CHARPTER 2
LITERATURE REVIEWS

2.1 Phytoplankton diversity and relations between species and environmental conditions

The study of phytoplanktons has been done to obtain the results as the principal organism group which could be important to be used for ecological and environmental assessment. Phytoplanktons are the key components in polluted water. The use of phytoplankton diversity, distribution and abundance as bioindicators of water quality has a long tradition. In addition, it has also been suggested that presence of phytoplankton may be the good monitoring organisms of the heavy metals and toxicities in water. Bioassay organisms may be useful for eutrophication tests and can be used to detect different types of pollution as well (Vymazal, 1995). Phytoplankton has evolved through various strategies to overcome nutrient depletion and grazing. These include production of special enzymes which release chemically bound nutrients or allow the phytoplankton to take up nutrients at low concentrations. While, movement by swimming or a change in cell density may allow them to reach new sources of nutrients. Some species form resting stages to overcome unfavourable conditions (Goldman and Horne, 1983). The driving force and mechanisms of seasonal changes are related to variations in the physical, chemical and biotic environments, e.g. changes in solar irradiance and nutrient levels (Harris, 1986). In recent years, the area occupied by algae and macrophytes has expanded and caused serious concern, given the known difficulties involved in controlling eutrophication and silting in urban lakes (Sperling, 1997). The consequences include a prolonged stable stratification and a persistent thermocline for water quality. In the hypolimnion, anoxia resulted in the accumulation of reduced ionic species, whilst in the epilimnion high algal production was probably sustained by nutrient inputs from the hypolimnion during drawdown (Hawkins, 1985).

Numerous water bodies are sufficiently rich in the essential nutrients as these nutrients do not become limiting factors in determining the abundance of phytoplanktons. It seems that the growth of phytoplanktons in tropical latitudes depends on encompassing nutrient levels more than on other environmental factors such as turbidity, water temperature and parasitism (Morris, 1980). Likewise, supply or loading ratios of biologically available nitrogen and phosphorus, N:P ratios have often been suggested as the major determinants for the presence or absence of N_2 fixing cyanobacteria in aquatic environments. Increasing evidence that some components of the dissolved organic nitrogen pool can play an active role in supplying N nutrition either directly or indirectly to phytoplankton which implies that this source of N must be considered in any attempt to apply the N:P resource ratio approach to predict or explain phytoplankton population composition (Berman, 2001). Whilst, nutrient supply ratios did not directly increase algal abundance and biomass, they were responsible for controlling the specifies composition of the phytoplankton. High total N:P ratios were found to result in an increase in cryptophyte and chrysophyte populations and this coupled with high Si:P ratios resulted in a marked increase in diatoms (Anton *et al*., 1995).

On the other hand, the influence of sediment at the lake bottom has been verified to significantly affect the species population dynamic. The shallow sediments are more important than deep ones for the recruitment and may still be important factors in the population dynamics of algae (Karlsson-Elfgren and Brunberg, 2004). Furthermore, the effects of materials released from the sediment on the growth of algae clearly varied from species to species of algae. The growth of green algae *Scenedesmus acutus* was influenced by nitrogen released from sediment and the growth of filamentous cyanobacterium *Oscillatoria agardhii* was mainly influenced by phosphorus released from the sediment (Tada *et al*., 2001). So the information obtained from both phytoplankton studies and investigations of the physico-chemical parameters of the water allow the evaluation of seasonal variations of water quality and of the mutual relationship between phytoplankton and its environment. Therefore, the use of phytoplankton species

composition, abundance and their relations to identify the trophic status of lakes is widely accepted.

Cyanobacteria or Cyanophytes are common in eutrophic natural waters. Being favored by warm, stable and nutrient-enriched waters they may constitute an important part of the phytoplankton community in wastewater treatment plants (Vasconcelos and Pereira, 2001). Whereas, the oligotrophic lakes are characterized by a desmid-diatom limnetic plankton of moderate diversity (12-35 species per lake). Of 144 taxa of phytoplankton recognised, 58% were desmids and 15% were diatoms (Hawkins *et al*., 1988). Most representatives of the algal, Family Desmidiaceae grow in slightly acidic, oligotrophic environments (Brook, 1981). Likely, the absence of eutrophic species in nutrient-poor environments may be due to a less efficient uptake of rare nutrients resulting in a lower growth rate than that of well adapted oligotrophic species with a high affinity to nutrients in low concentrations (Spijkerman and Coesel, 1998). It is clear why by far the great majority of the desmid taxa are bound to nutrient-poor water, because in more eutrophic environments, they are immediately ousted out by the fast reproducing Chlorococcaceae, Bacillariophyceae and/or Cyanophyceae (Coesel, 1983). Thus, it has been confirmed that phytoplankton species can be used as bioindicators because they respond to the physical and chemical changes of the habitat. So, qualitative and quantitative studies of phytoplankton can be regarded as essential tools for biological water quality assessment (Reynolds, 1984).

