Chong *et al.*, 2000; Bouaïcha and Maatouk, 2004).

Cytotoxicity of microcystins has been well documented both *in vivo* (Runnegar *et al.*, 1993) and *in vitro* (Honkanen *et al.*, 1990; MacKintosh *et al.*, 1990; Eriksson *et al.*, 1987;

Henning *et al.*, 1992). However, in Thailand the cytotoxic effect of microcystins from *Microcystis aeruginosa* Kütz. has not been reported. The MTT assay was chosen to investigate the cytotoxicity of microcystins on the primary rat hepatocytes in this study. The hypothesis of this study was; microcystin-LR would cause a cytotoxic effect on hepatocytes, and the intracellular microcystin-LR would correlate negatively with the extracellular microcystin-LR. Therefore, the objectives of this study were:

1. To identify and isolate microcystin-LR from *M. aeruginosa* collected from Huay Yuak reservoir, Chiang Mai province.
2. To investigate the correlation between microcystin-LR concentrations in *M. aeruginosa* cells and the culture media.
3. To investigate the cytotoxicity of microcystin-LR isolated from *M. aeruginosa* by using the MTT assay.