In the frame of favourable or limiting factors for desmids, there are no clear indications of the direct involvement of the ionic composition of lakes, though it has been observed that high levels of calcium can negatively affect the growth of some oligotrophic desmids (Martínez-Almeida and Tavera, 2005). However, some species of desmids (*Staurastrum chaetoceras*) are predominantly encountered in eutrophic lakes because of higher portions of extracellular relative to cellular alkaline phosphatase activity (Spijkerman and Coesel, 1998). Although it is assumed that the chemical and physical environment largely determines the amount of phytoplankton production, the exact relationships remain in many respects obscure (Palmer and Square, 1977). At the

present, there are many research projects being done on phytoplankton as the indicator for water quality and most of them have been done in temperate zones, so uncommonly little research has been undertaken in tropical zones. In Thailand, it is clear that there are few of papers dealing with ecological studies. Genarally, the literature is dominated by taxonomic studies primarily conducted by non-Thais, and surveys which are primarily lists of taxa collected at a particular locality or series of localities. The ecotoxicology is the most rare (4% of published papers) in this field (Campbell and Parnrong, 2000).

The present study was published on the diversity of phytoplankton of Banglang Reservoir, located on the Pattani River in southern Thailand and found 135 species in seven divisions. The greatest number of species were in Division Chlorophyta (50%) followed by Cyanophyta (21%), Bacillariophyta (13%), Pyrrophyta (6%), Cryptophyta (4%), Chrysophyta (3%) and Euglenophyta (3%). The most diverse genus was *Staurastrum* (15 species) (Ariyadej *et al*., 2004). Additionally, the various 186 species of phytoplankton in Division Cyanophyta, Chloroplhya, Euglenophyta, Chrysophyta, Pyrrhophyta and Crptophyta were found in Ang Kaew Reservoir, Chaing Mai, Thailand. The species which indicated eutrophication to hyper-eutrophication was *Anabaena catenula* (Kützing) Bornet&Flahault; the mesotrophication to eutrophication indicator species were *Euglena acus* Ehrenberg, *Phacus meson* Pochmann, *Phacus pleuronectus* Müller and *Trachelomonas volvocina* Ehrenberg, the mesotrophication indicator species were *Planktolyngbya limnetica* Lemmermann, *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba et Raju, *Aulacoseira granulata* (Ehrenberg) Simonsen and *Peridinium inconspicuum* lemmermann (Peerapornpisal *et al*., 2004).

2.2 Toxic cyanobacteria in water and their toxins

2.2.1 Microcystin production

The cyanobacterial toxins are divided into three groups by chemical structures, which are cyclic peptides, alkaloids and lipopolysaccharides (LPS). They are produced from several cyanobacterial species. Various cyanobacteria can produce secondary metabolites, which are toxic to other organisms. However, we can not exactly indicate which cyanobacterial cell is toxic or non-toxic by morphological study under microscopic identification but the chemical and molecular genetic techniques can be used for this purpose (Mur *et al*., 2004). Because the morphological classification of the genus *Microcystis* has sometimes been unclear, this is explicable by the present results. *Microcystis* possibly changes the colony forms in the field as well as in culture. Many strains maintained colony forms characteristic of their morphospecies, and others showed morphological variations, some of which were characteristic of other morphospecies (Otsuka *et al*., 2000). The production of toxins is an energy consuming and complicated process. Apparently, these toxins have a function, otherwise they would have been lost in evolution. The question on the function of toxin production is still not answered. Two putative roles can be differentiated; first, toxins have under certain conditions a function in the metabolism of the organism or second, toxins are of ecological relevance (Mur *et al*., 2004). The mechanisms of toxin production in the cyanobacterial cells or filaments were studied for obtaining the role of the original process under natural conditions.

The toxin-producing *M. aeruginosa* strain has a more efficient iron uptake system than the strain that does not produce toxins. The toxin could be an intracellular chelator which keeps the cellular level of free Fe^{2+} low. Fe^{3+} in the medium seems to be converted to Fe^{2+} by light before it is transported into algal cells. The non-toxin-producing *Microcystis* strain does not have this intracellular chelator (toxin) and must therefore have a lower cellular Fe^{2+} concentration; this is obtained by an iron uptake system less efficient than that in the toxin-producing *Microcystis* strain (Utkilen and

Gjølme, 1995). As the understanding that microcystin values are not linearly related to a single habitat component, but are rather the complex result of the interaction of many different factors, provides a new approach to addressing environmental influence on microcystin concentrations (Graham *et al*., 2004).

2.2.2 Cyanobacterial blooms in water environment

The first detailed scientific account of toxic cyanobacteria appeared in 1878. In a perceptive and prescient paper in *Nature*, the Adelaide assayer and chemist George Francis reported on stock deaths at Milang on the shores of Lake Alexandrina in South Australia (Codd *et al*., 1994). According to the reports throughout the world, it is found that the most frequent cyanobacterial genus causing the bloomings in fresh and brackish water is *Microcystis* (Botes *et al*., 1982), which produce cyclic peptide toxins named microcystins (Carmichael *et al*.,1988). Microcystins have been characterized from many genera such as *Microcystis*, *Anabaena*, *Oscillatoria*, *Nostoc* and *Anabaenopsis* (Chorus and Batram, 1999). The occurrences of cyanobacterial blooming have been reported from different places around the world. In Thailand, *M. aeruginosa* blooms were found in three water resources (Mahakhant *et al*., 1998) and six reservoirs as water sources for water supplies and fisheries. These bodies of water were found to be contaminated by toxic cyanobacteria, *M. aeruginosa* (Peerapornpisal *et al*., 2002). Similarly, *Microcystis aeruginosa* bloomed in the lower part of the Nakdong River, which was also investigated during the dry summer of 1994 (Ha *et al*., 1999).

2.2.3 *Microcystis* in sediments

The annual cycle of *Microcystis aeruginosa* colonies present that the colonies would be influenced from environmental conditions of seasonal changes affecting the collapsing of gas vacuoles in the cell resulting in a sinking down of *Microcystis* colonies to the sediment over winter (Reynolds *et al*., 1981). The extraction

of sediment cores which took place during total ice coverage of the investigation fishpond showed 49 species of cyanobacteria and algae in sediment. However, green algae were the most frequent and vital group (Hašler *et al*., 2004). The mucilage of *Microcystis* colony is important as the habitat with enhanced bacterial activity for both water column and sediment bacteria (Brunberg A-K., 1999). Microcystins are preserved within benthic *Microcystis* during autumn and winter because they could play an important role during the reinvasion phase, either by improving growth of the initial pelagic population or by activating resting colonies (Ihle *et al*., 2004).

2.2.4 Influences of environmental factors on cyanobacterial growth

Many investigations have reported that cyanobacterial toxin occuring in water regularly found in surface-bloom water is related to suitable water conditions for toxic cyanobacterial growth and they highlight the risk of direct cyanobacterial toxin exposure in bodies of water (Stirling and Quilliam, 2001). The importance of hydrodynamics and nutrient loading has reported that concentrations of dissolved inorganic nitrogen and total phosphorus during the bloom of *Microcystis* spp. were high. The pH was low (~7) until the initial stage but was high (pH>9) as the bloom formed. Elevated water temperature (>30°C) along with low discharge and high irradiance were major factors contributing to the *Microcystis* spp. bloom in Nakdong River, South Korea (Ha, *et al*., 1999).

Also, microcystins in the wastewater treatment pond were found during nutrient-rich conditions in water causing the main species of cyanobacteria, *Planktothrix mougeotii*, *Microcystis aeruginosa* and *Pseudanabaena mucicola* to be dominant in the water (Vasconcelos and Pereira, 2001). As in the results of Lake Suwa, Japan, non-toxic *M. ichthyoblabe* may more grow than toxic *M. aeruginosa* in phosphorus limited water, and toxic *M. aeruginosa* and *M. viridis* could be dominant in *Microcystis* bloom by high phosphorus loading (Honma and Park, 2004). Moreover, it was reported that physical and chemical environmental conditions of Lake Kasumigaura in Japan in the summer season

were suited for the growth of *Microcystis* and it was considered that an association with bacteria was of great advantage to the growth of *Microcystis* (Yagi *et al*., 1984). Also, in order to clarify the effect of the manganese released from lake sediment on the growth of *Microcystis aeruginosa,* it was considered that manganese might promote the growth of *M. aeruginosa* in eutrophic lakes (Tada *et al.*, 2002).

The carbohydrate accumulation is light dependent and consequently turbidity has a marked impact on buoyancy regulation of *Microcystis* colonies. A cell near the lake surface photosynthesizes and accumulates carbohydrates at a rate governed by its present light climate and previous light history. The increased density causes it to sink in the water column and fall out of the light zone. Below the depth corresponding to the compensation irradiance, respiration exceeds photosynthesis and buoyancy is restored. In turbid water, light penetration is reduced resulting in colonies being concentrated near the surface and increasing the probability of surface accumulations. Colony size has a marked influence on vertical migration. Larger colonies migrate to greater depth, spend varying lengths of time below the euphotic zone in possibly more nutrient rich conditions and travel further which may assist colonies to scavenge for nutrients at low concentrations (Brookes *et al*., 1998). In addition, the importance of colonies of *Microcystis* as a food resource for copepods has already been considered in several ways, although mainly through laboratory cultures of cyanobacteria. The present study showed that natural colonies could provide a food alternative for cyclopoids (Rietzler and Espíndola, 1998).

2.3 Effects of Cyanobacterial toxins on human and animal health

Cyanobacterial toxins have adverse effects on mammals, birds, crustaceans, protozoa and fish. They are being increasingly recognized as a potent stress factor and health hazard factor in aquatic ecosystem. All 126 patients who underwent dialysis at the IDR developed symptoms. At least 108 patients had evidence of liver injury, and 60 have subsequently died. More male than female patients were affected, and

many cases were in the 50-59 year age-group. Samples of serum, dialysis filters and water-treatment columns contained microcystins, the highly toxic low-molecular-weight hepatotoxins produced by cyanobacteria (Pouria *et al*., 1998). In Australia, toxic cyanobacterial blooms have occurred throughout recorded history, as in causes of livestock deaths and water unpalatability (Falconer, 2001). So that, the New South Wales Blue-Green Algae Task Force which recommended a level of 15,000 cells ml^{-1} as a threshold for acceptable contact exposure to cyanobacteria (Ressom *et al*., 1994) have now opted for a 20,000 cells ml^{-1} threshold but recognize that normal variations of cell density make the difference between the 15,000 and 20,000 levels and are immaterial from a practical point of view. Since 20,000 cells ml^{-1} gives a slight discoloration to the water, it is reasonable to accept that discolored water poses a health risk. Acute lethal toxicity of blooms usually occurs when microcystin levels exceed 1 mg g^{-1} of cells (Carlmichael, 1996). In case of the other cyanobacteria, determination of severe hepatotoxicity for mice caused by the tropical cyanobacterium, *Cylindrospermopsis raciborskii* isolated from the domestic water supply reservoir on Palm Island, Australia, has been associated with an incident of hepatoenteritis in humans living on the island (Hawkin *et al*., 1985). The LD_{50} (24h) for intraperitoneally administered *Cylindrospermopsis* preparations ranged from 50 to 110 mg dry weight of lysed cells per kg, whereas the LD_{50} (7 days) ranged between 20 and 65 mg kg^{-1}. In contrast, oral administration of 1,400 mg kg^{-1}, while inducing clear histological damage, was not fatal to any of the animals used in the study (Falconer *et al*., 1999). In the Africa, the presence of cyanobacteria, *Microcystis aeruginosa* and microcystins in water used for drinking in a North African country may be regarded as an health hazard. These results contribute to the knowledge of the biogeography of toxic cyanobacteria and their toxins, namely in north African countries (Oudra *et al*., 2001).

Harmful cyanobacteria pose a hazard to aquatic ecosystems due to toxins. Microcystins and nodularins may accumulate in aquatic organisms and result in higher trophic levels, and eventually affect vector animals and consumers. Moreover, problems caused from cyanobacterial toxin in water account for a serious economic loss for the

worldwide fisheries as reports that microcystins can accumulate in fish tissue used for human consumption. Rates of ingestion routinely exceed the TDI guidelines as set by the WHO for drinking water. Appropriate epidemiology and risk assessment should be undertaken so that an acceptable TDI and appropriate risk management decisions can be made regarding human consumption of fish which are harvested from cyanobacterial blooms that contain cyanotoxins (Magalhães *et al*., 2001). Additionally, exposure of fresh water rainbow trout to the cell contents of cyanobacteria, *Microcystis* PCC 7813 promotes osmoregulatory imbalance resulting from stimulation of the drinking response, increased volume of fluid in the gut and inability to remove excess water (Best *et al*., 2003). However, the respiratory system of eastern rainbow fish (*Melanotaenia duboulayi*) has been unaffected by short-term exposure to microcystins or other biologically active compounds produced by *M. aeruginosa* (Johnston *et al*., 1994). Statistically significant differences of haematological parameters among experimental and control juvenile carp (*Cyprinus carpio*) were ascribed to determine the effect of microcystins operating as co-factors of the toxic effect of ammonia (Kopp and Hete-A, 2000). Three juvenile *Tilapia rendalli* (Cichlidae) under laboratory conditions were observed to be able to accumucate microcystins and the availability of other feeding sources, besides toxic cells, probably interferes with the accumulation rate. Therefore, the occurrence of toxic cyanobacterial blooms producing microcystins in aquaculture ponds could represent a risk in the quality of fish to the consumers (Soares *et al*., 2004).

Likewise, not only fish but crustaceans also are affected by cyanobacterial toxins. Laboratory experiments indicated that in prawn hepatopancreas, hearts and brains were the primary organs for hepatotoxin bioaccumulation. Toxin concentrations in other organs, including muscles, was less effective. Although prawns may act as vectors for toxin transfer, they did not accumulate alerting amounts of hepatotoxins and were able to effectively detoxify themselves. Because bloom toxicity may vary, low-frequency toxin monitoring is recommended (Kankaanpää *et al*., 2004). Crustacean species, adult male *Chasmagnathus granulatus*, crabs exposed to microcystins also showed a significant increase in the total oxyradical scavenging capacity (TOSC) value against peroxyl

radicals, for both anterior and posterior gills. Lipid-peroxide levels did not change in both gill types after exposure to the toxin. The increased levels of TOSC suggest the occurrence of a crab response against oxidative stress induced by toxin injection, which prevents lipid peroxidation (Vinagre *et al*., 2003). The massive accumulation of toxic material affects the survival of several benthonic estuaring local organisms. The Tanaidaceae, *Kalliapseudes schubartii* is a benthonic estuarine species which occurs at high densities throughout the year in mixohaline areas of the Patos Lagoon, southern Brazil. This mirocrustacean is of high ecological relevance and plays and important role in the estuarine food web, as it is consumed on a large scale by estuarine fish. It verifies that the acute toxicity of aqueous extracts of *Microcystis aeruginosa* is a possible risk of intoxication to which the natural populations of *K. schubartii* were exposed in the environment and emphasize the importance of studies involving sublethal concentrations of *M. aeruginosa* to other organisms of the trophic web in the aquatic system (Montagnolli *et al*., 2004). Whilst, the other cyanobacteria, *Cylindrospermopsis raciborskii* was not lethally toxic to daphnia, it was inadequate as the sole food. The filtering rates of three daphnia species were reduced in the presence of the filaments (Hawkins and Lampert, 1989).

The cyanobacterial blooms were found to be specially flourishing in parallel with low fish productivity. *Microcystis aeruginosa* was investigated to some biological studies including, the antibacterial effects of its cell-free extract against gram positive and gram negative bacteria and the hepatotoxic effect of the cell-free extract on the liver of albino rats (Shaaban *et al*., 2001). Additionally, microcystins induce oxidative stress in a time-dependent manner and the type of administration of the cyanobacterial cells influences the extent of these effects. Thus, the crushed cyanobacterial cells (released toxins) induced the antioxidant defences studied and increased the level of lipid peroxidation (LPO) to a greater extent than the non-crushed cells. The liver was the most affected organ followed by kidney and gills (Jos *et al*., 2005).

In case of the effect on birds, the high amounts of cyanotoxins in the flamingo feed and livers therefore imply that cyanotoxins ingested with the daily food

can have chronic effects on health and contribute to the mass mortalities of Lesser Flamingos in the Kenya Rift Valley (Ballot *et al*., 2004). Not only the water resource but the crop areas are also affected. It is a surprising conclusion of the published paper that the colonies and single cells of *Microcystis aeruginosa* and the hepatotoxin were retained by salad lettuce after being grown with spray irrigation water containing the microcystin-producing cyanobacteria. These findings are discussed in terms of crop spray irrigation with water containing cyanobacteria and potential human exposure to cyanobacterial toxins via plant foods grown under such circumstances (Codd *et al.*, 1999). In the case of symbiotic cyanobacteria, the biomagnification of cyanobacterial *β*-methylamino-L-alanine (BMAA) was discovered via the neurodegenerative disease among the Chamorro people of Guam Island (Cox *et al*., 2003). As root symbionts of cycad trees, cyanobacteria of the genus *Nostoc* produce (BMAA), a neurotoxic nonprotein amino acid. The biomagnification of BMAA through the Guam ecosystem fits a classic triangle of increasing concentrations of toxic compounds up the food chain (Murch *et al*., 2004).

2.4 Determination methods for microcystins in cyanobacterial material, water and sediments

Cyanobacterial toxin analysis is currently constrained by the limited availability of purified reference and quantitative toxin standards. The recent guideline values of the World Health Organization, and those emerging at the national level, for cyanobacterial toxin concentrations in drinking and recreational water, and for dietary supplements, further emphasize the need for reliable, specific, and quantitative methods for cyanobacterial toxin analysis for the protection of health (Codd *et al*., 2001). Microcystins are a large group of toxic peptide of differing hydrophobicity that can be readily chromatographed by reversed-phase HPLC. Microcystins show two typical spectra, one with an absorption maximum at 238 nm, which is exhibited by all except a few microcystins that contain tryptophan and that give an absorption maximum at 222 nm. These characteristic spectra can be used in the identification of microcystins in

naturally occurring samples in the absence of a wide array of standards (Lawton *et al*., 1994). The raw extracts of cyanobacterial sample collected from the Bleiloch reservoir, in Germany, were cleaned by size-exclusion chromatography (SEC) or solid-phase extraction (SPE). The determination of microcystins was achieved by different HPLC separation followed by the application of alternative detection methods [UV, diode array detection (DAD), and mass spectrometry (MS), respectively]. Furthermore, the different results of clean-up by SPE and SEC are demonstrated. The identity of microcystins was verified by MS/MS measurements (Hummert *et al*., 2001).

The isolation and purification of toxins from the *Microcystis aeruginosa* dominated bloom of Lalla Takerkoust lake-reservoir in Morocco, was performed by reverse phase HPLC and then characterized by amino acid analysis and fast atom bombardment mass spectrometry (FAB-MS) (Oudra *et al*., 2001). Reversed-phase HPLC coupled with the atmospheric pressure ionization-electrospray ionization (API-ESI) MS was used for microcystin-LR detection and quantitation in samples of dried Microcystis aeruginosa cells. An alkaline linear gradient (20 mmol l^{-1} ammonium-nitrogen hydroxide acetonitrile, pH 9.7) was used for elution of the toxic peptides. Limit of detection was 1 µg ml^{-1} (20 ng per injection) in the scan mode of MS and 0.1 µg ml^{-1} (2 ng per injection) in the case of selective ion monitoring (Ruangyuttikarn *et al*., 2004). Microcystin-LR in aqueous solution is readily adsorbed by disposable polypropylene pipette tips commonly used in laboratory manipulations. Under the conditions tested, microcystin-LR adsorption to polypropylene tips was not affected by either the pH or salinity of the solution. Both methanol and acetonitrile concentratioins affected the determination of microcystin-LR concentration according to HPLD-PDA (Hyenstrand *et al*., 2001). However, problems could be found during laboratory manipulation, the effect of plastic and methanol on the loss of microcystin-LR from the solution was analysed by HPLC with photodiode array detection. With plastic disposable pipette tips, the loss from an aqueous microcystin-LR solution was 4.2% per tip operation. Using the same pipette tip, four operations were required to completely saturate a single tip with the toxin (Hyenstrand *et al*., 2001). Extraction and analysis of microcystins RR and LR in cyanobacteria using a cyano

cartridge produced better recoveries and better chromatograms were observed than with ODS cartridges (Pyo and Shin., 2002).

Responses of an Enzyme Link Immuno Sorpbent Assay (ELISA) to microcystins have been determined using the authentic toxin antigen, microcystin-LR, and conjugation products between the toxin and glutathione, cystein-glycine and cysteine. The antibodies against microcystin-LR crossreacted with the toxin conjugation products with similar affinities (92-112%) to that of microcystin-LR, when assayed at a concentration of 1 μg l^{-1}. Toxicity assessment of the conjugates, in comparison to microcystin-LR, indicated a reduction according to mouse bioassay. In vitro protein phosphatase inhibition assay indicated that the conjugates possessed approximately 3-9-fold lower toxicity than microcystin-LR (Metcalf *et al*., 2000a). Polyclonal antibodies showing good cross-reactivity against a range of purified microcystin variants were raised using this conjugate. An indirect competitive ELISA was developed, using the polyclonal antibodies. Microcystins were detected in laboratory isolates of cyanobacteria, using this method, and showed good correlation with HPLC detection. Preliminary investigations into the use of the ELISA for assessing the microcystin-LR content of spiked tap water were carried out. The results indicate that antibodies raised using this conjugate could be applied for sensitive detection of microcystins in water samples (Metcalf *et al*., 2000b).

Low-cost, straightforward methods for the extraction of microcystins and nodularins from cyanobacterial cells were developed using a microwave oven and boiling waterbath showing good correlation with results from lyophilisation and methanol extraction when extracts were analysed by high performance liquid chromatography with diode array detection ($R^2 \geq 0.92$). The microwave and boiling waterbath extraction methods also sterilised the environmental bloom samples, as evidenced by the abolition of heterotrophic bacterial growth (Metcalf and Codd, 2000). The estimation of the microcystin content in cyanobacterial field samples from German lakes using the colorimetric protein-phosphatase inhibition assay and reversed phase HPLC was done and has proved to be useful for a preliminary screening of cyanobacterial field samples: it requires little equipment and allows rapid detection and rough estimation of microcystin

content. Nevertheless, reversed-phase HPLC analysis remains necessary for a safe confirmation of micorcystins.

Improved sensitivity of the colorimetric PP-1 inhibition assay is desirable to make detection of lower microcystin concentrations possible e.g. in drinking water and to control the proposed guideline level of 1 µg microcystin per liter (Wirsing *et al*., 1999). However, the inhibitory activity against protein phosphatases is not always related to the apparent LD_{50} level, and that the appearance of toxicity by microcystins depends on the balance between accumulation and metabolism in the liver (Ito *et al*., 2002). Although ELISAs can be useful tools for the screening of water and cyanobacterial blooms for microcystins and nodularins, users should be aware that commercial kits can be susceptible to interference by commonly encountered environmental and laboratory conditions and materials. Methanol had the greatest effect, giving false positive microcystin concentrations with increasing methanol concentrations up to 30% (v/v) compared with the negative calibrators of each kit. False positive microcystin results were also produced with increasing salinity up to full strength seawater (Metcalf *et al*., 2000c).

After removal of *Microcystis* and microcystins using the ultrasonic radiation and jet circulation to the flushing treatment, a molecular genetic monitoring technique on the basis of DNA direct extraction from the sediment was applied. The DNA pattern representing *M. viridis* could not be detected in any of the sediment samples. Therefore, the results most likely indicated that *M. viridis* seems to have disappeared because of the addition of the ultrasonic radiation and jet circulation to the flushing treatment (Innok *et al*., 2005). Because it was very difficult to extract microcystins from sediments using conventional techniques, a physico-chemical screening method, the MMPB (2-methyl-3-methoxy-4-phenylbutyric acid) method, including ozonolysis and mass spectrometric detection was developed. The obtained results clearly indicated that the adsorption on sediments contributes to the detoxification of microcystins under natural conditions (Tsuji *et al*., 2001).

2.5 Removal of toxic cyanobacteria and their toxins in water

Several physico-chemical methods such as coagulations/flocculation, dissolved air flotation, activated carbon adsorption, rapid sand filtration, slow sand filtration, chlorination, decomposition by light, membrane processes and ozonation were applied to water treatment giving various efficacy in removal of cyanobacterial cells or filaments and toxins (Hitzfeld *et al*., 2000). The most consistently effective method for destruction of both intra- and extracellular microcystins appears to be ozonation which can rapidly achieve an essentially complete destruction of microcystins, nodularin and anatoxin-a (Keijola *et al*., 1988; Himberg *et al*., 1989; Rositano and Nicholson, 1994; Croll and Hart, 1996; Rositano *et al*., 1996 and Hart *et al*., 1997).

As provided, chlorine residual at 0.5 mg l^{-1} was present after 30 minutes contact time, chlorination was effective in the destruction of microcystins. Importantly, the destruction of these toxins was found to be pH dependent, with the efficiency declining at pH values above 8 (Nicholson *et al*., 1993). Activated carbon is, however, not always a very efficient method. The natural organic materials in natural water can interfere with the adsorption of target compounds using activated carbon. This can occur either by a direct competition, whereby the natural organic materials and target compound compete simultaneously for the same adsorption site (Newcombe *et al*., 1997).

As the same mechanism as an activated carbon, clay minerals have multiple potential applications in the control of algal and cyanobacterial blooms. They have been used to scavenge nutrients, in particular to reduce phosphorus concentrations and are known to bind to cyanobacterial and algal cells. Clays can also be used in water treatment processes as filter agents and flocculants (Morrison and Codd, 2004). Fine-grained particles known to have a high concentration of the clay mineral (montmorillonite) showed a very high percentage (81%) of adsorption for microcystin-LR (Morris *et al*., 2000).

The aerobic as well as anaerobic microorganisms in sediments of a water recharge facility can efficiently remove microcystins. Enrichment of anoxic sediment

slurries with nitrate-nitrogen indicated that the anaerobic degradation can be coupled to nitrate-nitrogen respiration (denitrification). Thus, the presence of nitrate in anoxic infiltration basins appears beneficial in removing and detoxifying microcystins (Holst *et al*., 2003). In the case of other toxins, clay flocculation of extra-cellular brevetoxins (neurotoxins), released from toxic dinoflagellate (*Karenia brevis*) cells ruptured by ultrasonication, removed 70% of the toxins. Addition of the chemical flocculant, polyaluminum chloride (PAC), removed all of the extra-cellular toxins (Pierce *et al*., 2004). Other practical methods were applied such as the ferrate treatment which is an effective and practical method for the removal of cyanobacterial peptide toxins from eutrophic waters, especially in water which holds high total organic carbon. The toxin was easily decomposed by oxidation with ferrate, and the removal efficiency depended on the dosage of ferrate, pH and contact time (Yuan *et al*., 2002).

Likewise, the filamentous cyanobacteria were reduced by calcite flushing also making precipitation of phosphorus (Rönicke *et al*., 1997). Likely, nutrients, toxic chemicals and toxin-forming microbes are found in much higher concentrations in sediments than in the overlying water column so that improvements in the quality of the overlying water and associated components of the aquatic ecosystem often cannot be achieved without some form of sediment treatment (Murphy *et al*., 1999). In addition, the biological water treatment technology has been developed to remove nutrients as the main cause of the eutrophication in water resources. It has been called the biopark which is a system that uses the rootstalks of cultivated plants as filter materials, making the organisms inhabiting the rootstalks perform bio-accumulation and shellfish gathering and proliferating, there it extracts nutrient salts and turbidity from the water and puts them to use. (Ministry of the Environment, Government of Japan, 2002)

In the case of biological method, some aquatic bacterial strains have been isolated and identified as microcystin degrading bacteria. *Sphingomonas* species and *Pseudomonas aeruginasa* were capable of degrading microcystins (Bourne *et al*., 1996; Bourne *et al*.2001; Park *et al* 2001; Watanabe, 1997). The decomposition rate of purified microcystin-YR and LR, compared in distilled water and culture medium, respectively,

indicated clearly that microcystin-YR was more labile for decomposition than microcystin-LR in the culture medium. At the end of the experiment (45^{th} day) microcystin-YR decreased to 58.6%, while 86.2% of microcystin-LR remained (Watanabe *et al*., 1992). Laboratory studies of dissolved radiolabelled microcystin-LR in lake water found that biodegradation of dissolved microcystin-LR occurred in water collected at the lake surface with carbon dioxide as a major end-product (Hyenstrand *et al*., 2003).

In the case of cell degradation, grazing on a *Microcystis* bloom by cladocerans and copepods in Tai Lake, China was observed and found clearly that the solution to eliminating cyanobacterial blooms lies not only in increased grazing using enhanced natural populations of herbivores, but also must include more large grazers, since filtering rates increase as does the square of grazer length (McNaught *et al*., 1997). However, persistent toxins in the organism can be detoxified as the present study shows the existence of a microcystin-LR glutathione conjugate formed enzymatically via soluble glutathione S-transferase in various aquatic organisms ranging from plants (*Ceratophyllum demersum*) and invertebrates (*Dreissena polymorpha* and *Daphnia magna*) up to fish eggs and fish (*Danio rerio*). The main derived conjugate was characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry yielding a mass of *mlz* 1302, which is equivalent to the mass assumed for a glutathione microcystin-LR conjugate. This conjugate appears to be the first step in the detoxication of a cyanobacterial toxin in aquatic organisms (Pflugmacher *et al*., 1998).